Adopted:
29 July 2016

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

PERFORMANCE-BASED TEST GUIDELINE FOR STABLY TRANSFECTED TRANSACTIVATION *IN VITRO* ASSAYS TO DETECT ESTROGEN RECEPTOR AGONISTS AND ANTAGONISTS

GENERAL INTRODUCTION

Performance-Based Test Guideline

1. This Performance-Based Test Guideline (PBTG) describes the methodology of Stably Transfected Transactivation *In Vitro* Assays to detect Estrogen Receptor Agonists and Antagonists (ER TA assays). It comprises several mechanistically and functionally similar test methods for the identification of estrogen receptor (i.e. ERα, and/or ERβ) agonists and antagonists and should facilitate the development of new similar or modified test methods in accordance with the principles for validation set forth in the OECD Guidance Document (GD) on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment (1). The fully validated reference test methods (Annex 2 and Annex 3) that provide the basis for this PBTG are:

- The Stably Transfected TA (STTA) assay (2) using the (h) ERα-HeLa-9903 cell line; and
- The VM7Luc ER TA assay (3) using the VM7Luc4E2 cell line[1] which predominately expresses hERα with some contribution from hERβ (4) (5).

For the development and validation of similar test methods for the same hazard endpoint, performance standards (PS) (6) (7) are available and should be used. They allow for timely amendment of this PBTG so that new similar test methods can be added to an updated PBTG; however, similar test methods will only be added after review and agreement that performance standards are met. The test methods included in this Test Guideline can be used indiscriminately to address countries' requirements for test results on estrogen receptor transactivation while benefiting from the Mutual Acceptance of Data.

[1] Before June 2016, this cell line was designated as BG1Luc cell line. BG-1 cells were originally described by Geisinger et al. (1998) (35) and were later characterized by researchers at the National Institute of Environmental Health Sciences (NIEHS) (36). Relatively recently, it was discovered that there exist two different variants of BG-1 cells being used by researchers, BG-1 Fr and BG-1 NIEHS. In-depth analysis, including DNA testing, of these two BG-1 variant cell lines carried out by Li and coworkers (2014) (37) showed that the BG-1 Fr was unique and that the BG-1 NIEHS, i.e. the original cell line used to develop the assay, was not the BG1 human ovarian carcinoma cell line, but was instead a variant of the MCF7 human breast cancer cell line. The cell line used in the assay, originally referred to as BG1Luc4E2 (38), will now be designated as VM7Luc4E2 ("V" = variant; "M7" = MCF7 cells). Likewise, the assay will now be designated as the VM7Luc ER TA. While this changes the origin of the cell line upon which the assay is based, it does not affect published validation studies nor the utility and application of this assay for screening of estrogenic/anti-estrogenic chemicals.

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

Background and principles of the test methods included in the PBTG

2. The OECD initiated a high-priority activity in 1998 to revise existing, and to develop new, Test Guidelines for the screening and testing of potential endocrine disrupting chemicals. The OECD conceptual framework (CF) for testing and assessment of potential endocrine disrupting chemicals was revised in 2012. The original and revised CFs are included as Annexes in the Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption (8). The CF comprises five levels, each level corresponding to a different level of biological complexity. The ER Transactivation (TA) assays described in this PBTG are level 2, which includes "*in vitro assays providing data about selected endocrine mechanism(s)/pathway(s).* This PBTG is for *in vitro* Transactivation (TA) test methods designed to identify estrogen receptor (ER) agonists and antagonists.

3. The interaction of estrogens with ERs can affect transcription of estrogen-controlled genes, which can lead to the induction or inhibition of cellular processes, including those necessary for cell proliferation, normal fetal development, and reproductive function (9) (10) (11). Perturbation of normal estrogenic systems may have the potential to trigger adverse effects on normal development (ontogenesis), reproductive health and the integrity of the reproductive system.

4. *In vitro* TA assays are based on a direct or indirect interaction of the substances with a specific receptor that regulates the transcription of a reporter gene product. Such assays have been used extensively to evaluate gene expression regulated by specific nuclear receptors, such as ERs (12) (13) (14) (15) (16). They have been proposed for the detection of estrogenic transactivation regulated by the ER (17) (18) (19). There are at least two major subtypes of nuclear ERs, α and β, which are encoded by distinct genes. The respective proteins have different biological functions as well as different tissue distributions and ligand binding affinities (20) (21) (22) (23) (24) (25) (26). Nuclear ERα mediates the classic estrogenic response (27) (28) (29) (30), and therefore most models currently being developed to measure ER activation or inhibition are specific to ERα. The assays are used to identify chemicals that activate (or inhibit) the ER following ligand binding, after which the receptor-ligand complex binds to specific DNA response elements and transactivates a reporter gene, resulting in increased cellular expression of a marker protein. Different reporter responses can be used in these test methods. In luciferase based systems, the luciferase enzyme transforms the luciferin substrate to a bioluminescent product that can be quantitatively measured with a luminometer. Other examples of common reporters are fluorescent protein and the *LacZ* gene, which encodes β-galactosidase, an enzyme that can transform the colourless substrate X-gal (5- bromo-4-chloro-indolyl-galactopyranoside) into a blue product that can be quantified with a spectrophotometer. These reporters can be evaluated quickly and inexpensively with commercially available test kits.

5. Validation studies of the STTA and the VM7Luc TA assays have demonstrated their relevance and reliability for their intended purpose (3) (4) (5) (30). Performance standards for luminescence-based ER TA assays using breast cells lines are included in ICCVAM Test Method Evaluation Report on the LUMI-CELL® ER (VM7Luc ER TA) Test Method: An *In Vitro* Assay for Identifying Human Estrogen Receptor Agonist and Antagonist Activity of Chemicals (3). These performance standards have been modified to be applicable to both the STTA and VM7Luc TA test methods (2).

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

Scope and limitations related to the TA assays

7. These test methods are being proposed for screening and prioritisation purposes, but can also provide mechanistic information that can be used in a weight of evidence approach. They address TA

induced by chemical binding to the ERs in an *in vitro* system. Thus, results should not be directly extrapolated to the complex signaling and regulation of the intact endocrine system *in vivo*.

8. TA mediated by the ERs is considered one of the key mechanisms of endocrine disruption (ED), although there are other mechanisms through which ED can occur, including (i) interactions with other receptors and enzymatic systems within the endocrine system, (ii) hormone synthesis, (iii) metabolic activation and/or inactivation of hormones, (iv) distribution of hormones to target tissues, and (v) clearance of hormones from the body. None of the test methods under this PBTG addresses these modes of action.

9. This PBTG addresses the ability of chemicals to activate (i.e. act as agonists) and also to suppress (i.e. act as antagonists) ER- dependent transcription. Some chemicals may, in a cell type-dependent manner, display both agonist and antagonist activity and are known as selective estrogen receptor modulators (SERMs). Chemicals that are negative in these test methods could be evaluated in an ER binding assay before concluding that the chemical does not bind to the receptor. 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. Considering that only single substances were used during the validation, the applicability to test mixtures has not been addressed. The test method is nevertheless theoretically applicable to the testing of multi-constituent substances and mixtures. Before use of the Test Guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

10. For informational purposes, Table 1 provides the agonist test results for the 34 substances that were tested in both of the fully validated reference test methods described in this PBTG. Of these substances, 26 are classified as definitive ER agonists and 8 negatives based upon published reports, including *in vitro* assays for ER binding and TA, and/or the uterotrophic assay (2) (3) (18) (31) (32) (33) (34). Table 2 provides the antagonist test results for the 15 substances that were tested in both of the fully validated reference test methods described in this PBTG. Of these substances, 4 are classified as definitive/presumed ER antagonists and 10 negatives based upon published reports, including in vitro assays for ER binding and TA (2) (3) (18) (31). In reference to the data summarised in Table 1 and Table 2, there was 100% agreement between the two reference test methods on the classifications of all the substances except for one substance (Mifepristone) for antagonist assay, and each substance was correctly classified as an ER agonist/antagonist or negative. Supplementary information on this group of chemicals as well as additional chemicals tested in the STTA and VM7Luc ER TA test methods during the validation studies is provided in the Performance Standards for the ERTA (6) (7), Annex 2 (Tables 1, 2 and 3).

TABLE 1: OVERVIEW OF THE RESULTS FROM STTA AND VM7LUC ER TA ASSAYS FOR SUBSTANCES TESTED IN BOTH AGONIST ASSAYS AND CLASSIFIED AS ER AGONISTS (POS) OR NEGATIVES (NEG)

	Substance	CASRN	STTA Assay[1]			VM7Luc ER TA Assay[2]		Data Source For Classification[4]		
			ER TA Activity	PC_{10} Value (M)	PC_{50} Value[b] (M)	ER TA Activity	EC_{50} Value[b,3] (M)	Other ER TAs[C]	ER Binding	Uterotrophic
1	17ß-estradiol[a]	50-28-2	POS	$<1.00 \times 10^{-11}$	$<1.00 \times 10^{-11}$	POS	5.63×10^{-12}	POS (227/227)	POS	POS
2	17α-estradiol[a]	57-91-0	POS	7.24×10^{-11}	6.44×10^{-10}	POS	1.40×10^{-9}	POS(11/11)	POS	POS
3	17α-ethinyl estradiol[a]	57-63-6	POS	$<1.00 \times 10^{-11}$	$<1.00 \times 10^{-11}$	POS	7.31×10^{-12}	POS(22/22)	POS	POS
4	17β-trenbolone	10161-33-8	POS	1.78×10^{-8}	2.73×10^{-7}	POS	4.20×10^{-8}	POS (2/2)	NT	NT
5	19-nortestosterone[a]	434-22-0	POS	9.64×10^{-9}	2.71×10^{-7}	POS	1.80×10^{-6}	POS(4/4)	POS	POS
6	4-cumylphenol[a]	599-64-4	POS	1.49×10^{-7}	1.60×10^{-6}	POS	3.20×10^{-7}	POS(5/5)	POS	NT
7	4-*tert*-octylphenol[a]	140-66-9	POS	1.85×10^{-9}	7.37×10^{-8}	POS	3.19×10^{-8}	POS(21/24)	POS	POS
8	Apigenin[a]	520-36-5	POS	1.31×10^{-7}	5.71×10^{-7}	POS	1.60×10^{-6}	POS(26/26)	POS	NT
9	Atrazine[a]	1912-24-9	NEG	-	-	NEG	-	NEG (30/30)	NEG	NT
10	Bisphenol A[a]	80-05-7	POS	2.02×10^{-8}	2.94×10^{-7}	POS	5.33×10^{-7}	POS(65/65)	POS	POS
11	Bisphenol B[a]	77-40-7	POS	2.36×10^{-8}	2.11×10^{-7}	POS	1.95×10^{-7}	POS(6/6)	POS	POS
12	Butylbenzyl phthalate[a]	85-68-7	POS	1.14×10^{-6}	4.11×10^{-6}	POS	1.98×10^{-6}	POS(12/14)	POS	NEG
13	Corticosterone[a]	50-22-6	NEG	-	-	NEG	-	NEG(6/6)	NEG	NT
14	Coumestrol[a]	479-13-0	POS	1.23×10^{-9}	2.00×10^{-8}	POS	1.32×10^{-7}	POS(30/30)	POS	NT
15	Daidzein[a]	486-66-8	POS	1.76×10^{-8}	1.51×10^{-7}	POS	7.95×10^{-7}	POS(39/39)	POS	POS
16	Diethylstilbestrol[a]	56-53-1	POS	$<1.00 \times 10^{-11}$	2.04×10^{-11}	POS	3.34×10^{-11}	POS(42/42)	POS	NT
17	Di-n-butyl phthalate	84-74-2	POS	4.09×10^{-6}		POS	4.09×10^{-6}	POS(6/11)	POS	NEG

18	Ethyl paraben	120-47-8	POS	5.00×10^{-6}	(no PC_{50})	POS	2.48×10^{-5}	POS		NT
19	Estrone[a]	53-16-7	POS	3.02×10^{-11}	5.88×10^{-10}	POS	2.34×10^{-10}	POS(26/28)	POS	POS
20	Genistein[a]	446-72-0	POS	2.24×10^{-9}	2.45×10^{-8}	POS	2.71×10^{-7}	POS(100/102)	POS	POS
21	Haloperidol	52-86-8	NEG	-	-	NEG	-	NEG (2/2)	NEG	NT
22	Kaempferol[a]	520-18-3	POS	1.36×10^{-7}	1.21×10^{-6}	POS	3.99×10^{-6}	POS(23/23)	POS	NT
23	Kepone[a]	143-50-0	POS	7.11×10^{-7}	7.68×10^{-6}	POS	4.91×10^{-7}	POS(14/18)	POS	NT
24	Ketoconazole	65277-42-1	NEG	-	-	NEG	-	NEG (2/2)	NEG	NT
25	Linuron[a]	330-55-2	NEG	-	-	NEG	-	NEG (8/8)	NEG	NT
26	*meso*-Hexestrol[a]	84-16-2	POS	$<1.00 \times 10^{-11}$	2.75×10^{-11}	POS	1.65×10^{-11}	POS(4/4)	POS	NT
27	Methyl testosterone[a]	58-18-4	POS	1.73×10^{-7}	4.11×10^{-6}	POS	2.68×10^{-6}	POS(5/6)	POS	NT
28	Morin	480-16-0	POS	5.43×10^{-7}	4.16×10^{-6}	POS	2.37×10^{-6}	POS(2/2)	POS	NT
29	Norethynodrel[a]	68-23-5	POS	1.11×10^{-11}	1.50×10^{-9}	POS	9.39×10^{-10}	POS(5/5)	POS	NT
30	*p,p*'-Methoxychlor[a]	72-43-5	POS	1.23×10^{-6}	(no PC_{50})[b]	POS	1.92×10^{-6}	POS(24/27)	POS	POS
31	Phenobarbital[a]	57-30-7	NEG	-	-	NEG	-	NEG(2/2)	NEG	NT
32	Reserpine	50-55-5	NEG	-	-	NEG	-	NEG(4/4)	NEG	NT
33	Spironolactone[a]	52-01-7	NEG	-	-	NEG	-	NEG(4/4)	NEG	NT
34	Testosterone	58-22-0	POS	2.82×10^{-8}	9.78×10^{-6}	POS	1.75×10^{-5}	POS(5/10)	POS	NT

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; M = molar; EC_{50} = half maximal effective concentration of test substance; NEG = negative; POS = positive; NT = Not tested; PC_{10} (and PC_{50}) = the concentration of a test substance at which the response is 10% (or 50 % for PC_{50}) of the response induced by the positive control (E2, 1nM) in each plate.

[a]Common substances tested in the STTA and VM7Luc ER TA assays that were designated as ER agonists or negatives and used to evaluate accuracy in the VM7Luc ER TA validation study (ICCVAM VM7Luc ER TA Evaluation Report, Table 4-1 (3).

[b]Maximum concentration tested in the absence of limitations due to cytotoxicity or insolubility was 1×10^{-5} M (STTA Assay) and 1×10^{-3} M (VM7Luc ER TA Assay).

[c]Number in parenthesis represents the test results classified as positive (POS) or negative (NEG) over the total number of referenced studies.

[1]Values reported in Draft Report of Pre-validation and Inter-laboratory Validation For Stably Transfected Transcriptional Activation (TA) Assay to Detect Estrogenic Activity - The Human Estrogen Receptor Alpha Mediated Reporter Gene Assay Using hER-HeLa-9903 Cell Line (2)

[2]ICCVAM Test Method Evaluation Report on the LUMI-CELL® ER (VM7Luc ER TA) Test Method: An *In Vitro* Method for Identifying ER Agonists and Antagonists (3)

[3]Mean EC_{50} values were calculated with values reported by the laboratories of the VM7Luc ER TA validation study (XDS, ECVAM, and Hiyoshi) (3).

[4]Classification as an ER agonist or negative was based upon information in the ICCVAM Background Review Documents (BRD) for ER Binding and TA test methods (31) as well as information obtained from publications published and reviewed after the completion of the ICCVAM BRDs (2) (3) (18) (31) (33) (34).

Notes: Each test method within this PBTG does not have the same measurements. In some situations the EC50 cannot be calculated because a full dose response curve is not generated. Whilst with the STTA test method, the PC10 value is a key measurement, there may also be further examples where a PCx will provide useful information.

Table 2: Comparison of Results from STTA and VM7Luc ER TA Assays for Substances Tested in Both Antagonist Assays and Classified as ER Antagonists (POS) or Negatives (NEG)

	Substance[a]	CASRN	ER STTA assay[1]		VM7Luc ER TA assay[2]		ER STTA candidate effects[4]	ICCVAM [5] Consensus Classification	MeSH[6] Chemical Class	Product Class[7]
			ER TA Activity	IC_{50} Value[b] (M)	ER TA Activity	IC_{50} Value[b,3] (M)				
1	4-hydroxytamoxifen	68047-06-3	POS	3.97×10^{-9}	POS	2.08×10^{-7}	moderate POS	POS	Hydrocarbon (Cyclic)	Pharmaceutical
2	Dibenzo[a.h] anthracene	53-70-3	POS	No IC_{50}	POS	No IC_{50}	POS	PP	Polycyclic Compound	Laboratory Chemical, Natural Product
3	Mifepristone	84371-65-3	POS	5.61×10^{-6}	NEG	-	mild POS	NEG	Steroid	Pharmaceutical
4	Raloxifene HCl	82640-04-8	POS	7.86×10^{-10}	POS	1.19×10^{-9}	moderate POS	POS	Hydrocarbon (Cyclic)	Pharmaceutical
5	Tamoxifen	10540-29-1	POS	4.91×10^{-7}	POS	8.17×10^{-7}	POS	POS	Hydrocarbon (Cyclic)	Pharmaceutical
6	17β-estradiol	50-28-2	NEG	-	NEG	-	PN	PN	Steroid	Pharmaceutical, Veterinary Agent
7	Apigenin	520-36-5	NEG	-	NEG	-	NEG	NEG	Heterocyclic Compound	Dye, Natural Product, Pharmaceutical Intermediate
8	Atrazine	1912-24-9	NEG	-	NEG	-	NEG	PN	Heterocyclic Compound	Herbicide
9	Di-n-butyl phthalate	84-74-2	NEG	-	NEG	-	NEG	NEG	Ester, Phthalic Acid	Cosmetic Ingredient, Industrial Chemical, Plasticizer
10	Fenarimol	60168-88-9	NEG	-	NEG	-	not tested	PN	Heterocyclic Compound, Pyrimidine	Fungicide
11	Flavone	525-82-6	NEG	-	NEG	-	PN	PN	Flavonoid, Heterocyclic Compound	Natural Product, Pharmaceutical
12	Flutamide	13311-84-7	NEG	-	NEG	-	NEG	PN	Amide	Pharmaceutical, Veterinary Agent
13	Genistein	446-72-0	NEG	-	NEG	-	PN	NEG	Flavonoid, Heterocyclic Compound	Natural Product, Pharmaceutical
14	p-n-nonylphenol	104-40-5	NEG	-	NEG	-	not tested	NEG	Phenol	Chemical Intermediate
15	Resveratrol	501-36-0	NEG	-	NEG	-	PN	NEG	Hydrocarbon (Cyclic)	Natural Product

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; M = molar; IC_{50} = half maximal inhibitory concentration of test substance; NEG = negative; PN = presumed negative; POS = positive; PP = presumed positive.

[a] Common substances tested in the STTA and VM7Luc ER TA assays that were designated as ER antagonists or negatives and used to evaluate accuracy in the VM7Luc ER TA validation study (2) (3).

[b] Maximum concentration tested in the absence of limitations due to cytotoxicity or insolubility was 1 x 10-3 M (STTA Assay) and 1 x 10-5 M (VM7Luc ER TA Assay).

[1] The Validation Report of the Stably transfected Transcriptional Activation Assay to Detect ER mediated activity, Part B (2)

[2] ICCVAM Test Method Evaluation Report on the LUMI-CELL ER (VM7Luc ER TA) Test Method: An In Vitro Method for Identifying ER Agonists and Antagonists (3).

[3] Mean IC_{50} values were calculated with values reported by the laboratories of the VM7Luc ER TA validation study (XDS, ECVAM, and Hiyoshi) (3).

[4] ER STTA activity assumed from their reported effects known from the CERI historical data of ER receptor binding assay, the uterotrophic assay and information collated from the open literature (2)

[5] Classification as an ER antagonist or negative was based upon information in the ICCVAM Background Review Documents (BRD) for ER Binding and TA test methods (31) as well as information obtained from publications published and reviewed after the completion of the ICCVAM BRDs (2) (3) (18) (31).

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

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

ER TA TEST METHOD COMPONENTS

Essential Test Method Components

11. This PBTG applies to methods using a stably transfected or endogenous ERα receptor and stably transfected reporter gene construct under the control of one or more estrogen response elements; however, other receptors such as ERβ may be present. These are essential test method components.

Control substances

12. The basis for the proposed concurrent reference standards for each of agonist and antagonist assay should be described. Concurrent controls (negative, solvent, and positive), as appropriate, serve as an indication that the test method is operative under the test conditions and provide a basis for experiment-to-experiment comparisons; they are usually part of the acceptability criteria for a given experiment (1).

Standard Quality Control Procedures

13. Standard quality control procedures should be performed as described for each assay to ensure the cell line remains stable through multiple passages, remains mycoplasma-free (i.e. free of bacterial contamination), and retains the ability to provide the expected ER-mediated responses over time. Cell lines should be further checked for their correct identity as well as for other contaminants (e.g. fungi, yeast and viruses).

Demonstration of Laboratory Proficiency

14. Prior to testing unknown chemicals with any of the test methods under this PBTG, each laboratory should demonstrate proficiency in using the test method. To demonstrate proficiency, each laboratory should test the 14 proficiency substances listed in Table 3 for the agonist assay and 10 proficiency substances in Table 4 for the antagonist assay. This proficiency testing will also confirm the responsiveness of the test system. The list of proficiency substances is a subset of the reference substances provided in the Performance Standards for the ER TA assays (6). These substances are commercially available, represent the classes of chemicals commonly associated with ER agonist or antagonist activity, exhibit a suitable range of potency expected for ER agonists/antagonists (i.e. strong to weak) and include negatives. Testing of the proficiency substances should be replicated at least twice, on different days. Proficiency is demonstrated by correct classification (positive/negative) of each proficiency substance. Proficiency testing should be repeated by each technician when learning the test methods. Dependent on

cell type, some of these proficiency substances may behave as SERMs and display activity as both agonists and antagonists. However, the proficiency substances are classified in Tables 3 and 4 by their known predominant activity which should be used for proficiency evaluation.

15. To demonstrate performance and for quality control purposes each laboratory should compile agonist and antagonist historical databases with reference standard (e.g. 17β-estradiol and tamoxifen), positive and negative control chemicals and solvent control (e.g. DMSO) data. As a start, the database should be generated from at least 10 independent agonist (e.g. 17β-estradiol) and 10 independent antagonist (e.g. tamoxifen) runs. Results from future analyses of these reference standards and solvent controls should be added to enlarge the database to ensure consistency and performance of the bioassay by the laboratory over time.

Table 3: List of (14) Proficiency Substances for agonist assay[8]

N°[7]	Substance	CASRN	Expected Response[1]	STTA Assay			VM7Luc ER TA Assay		MeSH Chemical Class[5]	Product Class[6]
				PC_{10} Value (M)[2]	PC_{50} Value (M)[2]	Test Conc. Range (M)	VM7Luc EC_{50} Value (M)[3]	Highest Conc. for Range Finder (M)[4]		
14	Diethylstilbestrol	56-53-1	POS	$<1.00 \times 10^{-11}$	2.04×10^{-11}	$10^{-14} – 10^{-8}$	3.34×10^{-11}	3.73×10^{-4}	Hydrocarbon (Cyclic)	Pharmaceutical Veterinary Agent
12	17α-estradiol	57-91-0	POS	4.27×10^{-11}	6.44×10^{-10}	$10^{-11} – 10^{-5}$	1.40×10^{-9}	3.67×10^{-3}	Steroid	Pharmaceutical, Veterinary Agent
15	*meso*-Hexestrol	84-16-2	POS	$<1.00 \times 10^{-11}$	2.75×10^{-11}	$10^{-11} – 10^{-5}$	1.65×10^{-11}	3.70×10^{-3}	Hydrocarbon (Cyclic), Phenol	Pharmaceutical, Veterinary Agent
11	4-*tert*-Octylphenol	140-66-9	POS	1.85×10^{-9}	7.37×10^{-8}	$10^{-11} – 10^{-5}$	3.19×10^{-8}	4.85×10^{-3}	Phenol	Chemical Intermediate
9	Genistein	446-72-0	POS	2.24×10^{-9}	2.45×10^{-8}	$10^{-11} – 10^{-5}$	2.71×10^{-7}	3.70×10^{-4}	Flavonoid, Heterocyclic Compound	Natural Product, Pharmaceutical
6	Bisphenol A	80-05-7	POS	2.02×10^{-8}	2.94×10^{-7}	$10^{-11} – 10^{-5}$	5.33×10^{-7}	4.38×10^{-3}	Phenol	Chemical Intermediate
2	Kaempferol	520-18-3	POS	1.36×10^{-7}	1.21×10^{-6}	$10^{-11} – 10^{-5}$	3.99×10^{-6}	3.49×10^{-3}	Flavonoid, Heterocyclic Compound	Natural Product
3	Butylbenzyl phthalate	85-68-7	POS	1.14×10^{-6}	4.11×10^{-6}	$10^{-11} – 10^{-5}$	1.98×10^{-6}	3.20×10^{-4}	Carboxylic Acid, Ester, Phthalic Acid	Plasticizer, Industrial Chemical
4	*p,p'*- Methoxychlor	72-43-5	POS	1.23×10^{-6}	-	$10^{-11} – 10^{-5}$	1.92×10^{-6}	2.89×10^{-3}	Hydrocarbon (Halogenated)	Pesticide, Veterinary Agent
1	Ethyl paraben	120-47-8	POS	5.00×10^{-6}	-	$10^{-11} – 10^{-5}$	2.48×10^{-5}	6.02×10^{-3}	Carboxylic Acid, Phenol	Pharmaceutical, Preservative
17	Atrazine	1912-24-9	NEG	-	-	$10^{-10} – 10^{-4}$	-	4.64×10^{-4}	Heterocyclic Compound	Herbicide
20	Spironolactone	52-01-7	NEG	-	-	$10^{-11} – 10^{-5}$	-	2.40×10^{-3}	Lactone, Steroid	Pharmaceutical
21	Ketoconazole	65277-42-1	NEG	-	-	$10^{-11} – 10^{-5}$	-	9.41×10^{-5}	Heterocyclic Compound	Pharmaceutical
22	Reserpine	50-55-5	NEG	-	-	$10^{-11} – 10^{-5}$	-	1.64×10^{-3}	Heterocyclic Compound, Indole	Pharmaceutical, Veterinary Agent

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; EC_{50} = half maximal effective concentration of test substance; NEG = negative; POS = positive; PC_{10} (and PC_{50}) = the concentration of a test substance at which the response is 10% (or 50 % for PC_{50}) of the response induced by the positive control (E2, 1nM) in each plate.

[1]Classification as positive or negative for ER agonist activity was based upon the ICCVAM Background Review Documents (BRD) for ER Binding and TA test methods (31) as well as empirical data and other information obtained from referenced studies published and reviewed after the completion of the ICCVAM BRDs (2) (3) (18) (31) (32) (33) (34).

[2]Values reported in Draft Report of Pre-validation and Inter-laboratory Validation For Stably Transfected Transcriptional Activation (TA) Assay to Detect Estrogenic Activity - The Human Estrogen Receptor Alpha Mediated Reporter Gene Assay Using hER-HeLa-9903 Cell Line (30).

[3]Mean EC_{50} values were calculated with values reported by the laboratories of the VM7Luc ER TA validation study (XDS, ECVAM, and Hiyoshi) (3).

[4]Concentrations reported were the highest concentrations tested (range finder) during the validation of the VM7Luc ER TA Assay. If concentrations differed between the laboratories, the highest concentration is reported. See table 4-10 of ICCVAM Test Method Evaluation Report; The LUMI-Cell®ER (VM7Luc ER TA) Test Method: An *In Vitro* Assay for Identifying Human Estrogen Receptor Agonist and Antagonist Activity of Chemicals (3).

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

[7]From Table 1 (List of Reference Chemicals (22) for Evaluation of ER Agonist Accuracy) of the Performance Standards (6)

[8]If a proficiency substance is no longer commercially available, a substance with the same classification and, comparable potency, mode of action and chemical class can be used.

Table 4: List of (10) Proficiency Substances for antagonist assay

	Substance[a]	CASRN	ER STTA assay[1]			VM7Luc ER TA assay[2]			ER STTA[1] Candidate Effects	ICCVAM[5] Consensus Classification	MeSH[6] Chemical Class	Product Class[7]
			ER TA Activity	IC_{50} (M)	Test Conc. range (M)	ER TA Activity	IC_{50}[3] (M)	Highest Conc. for Range Finder (M)[4]				
1	4-hydroxytamoxifen	68047-06-3	POS	3.97×10^{-9}	$10^{-12} – 10^{-7}$	POS	2.08×10^{-7}	2.58×10^{-4}	moderate POS	POS	Hydrocarbon (Cyclic)	Pharmaceutical
2	Raloxifene HCl	82640-04-8	POS	7.86×10^{-10}	$10^{-12} – 10^{-7}$	POS	1.19×10^{-9}	1.96×10^{-4}	moderate POS	POS	Hydrocarbon (Cyclic)	Pharmaceutical
3	Tamoxifen	10540-29-1	POS	4.91×10^{-7}	$10^{-10} – 10^{-5}$	POS	8.17×10^{-7}	2.69×10^{-4}	POS	POS	Hydrocarbon (Cyclic)	Pharmaceutical
4	17β-estradiol	50-28-2	NEG	-	$10^{-9} – 10^{-4}$	NEG	-	3.67×10^{-3}	to be negative*	PN	Steroid	Pharmaceutical, Veterinary Agent
5	Apigenin	520-36-5	NEG	-	$10^{-9} – 10^{-4}$	NEG	-	3.70×10^{-4}	NEG	NEG	Heterocyclic Compound	Dye, Natural Product, Pharmaceutical Intermediate
6	Di-n-butyl phthalate	84-74-2	NEG	-	$10^{-8} – 10^{-3}$	NEG	-	3.59×10^{-3}	NEG	NEG	Ester, Phthalic Acid	Cosmetic Ingredient, Industrial Chemical, Plasticizer
7	Flavone	525-82-6	NEG	-	$10^{-8} – 10^{-3}$	NEG	-	4.50×10^{-4}	to be negative*	PN	Flavonoid, Heterocyclic Compound	Natural Product, Pharmaceutical
8	Genistein	446-72-0	NEG	-	$10^{-9} – 10^{-4}$	NEG	-	3.70×10^{-4}	to be negative*	NEG	Flavonoid, Heterocyclic Compound	Natural Product, Pharmaceutical
9	p-n-nonylphenol	104-40-5	NEG	-	$10^{-9} – 10^{-4}$	NEG	-	4.54×10^{-4}	not tested	NEG	Phenol	Chemical Intermediate
10	Resveratrol	501-36-0	NEG	-	$10^{-8} – 10^{-3}$	NEG	-	4.38×10^{-4}	to be negative*	NEG	Hydrocarbon (Cyclic)	Natural Product

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; M = molar; IC_{50} = half maximal inhibitory concentration of test substance; NEG = negative; PN = presumed negative; POS = positive.

* classified negative according to literature review (2).

[a] Common substances tested in the STTA and VM7Luc ER TA assays that were designated as ER antagonists or negatives and used to evaluate accuracy in the VM7Luc ER TA validation study (2) (3).

[1] The Validation Report of the Stably transfected Transcriptional Activation Assay to Detect ER mediated activity, Part B (2)

[2] ICCVAM Test Method Evaluation Report on the LUMI-CELL ER (VM7Luc ER TA) Test Method: An In Vitro Method for Identifying ER Agonists and Antagonists (3).

[3] Mean IC_{50} values were calculated with values reported by the laboratories of the VM7Luc ER TA validation study (XDS, ECVAM, and Hiyoshi) (3).

[4]Concentrations reported were the highest concentrations tested (range finder) during the validation of the VM7Luc ER TA Assay. If concentrations differed between the laboratories, the highest concentration is reported. See table 4-11 of ICCVAM Test Method Evaluation Report; The LUMI-Cell®ER (VM7Luc ER TA) Test Method: An *In Vitro* Assay for Identifying Human Estrogen Receptor Agonist and Antagonist Activity of Chemicals (3).

[5] Classification as an ER antagonist or negative was based upon information in the ICCVAM Background Review Documents (BRD) for ER Binding and TA test methods (31) as well as information obtained from publications published and reviewed after the completion of the ICCVAM BRDs (2) (3) (18) (31).

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

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

Test Run Acceptability Criteria

16. Acceptance or rejection of a test run is based on the evaluation of results obtained for the reference standards and controls used for each experiment. Values for the PC_{50} (EC_{50}) or IC_{50} for the reference standards should meet the acceptability criteria as provided for the selected test method (for STTA see Annex 2, for VM7Luc ER TA see Annex 3), and all positive/negative controls should be correctly classified for each accepted experiment. The ability to consistently conduct the test method should be demonstrated by the development and maintenance of a historical database for the reference standards and controls (see paragraph 15). Standard deviations (SD) or coefficients of variation (CV) for the means of reference standards curve fitting parameters from multiple experiments may be used as a measure of within-laboratory reproducibility. In addition, the following principles regarding acceptability criteria should be met:

- Data should be sufficient for a quantitative assessment of ER activation (for agonist assay) or suppression (for antagonist assay) (i.e. efficacy and potency).

- The mean reporter activity for the reference concentration of reference estrogen should be at least the minimum specified in the test methods relative to that of the vehicle (solvent) control to ensure adequate sensitivity. For the STTA and VM7Luc ER TA test methods, this is four times that of the mean vehicle control on each plate.

- The concentrations tested should remain within the solubility range of the test chemicals and not demonstrate cytotoxicity.

Analysis of data

17. The defined data interpretation procedure for each test method should be used for classifying a positive and negative response.

18. Meeting the acceptability criteria (paragraph 16) indicates the test method is operating properly, but it does not ensure that any particular test run will produce accurate data. Replicating the results of the first run is the best indication that accurate data were produced. If two runs give reproducible results (e.g. both test run results indicate a test chemical is positive), it is not necessary to conduct a third run.

19. If two runs do not give reproducible results (e.g. a test chemical is positive in one run and negative in the other run), or if a higher degree of certainty is required regarding the outcome of this assay, at least three independent runs should be conducted. In this case the classification is based on the two concordant results out of the three.

General Data Interpretation Criteria

20. There is currently no universally agreed method for interpreting ER TA data. However, both qualitative (e.g. positive/negative) and/or quantitative (e.g. EC_{50}, PC_{50}, IC_{50}) assessments of ER-mediated

activity should be based on empirical data and sound scientific judgment. Where possible, positive results should be characterised by both the magnitude of the effect as compared to the vehicle (solvent) control or reference estrogen and the concentration at which the effect occurs (e.g. an EC_{50}, PC_{50}, RPC_{Max}, IC_{50} , etc.).

Test Report

21. The test report should include the following information:

Test method:

- Test method used;

Control/Reference standard/Test chemical

- source, lot number, limit date for use, if available
- stability of the test chemical itself, if known;
- solubility and stability of the test chemical in solvent, if known.
- measurement of pH, osmolality and precipitate in the culture medium to which the test chemical was added, as appropriate.

Mono-constituent substance:

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

Multi-constituent substance, UVCBs and mixtures:

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

Solvent/Vehicle:

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

Cells:

- type and source of cells:
  - Is ER endogenously expressed? If not, which receptor(s) were Transfected?
  - Reporter construct(s) used (including source species);
  - Transfection method;
  - Selection method for maintenance of stable transfection (where applicable);
  - Is the transfection method relevant for stable lines?

- number of cell passages (from thawing);
- passage number of cells at thawing;
- methods for maintenance of cell cultures;

Test conditions:

- solubility limitations;
- description of the methods of assessing viability applied;
- composition of media, CO_2 concentration;
- concentrations of test chemical;
- volume of vehicle and test chemical added;
- incubation temperature and humidity;
- duration of treatment;
- cell density at the start of - and during treatment;
- positive and negative reference standards;
- reporter reagents (product name, supplier and lot);
- criteria for considering test runs as positive, negative or equivocal;

Acceptability check:

- fold inductions for each assay plate and whether they meet the minimum required by the test method based on historical controls;
- actual values for acceptability criteria, e.g. $\log_{10}EC_{50}$, $\log_{10}PC_{50}$, $\log IC_{50}$ and Hillslope values, for concurrent positive controls/reference standards;

Results:

- raw and normalised data;
- the maximum fold induction level;
- cytotoxicity data;
- if it exists, the lowest effective concentration (LEC);
- RPC_{Max}, PC_{Max}, PC_{50}, IC_{50} and/or EC_{50} values, as appropriate;
- concentration-response relationship, where possible;
- statistical analyses, if any, together with a measure of error and confidence (e.g. SEM, SD, CV or 95% CI) and a description of how these values were obtained;

Discussion of the results

Conclusion

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ANNEX 1

Definitions and Abbreviations

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.

Accuracy (concordance): The closeness of agreement between test method results and an accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with "concordance" to mean the proportion of correct outcomes of a test method (1).

Agonist: A substance that produces a response, e.g. transcription, when it binds to a specific receptor.

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-estrogenic activity, the capability of a chemical to suppress the action of 17β-estradiol mediated through estrogen receptors.

Cell morphology: The shape and appearance of cells grown in a monolayer in a single well of a tissue culture plate. Cells that are dying often exhibit abnormal cell morphology.

CF: The OECD Conceptual Framework for the Testing and Evaluation of Endocrine Disrupters.

Charcoal/dextran treatment: Treatment of serum used in cell culture. Treatment with charcoal/dextran (often referred to as "stripping") removes endogenous hormones and hormone-binding proteins.

Cytotoxicity: Harmful effects to cell structure or function that can ultimately cause cell death and 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.

CV: Coefficient of variation

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

DMEM: Dulbecco's Modification of Eagle's Medium

DMSO: Dimethyl sulfoxide

E2: 17β-estradiol

EC_{50}: The half maximal effective concentration of a test chemical.

ED: Endocrine disruption

hERα: Human estrogen receptor alpha

hERß: Human estrogen receptor beta

EFM: Estrogen-free medium. Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 4.5% charcoal/dextran-treated FBS, 1.9% L-glutamine, and 0.9% Pen-Strep.

ER: Estrogen receptor

ERE: Estrogen response element

Estrogenic activity: The capability of a chemical to mimic 17β-estradiol in its ability to bind to and activate estrogen receptors. hERα-mediated estrogenic activity can be detected with this PBTG.

ERTA: Estrogen Receptor Trans Activation

FBS: Fetal bovine serum

HeLa: An immortal human cervical cell line

HeLa9903: A HeLa cell subclone into which hERα and a luciferase reporter gene have been stably transfected

IC_{50}: The half maximal effective concentration of an inhibitory test chemical.

ICCVAM: The Interagency Coordinating Committee on the Validation of Alternative Methods.

Inter-laboratory reproducibility: A measure of the extent to which different qualified laboratories, using the same protocol and testing the same substances, can produce qualitatively and quantitatively similar results. Interlaboratory reproducibility is determined during the prevalidation and validation processes, and indicates the extent to which a test method can be successfully transferred between laboratories, also referred to as between-laboratory reproducibility (1).

Intra-laboratory reproducibility: A determination of the extent that qualified people within the same laboratory can successfully replicate results using a specific protocol at different times. Also referred to as "within-laboratory reproducibility" (1).

LEC: Lowest effective concentration is the lowest concentration of test chemical that produces a response (i.e. the lowest test chemical concentration at which the fold induction is statistically different from the concurrent vehicle control).

Me-too test: A colloquial expression for a test method that is structurally and functionally similar to a validated and accepted reference test method. Interchangeably used with similar test method

MT: Metallothionein

MMTV: Mouse Mammary Tumor Virus

OHT: 4-Hydroxytamoxifen

PBTG: Performance-Based Test Guideline

PC (Positive control)**:** a strongly active substance, preferably 17ß-estradiol that is included in all tests to help ensure proper functioning of the assay.

PC_{10}: the concentration of a test chemical at which the measured activity in an agonist assay is 10% of the maximum activity induced by the PC (E2 at 1nM for the STTA assay) in each plate.

PC_{50}: the concentration of a test chemical at which the measured activity in an agonist assay is 50% of the maximum activity induced by the PC (E2 at the reference concentration specified in the test method) in each plate.

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

Performance standards: Standards, based on a validated test method, that provide a basis for evaluating the comparability of a proposed test method that is mechanistically and functionally similar. Included are (1) essential test method components; (2) a minimum list of reference chemicals selected from among the chemicals used to demonstrate the acceptable performance of the validated test method; and (3) the comparable levels of accuracy and reliability, based on what was obtained for the validated test method, that the proposed test method should demonstrate when evaluated using the minimum list of reference chemicals (1).

Proficiency substances: A subset of the reference substances included in the Performance Standards that can be used by laboratories to demonstrate technical competence with a standardised test method. Selection criteria for these substances typically include that they represent the range of responses, are commercially available, and have high quality reference data available.

Proficiency: The demonstrated ability to properly conduct a test method prior to testing unknown substances.

Reference estrogen (Positive control, PC): 17β-estradiol (E2, CAS 50-28-2).

Reference standard: a reference substance used to demonstrate the adequacy of a test method. 17β-estradiol is the reference standard for the STTA and VM7Luc ER TA assays.

Reference test methods: The test methods upon which this PBTG is based.

Relevance: Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the

biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (1).

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 intra- and inter-laboratory reproducibility.

RLU: Relative Light Units

RNA: Ribonucleic Acid

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

RPMI: RPMI 1640 medium supplemented with 0.9% Pen-Strep and 8.0% fetal bovine serum (FBS)

Run: An individual experiment 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.

Independent run: A separate, independent experiment that evaluates chemical action on the biological outcome of the test method, using cells from a different pool, freshly diluted chemicals, conducted on different days or on the same day by different staff.

SD: Standard deviation.

Sensitivity: The proportion of all positive/active substances that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (1).

Specificity: The proportion of all negative/inactive substances that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (1).

Stable transfection: When DNA is transfected into cultured cells in such a way that it is stably integrated into the cells genome, resulting in the stable expression of transfected genes. Clones of stably transfected cells are selected by stable markers (e.g. resistance to G418).

STTA Assay: Stably Transfected Transactivation Assay, the ERα transcriptional activation assay using the HeLa 9903 Cell Line.

Study: The full range of experimental work performed to evaluate a single, specific substance using a specific Test Method. A study comprises all steps including tests of dilution of test substance in the test media, preliminary range finding runs, all necessary comprehensive runs, data analyses, quality assurance, cytotoxicity assessments, etc. Completion of a study allows the classification of the test chemical activity on the toxicity target (i.e. active, inactive or inconclusive) that is evaluated by the test method used and an estimate of potency relative to the positive reference chemical.

Substance: Used in the context of the UN GHS (1) as chemical elements and their compounds in the natural state or obtained by any production process, including any additive necessary to preserve the stability of the product and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition.

TA (Transactivation): The initiation of mRNA synthesis in response to a specific chemical signal, such as a binding of an estrogen to the estrogen receptor

Test Method: Within the context of a PBTG, 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.

Transcription: mRNA synthesis

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

Validated test method: A test method for which validation studies have 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 (1).

Validation: The process by which the reliability and relevance of a particular approach, method, process or assessment is established for a defined purpose (1).

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

VM7: An immortalised adenocarcinoma cell that endogenously express estrogen receptor.

VM7Luc4E2: The VM7Luc4E2 cell line was derived from VM7 immortalised human-derived adenocarcinoma cells that endogenously express both forms of the estrogen receptor (ERα and ERβ) and have been stably transfected with the plasmid pGudLuc7.ERE. This plasmid contains four copies of a synthetic oligonucleotide containing the estrogen response element upstream of the mouse mammary tumor viral (MMTV) promoter and the firefly luciferase gene.

Weak positive control: A weakly active substance selected from the reference chemicals list that is included in all tests to help ensure proper functioning of the assay.

ANNEX 2

Stably Transfected Human Estrogen Receptor-α Transactivation Assay for Detection of Estrogenic Agonist and antagonist Activity of Chemicals using the hERα-HeLa-9903 cell line

INITIAL CONSIDERATIONS AND LIMITATIONS (See also **GENERAL INTRODUCTION**, page 1**)**

1. This transactivation (TA) assay uses the hERα-HeLa-9903 cell line to detect estrogenic agonist activity mediated through human estrogen receptor alpha (hERα). The validation study of the Stably Transfected Transactivation (STTA) Assay by the Japanese Chemicals Evaluation and Research Institute (CERI) using the hERα-HeLa-9903 cell line to detect estrogenic agonist and antagonist activity mediated through human estrogen receptor alpha (hERα) demonstrated the relevance and reliability of the assay for its intended purpose (1).

2. This test method is specifically designed to detect hERα-mediated TA by measuring chemiluminescence as the endpoint. However, non-receptor-mediated luminescence signals have been reported at phytoestrogen concentrations higher than 1 μM due to the over-activation of the luciferase reporter gene (2) (3). While the dose-response curve indicates that true activation of the ER system occurs at lower concentrations, luciferase expression obtained at high concentrations of phytoestrogens or similar compounds suspected of producing phytoestrogen-like over-activation of the luciferase reporter gene needs to be examined carefully in stably transfected ER TA assay systems (Appendix 1).

3. The "**GENERAL INTRODUCTION**" and "**ER TA TEST METHOD COMPONENTS"** (pages 1-14) should be read before using this test method for regulatory purposes. Definitions and abbreviations used in this TG are described in Annex 1.

PRINCIPLE OF THE TEST METHOD (See also **GENERAL INTRODUCTION**, page 1)

4. The assay is used to signal binding of the estrogen receptor with a ligand. Following ligand binding, the receptor-ligand complex translocates to the nucleus where it binds specific DNA response elements and transactivates a firefly luciferase reporter gene, resulting in increased cellular expression of luciferase enzyme. Luciferin is a substrate that is transformed by the luciferase enzyme to a bioluminescence product that can be quantitatively measured with a luminometer. Luciferase activity can be evaluated quickly and inexpensively with a number of commercially available test kits.

5. The test system utilises the hERα-HeLa-9903 cell line, which is derived from a human cervical tumor, with two stably inserted constructs: (i) the hERα expression construct (encoding the full-length human receptor), and (ii) a firefly luciferase reporter construct bearing five tandem repeats of a vitellogenin Estrogen-Responsive Element (ERE) driven by a mouse metallothionein (MT) promoter TATA element.

The mouse MT TATA gene construct has been shown to have the best performance, and so is commonly used. Consequently this hERα-HeLa-9903 cell line can measure the ability of a test chemical to induce hERα-mediated transactivation of luciferase gene expression.

6. In case of ER agonist assay, data interpretation is based upon whether or not the maximum response level induced by a test chemical equals or exceeds an agonist response equal to 10% of that induced by a maximally inducing (1 nM) concentration of the positive control (PC) 17β-estradiol (E2) (i.e. the PC_{10}). In case of ER antagonist assay, data interpretation is based upon whether or not the response shows at least a 30% reduction in activity from the response induced by the spike in control (25 pM of E2) without cytotoxicity. Data analysis and interpretation are discussed in detail in paragraphs 34 - 48.

PROCEDURE

Cell Lines

7. The stably transfected hERα-HeLa-9903 cell line should be used for the assay. The cell line can be obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank[2], upon signing a Material Transfer Agreement (MTA).

8. Only cells characterised as mycoplasma-free should be used in testing. RT-PCR (Real Time Polymerase Chain Reaction) is the method of choice for a sensitive detection of mycoplasma infection (4) (5) (6).

Stability of the cell line

9. To monitor the stability of the cell line, E2, 17α-estradiol, 17α-methyltestosterone and corticosterone should be used as the reference standards for agonist assay and a complete concentration-response curve in the test concentration range provided in Table 1 should be measured at least once each time the assay is performed, and the results should be in agreement with the results provided in Table 1.

10. In case of antagonist assay, complete concentration curves for two reference standards, tamoxifen and flutamide, should be measured simultaneously with each run. Correct qualitative classification as positive or negative for the two chemicals should be monitored.

Cell Culture and Plating Conditions

11. Cells should be maintained in Eagle's Minimum Essential Medium (EMEM) without phenol red, supplemented with 60 mg/L of antibiotic kanamycine and 10% dextran-coated-charcoal-treated fetal bovine

[2] JCRB Cell Bank : National Institute of Biomedical Innovation, 7-6-8 Asagi Saito, Ibaraki-shi, Osaka 567-0085, Japan Fax: +81-72-641-9812

serum (DCC-FBS), in a CO_2 incubator (5% CO_2) at 37±1˚C. Upon reaching 75 -90% confluency, cells can be subcultured at 10 mL of 0.4 x 10^5 – 1 x 10^5 cells/mL for 100 mm cell culture dish. Cells should be suspended with 10% FBS-EMEM (which is the same as EMEM with DCC-FBS) and then plated into wells of a microplate at a density of 1 x 10^4 cells/(100 µL x well). Next, the cells should be pre-incubated in a 5% CO_2 incubator at 37˚±1˚C for 3 hours before the chemical exposure. The plastic-ware should be free of estrogenic activity.

12. To maintain the integrity of the response, the cells should be grown for more than one passage from the frozen stock in the conditioned media and should not be cultured for more than 40 passages. For the hERα-HeLa-9903 cell line, this will be less than three months. However the performance of cells may be reduced if they are grown in inappropriate culture conditions.

13. The DCC-FBS can be prepared as described in Appendix 2, or obtained from commercial sources.

Acceptability criteria

Positive and negative reference standards for ER agonist assay

14. Prior to and during the study, the responsiveness of the test system should be verified using the appropriate concentrations of a strong estrogen: E2, a weak estrogen (17α-estradiol), a very weak agonist (17α-methyltestosterone), and a negative substance (corticosterone). Acceptable range values derived from the validation study (1) are given in Table 1. These 4 concurrent reference standards should be included with each experiment and the results should fall within the given acceptable limits. If this is not the case, the cause for the failure to meet the acceptability criteria should be determined (e.g. cell handling, and serum and antibiotics for quality and concentration) and the assay repeated. Once the acceptability criteria have been achieved, to ensure minimum variability of EC_{50}, PC_{50} and PC_{10} values, consistent use of materials for cell culturing is essential. The four concurrent reference standards, which should be included in each experiment (conducted under the same conditions including the materials, passage level of cells and technicians), can ensure the sensitivity of the assay because the PC_{10}s of the three positive reference standards should fall within the acceptable range, as should the PC_{50}s and EC_{50}s where they can be calculated (see Table 1).

Table 1. Acceptable range values of the four reference standards for the ER agonist assay

Name	logPC_{50}	logPC_{10}	logEC_{50}	Hill slope	Test range
17β-estradiol (E2) CAS No: 50-28-2	-11.4 ~ -10.1	<-11	-11.3 ~ -10.1	0.7 ~ 1.5	10^{-14} ~ 10^{-8} M
17α-estradiol CAS No: 57-91-0	-9.6 ~ -8.1	-10.7 ~ -9.3	-9.6 ~ -8.4	0.9 ~ 2.0	10^{-12} ~ 10^{-6} M
Corticosterone CAS No: 50-22-6	–	–	–	–	10^{-10} ~ 10^{-4}M
17α-methyltestosterone CAS No: 58-18-4	-6.0 ~ -5.1	-8.0 ~ -6.2	–	–	10^{-11} ~ 10^{-5} M

Positive and negative reference standards for ER antagonist assay

15. Prior to and during the study, the responsiveness of the test system should be verified using the appropriate concentrations of a positive substance (Tamoxifen), and a negative substance (Flutamide). Acceptable range values derived from the validation study (1) are given in Table 2. These two concurrent reference standards should be included with each experiment and the results should be judged correctly as shown in the criteria. If this is not the case, the cause for the failure to meet the criteria should be determined (e.g. cell handling, and serum and antibiotics for quality and concentration) and the assay repeated. In addition, IC_{50} values for a positive substance (Tamoxifen) should be calculated and the results should fall within the given acceptable limits. Once the acceptability criteria have been achieved, to ensure minimum variability of IC_{50} values, consistent use of materials for cell culturing is essential. The two concurrent reference standards, which should be included in each experiment (conducted under the same conditions including the materials, passage level of cells and technicians), can ensure the sensitivity of the assay (see Table 2).

Table 2. Criteria and acceptable range values of the two reference standards for the ER antagonist assay

Name	Criteria	$LogIC_{50}$	Test range
Tamoxifen CAS No: 10540-29-1	Positive: IC_{50} should be calculated	-5.942～ -7.596	10^{-10}～10^{-5} M
Flutamide CAS No: 13311-84-7	Negative: IC_{30} should not be calculated	-	10^{-10} ～10^{-5} M

Positive and Vehicle Controls

16. The positive control (PC) for ER agonist assay (1 nM of E2) and for ER antagonist assay (10μM TAM) should be tested at least in triplicate in each plate. The vehicle that is used to dissolve a test chemical should be tested as a vehicle control (VC) at least in triplicate in each plate. In addition to this VC, if the PC uses a different vehicle than the test chemical, another VC should be tested at least in triplicate on the same plate with the PC.

Quality criteria for ER agonist assay

17. The mean luciferase activity of the positive control (1 nM E2) should be at least 4-fold that of the mean VC on each plate. This criterion is established based on the reliability of the endpoint values from the validation study (historically between four- and 30-fold).

18. With respect to the quality control of the assay, the fold-induction corresponding to the PC_{10} value of the concurrent PC (1 nM E2) should be greater than 1+2SD of the fold-induction value (=1) of the concurrent VC. For prioritisation purposes, the PC_{10} value can be useful to simplify the data analysis required compared to a statistical analysis. Although a statistical analysis provides information on significance, such an analysis is not a quantitative parameter with respect to concentration-based potential, and so is less useful for prioritisation purposes.

Quality criteria for ER antagonist assay

19. The mean luciferase activity of the spike in control (25 pM E2) should be at least 4-fold that of the mean VC on each plate. This criterion is established based on the reliability of the endpoint values from the validation study.

20. With respect to the quality control of the assay, relative transcriptional activation (RTA) of 1 nM E2 should be greater than 100%, RTA of 1μM 4-Hydroxytamoxifen (OHT) should be less than 40.6% and RTA of 100 μM Digitonin (Dig) should be less than 0%.

Demonstration of Laboratory Proficiency (see paragraph 14 and Tables 3 and 4 in « ER TA TEST METHOD COMPONENTS» of this Test Guideline (pages 8-15)).

Vehicle

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

Preparation of Test Chemicals

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

Solubility and Cytotoxicity: Considerations for Range Finding.

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

24. For ER agonists and 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 testing methods that can provide information regarding 80% cell viability should be used, utilising an appropriate assay based upon laboratory experience.

25. Should the results of the cytotoxicity test show that the concentration of the test chemical has reduced the cell number by 20% or more, this concentration should be regarded as cytotoxic, and the

concentrations at or above the cytotoxic concentration should be excluded from the evaluation.

Chemical Exposure and Assay Plate Organisation

26. The procedure for chemical dilutions (Steps-1 and 2) and exposure to cells (Step-3) can be conducted as follows:

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

Step-2: Chemical dilution: First dilute 1.5 µL of the test chemical in the solvent to a concentration of 500 µL of media.

Step-3: Chemical exposure of the cells: Add 50 µL of dilution with media (prepared in Step-2) to an assay well containing 10^4 cells/100 µL/well.

The recommended final volume of media required for each well is 150 µL. Test samples and reference standards can be assigned as shown in Table 3 and Table 4.

Table 3: Example of plate concentration assignment of the reference standards in the assay plate in ER agonist assay

Row	17α-methyltestosterone			Corticosterone			17α-estradiol			E2		
	1	2	3	4	5	6	7	8	9	10	11	12
A	conc 1 (10 µM)	→	→	100 µM	→	→	1 µM	→	→	10 nM	→	→
B	conc 2 (1 µM)	→	→	10 µM	→	→	100 nM	→	→	1 nM	→	→
C	conc 3 (100 nM)	→	→	1 µM	→	→	10 nM	→	→	100 pM	→	→
D	conc 4 (10 nM)	→	→	100 nM	→	→	1 nM	→	→	10 pM	→	→
E	conc 5 (1 nM)	→	→	10 nM	→	→	100 pM	→	→	1 pM	→	→
F	conc 6 (100 pM)	→	→	1 nM	→	→	10 pM	→	→	0.1 pM	→	→
G	conc 7 (10 pM)	→	→	100 pM	→	→	1 pM	→	→	0.01 pM	→	→
H	VC	→	→	→	→	→	PC	→	→	→	→	→

VC: Vehicle control (0.1% DMSO); PC: Positive control (1 nM E2)

27. The reference standards (E2, 17α-estradiol, 17α-methyl testosterone and corticosterone) should be tested in every run (Table 3). PC wells treated with 1 nM of E2 that can produce maximum induction of E2 and VC wells treated with DMSO (or appropriate solvent) alone should be included in each test assay plate (Table 4). If cells from different sources (e.g. different passage number, different lot, etc.) are used in the same experiment, the reference standards should be tested for each cell source.

Table 4: Example of plate concentration assignment of test and plate control chemicals in the assay plate in ER 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	→	→	→	→	→

VC: Vehicle control (0.1% DMSO); PC: Positive control (1 nM E2)

Table 5: Example of plate concentration assignment of the reference standards in the assay plate in ER antagonist assay

Row	Tamoxifen			Flutamide			Test Chemical 1			Test Chemical 2		
	1	2	3	4	5	6	7	8	9	10	11	12
A	**conc 1 (10 µM)**	→	→	**10 µM**	→	→	**10 µM**	→	→	**10 µM**	→	→
B	**conc 2 (1 µM)**	→	→	**1 µM**	→	→	**1 µM**	→	→	**1 µM**	→	→
C	**conc 3 (100 nM)**	→	→	**100 nM**	→	→	**100 nM**	→	→	**100 nM**	→	→
D	**conc 4 (10 nM)**	→	→	**10 nM**	→	→	**10 nM**	→	→	**10 nM**	→	→
E	**conc 5 (1 nM)**	→	→	**1 nM**	→	→	**1 nM**	→	→	**1 nM**	→	→
F	**conc 6 (100 pM)**	→	→	**100 pM**	→	→	**100 pM**	→	→	**100 pM**	→	→
G	**0.1% DMSO**	→	→	→	→	→	**1 µM OHT**	→	→	**100 µM Dig**	→	→
H	**VC**	→	→	→	→	→	**PC**	→	→	→	→	→

VC: Vehicle control (0.1% DMSO), PC: Positive control (1 nM E2), OHT :4-Hydroxytamoxifen, Dig: Digitonin.

: Spiked with 25pM E2

28. To evaluate the antagonist activity of chemicals, assay wells located in rows from A to G should be spiked with 25pM E2. The reference standards (Tamoxifen and Flutamide) should be tested in every run. PC wells treated with 1 nM of E2 that can be control quality of hERα-HeLa-9903 cell line, VC wells treated with DMSO (or appropriate solvent), 0.1% DMSO wells treated with DMSO addition to the spiked E2 corresponding to "Spike-in-control", wells treated with final concentration 1 µM OHT and wells treated with 100 µM Dig should be included in each test assay plate (Table 5). Subsequent assay plate should follow the same plate layout without reference standards wells (Table 6). If cells from different sources (e.g. different passage number, different lot, etc.) are used in the same experiment, the reference standards should be tested for each cell source.

Table 6: Example of plate concentration assignment of test and plate control chemicals in the assay plate in ER 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)	→	→	10 µM	→	→	10 µM	→	→	10 µM	→	→
B	conc 2 (1 µM)	→	→	1 µM	→	→	1 µM	→	→	1 µM	→	→
C	conc 3 (100 nM)	→	→	100 nM	→	→	100 nM	→	→	100 nM	→	→
D	conc 4 (10 nM)	→	→	10 nM	→	→	10 nM	→	→	10 nM	→	→
E	conc 5 (1 nM)	→	→	1 nM	→	→	1 nM	→	→	1 nM	→	→
F	conc 6 (100 pM)	→	→	100 pM	→	→	100 pM	→	→	100 pM	→	→
G	0.1% DMSO	→	→	→	→	→	1 µM OHT	→	→	100 µM Dig	→	→
H	VC	→	→	→	→	→	PC	→	→	→	→	→

VC: Vehicle control (0.1% DMSO), PC: Positive control (1 nM E2), OHT: 4-Hydroxytamoxifen, Dig: Digitonin.

: Spiked with 25pM E2

29. 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.

30. 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.

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

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

Luciferase assay

33. A commercial 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 for the assay, as long as the acceptability criteria are met. The assay reagents should be selected based on the sensitivity of the luminometer to be used. When using the standard luciferase assay system, Cell Culture Lysis Reagent (Promega, E1531, or equivalents) should be used before adding the substrate. The luciferase reagent should be applied following the manufacturers' instructions.

ANALYSIS OF DATA

ER agonist assay

34. In case of ER agonist assay, to obtain the relative transcriptional activity to PC (1 nM of E2), 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 to normalise the data.

Step 3. Calculate the mean for the normalised PC.

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

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

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

EC_{50}, PC_{50} and PC_{10} induction considerations

35. 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).

36. If the Hill's logistic equation is applicable to the concentration response data, the EC_{50} should be calculated by the following equation (7):

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

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.

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

(i) The RPC_{Max} which is the maximum level of response induced by a test chemical, expressed as a percentage of the response induced by 1 nM E2 on the same plate, as well as the PCMax (concentration associated with the RPC_{Max}); and

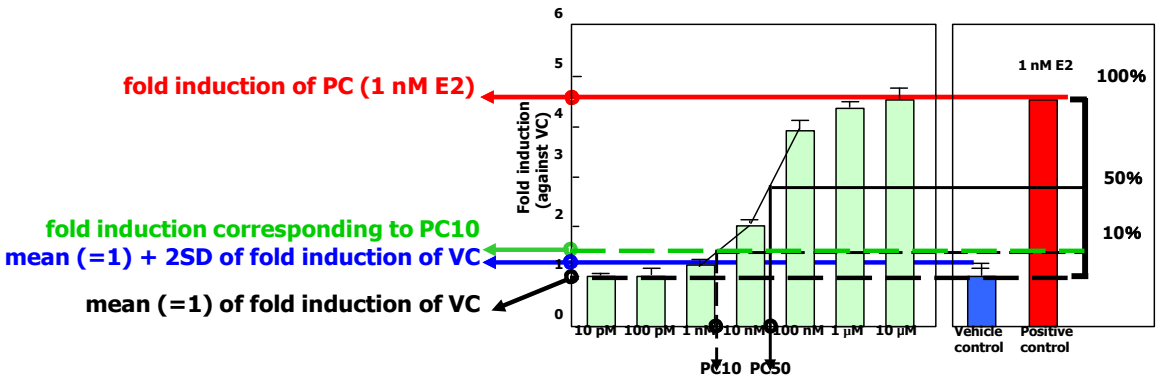
(ii) For positive chemicals, the concentrations that induce the PC_{10} and, if appropriate, the PC_{50}.

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

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

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

Figure 1: Example of how to derive PC-values. The PC (1 nM of E2) is included on each assay plate



ER antagonist assay

40. In case of ER antagonist assay, to obtain the relative transcriptional activity (RTA) to spike in control (25 pM of E2), 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 to normalise the data.

Step 3. Calculate the mean for the normalised spike in control.

Step 4. Divide the normalised value of each well in the plate by the mean value of the normalised spike in control (spike in control=100%).

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

Step 5. Calculate the mean value of the relative transcriptional activity for each treatment.

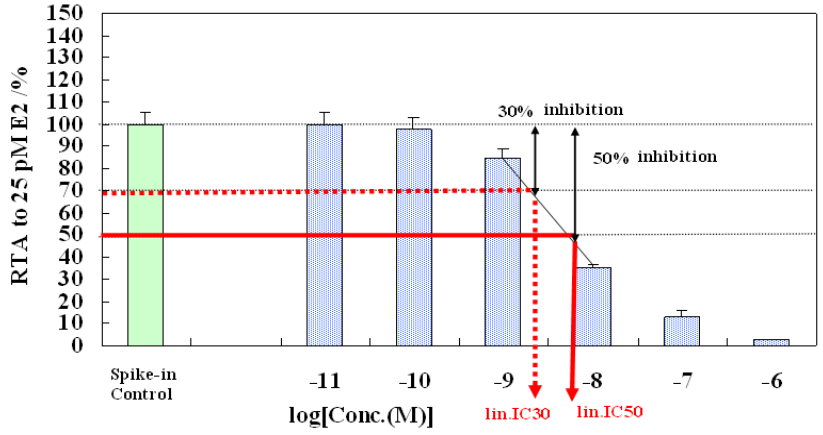
IC_{30} and IC_{50} induction considerations

41. For positive chemicals, the concentrations that induce the IC30 and, if appropriate, the IC50 should be provided.

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

$$\text{lin ICx} = a-(b-(100-x))\ (a-c)\ /(b-d)$$

Figure 2: Example of how to derive IC-values. The spike in control (25 pM of E2) is included on each assay plate



RTA: relative transcriptional activity

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

- Meet the acceptability criteria (see Acceptability criteria para 14-20),
- Be reproducible.

Data Interpretation Criteria

Table 7: Positive and negative decision criteria in ER agonist assay

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

Table 8: Positive and negative decision criteria in ER antagonist assay

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

44. Data interpretation criteria are shown in Tables 7 and 8. 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% (PC50) or 10% (PC10) of PC values are reached for the agonist assay, and 50% (IC50) or 30% (IC30) of the spike-in control value is inhibited for the antagonist assay, accomplishes both of these goals. However, a test chemical is determined to be positive, if the maximum response induced by the test chemical (RPCMax) is equal to or exceeds 10% of the response of the PC in at least two of two or two of three runs, while a test chemical is considered negative if the RPCMax fails to achieve at least 10% of the response of the positive control in two of two or two of three runs.

45. The calculations of PC_{10}, PC_{50} and PC_{Max} in ER agonist assay and IC_{30} and IC_{50} in ER antagonist assay can be made by using a spreadsheet available with the Test Guideline on the OECD public website[3].

46. It should be sufficient to obtain PC_{10} or PC_{50} and IC_{30} or IC_{50} values at least twice. However, should the resulting base-line for data in the same concentration range show variability with an unacceptably high coefficient of variation (CV; %) the data may not be considered reliable and the source of the high variability should be identified. The CV of the raw data triplicates (i.e. luminescence intensity data) of the data points that are used for the calculation of PC_{10} should be less than 20%.

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

48. In case of ER agonist assay, where more information is required in addition to the screening and prioritisation purposes of this TG for positive test chemicals, particularly for PC10-PC49 chemicals, as well as chemicals suspected to over-stimulate luciferase, it can be confirmed that the observed luciferase-activity is solely an ERα-specific response, using an ERα antagonist (see Appendix 1).

TEST REPORT

49. See paragraph 20 of "**ER TA TEST METHOD COMPONENTS**" (Pages 8-15 of this Test Guideline).

[3] http://www.oecd.org/env/testguidelines

LITERATURE (2)

1. OECD (2015). Report of the Inter-Laboratory Validation for Stably Transfected Transactivation Assay to detect Estrogenic and Anti-estrogenic Activity. Environment, Health and Safety Publications, Series on Testing and Assessment (No.225), Organisation for Economic Cooperation and Development, Paris.
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APPENDIX 1

False positives: Assessment of non-receptor mediated luminescence signals

1. False positives in the ER agonist assay might be generated by non-ER-mediated activation of the luciferase gene, or direct activation of the gene product or unrelated fluorescence. Such effects are indicated by an incomplete or unusual dose-response curve. If such effects are suspected, the effect of an ER antagonist (e.g. 4- hydroxytamoxifen (OHT) at non-toxic concentration) on the response should be examined. The pure antagonist ICI 182780 may not be suitable for this purpose as a sufficient concentration of ICI 182780 may decrease the VC value, and this will affect the data analysis.

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

- Agonistic activity of the unknown chemical with / without 10 µM of OHT
- VC (in triplicate)
- OHT (in triplicate)
- 1 nM of E2 (in triplicate) as agonist PC
- 1 nM of E2 + OHT (in triplicate)

3. **Data interpretation criteria**

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

- If the agonistic activity of the unknown chemical is NOT affected by the treatment with ER antagonist, it is classified as "Negative".
- If the agonistic activity of the unknown chemical is completely inhibited, apply the decision criteria.
- If the agonistic activity at the lowest concentration is equal to, or is exceeding, PC_{10} response the unknown chemical is inhibited equal to or exceeding PC_{10} response. The difference in the responses between the non-treated and treated wells with the ER antagonist is calculated and 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.

4. **Data analysis**

Check the performance standard.

Check the CV between wells treated under the same conditions.

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

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

APPENDIX 2

Preparation of Serum treated with Dextran Coated Charcoal (DCC)

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

COMPONENTS

2. The following materials and equipment will be required:

Materials

Activated charcoal
Dextran

Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)
Sucrose

1 M HEPES buffer solution (pH 7.4)
Ultrapure water produced from a filter system

Equipment

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

PROCEDURE

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

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

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

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

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

ANNEX 3

VM7Luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists

INITIAL CONSIDERATIONS AND LIMITATIONS (See also **GENERAL INTRODUCTION**, page 1)

1. This assay uses the VM7Luc4E2 cell line[4]. It has been validated by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) (1). The VM7Luc cell lines predominantly express endogenous ERα and a minor amount of endogenous ERβ (2) (3) (4).

2. This assay is applicable to a wide range of substances, provided they can be dissolved in dimethyl sulfoxide (DMSO; CASRN 67-68-5), do not react with DMSO or the cell culture medium, and are not cytotoxic at the concentrations being tested. If use of DMSO is not possible, another vehicle such as ethanol or water may be used (see paragraph 12). The demonstrated performance of the VM7Luc ER TA (ant)agonist test method suggests that data generated with this test method may inform upon ER mediated mechanisms of action and could be considered for prioritisation of substances for further testing.

3. This test method is specifically designed to detect hERα and hERß-mediated TA by measuring chemiluminescence as the endpoint. Chemiluminescence use in bioassays is widespread because luminescence has a high signal-to-background ratio (10). However, the activity of firefly luciferase in cell-based assays can be confounded by substances that inhibit the luciferase enzyme, causing both apparent inhibition or increased luminescence due to protein stabilisation (10). In addition, in some luciferase-based ER reporter gene assays, non-receptor-mediated luminescence signals have been reported at phytoestrogen concentrations higher than 1 μM due to the over-activation of the luciferase reporter gene (9) (11). While the dose-response curve indicates that true activation of the ER system occurs at lower concentrations, luciferase expression obtained at high concentrations of phytoestrogens or similar compounds suspected of producing phytoestrogen-like over-activation of the luciferase reporter gene needs to be examined carefully in stably transfected ER TA assay systems (see Annex 2).

[4] Before June 2016, this cell line was designated as BG1Luc cell line. BG-1 cells were originally described by Geisinger et al. (1998) (12) and were later characterized by researchers at the National Institute of Environmental Health Sciences (NIEHS) (13). Relatively recently, it was discovered that there exist two different variants of BG-1 cells being used by researchers, BG-1 Fr and BG-1 NIEHS. In-depth analysis, including DNA testing, of these two BG-1 variant cell lines carried out by Li and coworkers (2014) (14) showed that the BG-1 Fr was unique and that the BG-1 NIEHS, i.e. the original cell line used to develop the assay, was not the BG1 human ovarian carcinoma cell line, but was instead a variant of the MCF7 human breast cancer cell line. The cell line used in the assay, originally referred to as BG1Luc4E2 (15), will now be designated as VM7Luc4E2 ("V" = variant; "M7" = MCF7 cells). Likewise, the assay will now be designated as the VM7Luc ER TA. While this changes the origin of the cell line upon which the assay is based, it does not affect published validation studies nor the utility and application of this assay for screening of estrogenic/anti-estrogenic chemicals.

4. The "**GENERAL INTRODUCTION**" and "**ER TA TEST METHOD COMPONENTS"** (pages 1-15) should be read before using this test method for regulatory purposes. Definitions and abbreviations used in this TG are described in Annex 1.

PRINCIPLE OF THE TEST METHOD (See also **GENERAL INTRODUCTION**, page 1)

5. The assay is used to indicate ER ligand binding, followed by translocation of the receptor-ligand complex to the nucleus. In the nucleus, the receptor-ligand complex binds to specific DNA response elements and transactivates the reporter gene (*luc*), resulting in the production of luciferase and the subsequent emission of light, which can be quantified using a luminometer. Luciferase activity can be quickly and inexpensively evaluated with a number of commercially available kits. The VM7Luc ER TA utilises an ER responsive human breast adenocarcinoma cell line, VM7, which has been stably transfected with a firefly *luc* reporter construct under control of four estrogen response elements placed upstream of the mouse mammary tumour virus promoter (MMTV), to detect substances with *in vitro* ER agonist or antagonist activity. This MMTV promoter exhibits only minor cross-reactivity with other steroid and non-steroid hormones (8). Criteria for data interpretation are described in detail in paragraph 41. Briefly, a positive response is identified by a concentration-response curve containing at least three points with non-overlapping error bars (mean ± SD), as well as a change in amplitude (normalised relative light unit [RLU]) of at least 20% of the maximal value for the reference standard (17β-estradiol [E2; CASRN 50-28-2] for the agonist assay, raloxifene HCl [Ral; CASRN 84449-90-1]/E2 for the antagonist assay).

PROCEDURE

Cell Line

6. The stably transfected VM7Luc4E2 cell line should be used for the assay. The cell line is currently only available with a technical licensing agreement from the University of California, Davis, California, USA[5], and from Xenobiotic Detection Systems Inc., Durham, North Carolina, USA[6].

Stability of the Cell Line

7. To maintain the stability and integrity of the cell line, the cells should be grown for more than one passage from the frozen stock in cell maintenance media (see paragraph 9). Cells should not be cultured for more than 30 passages. For the VM7Luc4E2 cell line, 30 passages will be approximately three months.

Cell Culture and Plating Conditions

8. Procedures specified in the Guidance on Good Cell Culture Practice (5) (6) should be followed to assure the quality of all materials and methods in order to maintain the integrity, validity, and reproducibility of any work conducted.

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[6] Xenobiotic Detection Systems Inc. 1601 East Geer Street, Suite S, Durham NC, 27704 USA, email: info@dioxins.com, Telephone: 919-688-4804, Fax: 919-688-4404

9. VM7Luc4E2 cells are maintained in RPMI 1640 medium supplemented with 0.9% Pen-Strep and 8.0% fetal bovine serum (FBS) in a dedicated tissue culture incubator at 37ºC ± 1ºC, 90% ± 5% humidity, and 5.0% ± 1% CO_2/air.

10. Upon reaching ~80% confluence, VM7Luc4E2 cells are subcultured and conditioned to an estrogen-free environment for 48 hours prior to plating the cells in 96-well plates for exposure to test chemicals and analysis of estrogen dependent induction of luciferase activity. The estrogen-free medium (EFM) contains Dulbecco's Modification of Eagle's Medium (DMEM) without phenol red, supplemented with 4.5% charcoal/dextran-treated FBS, 1.9% L-glutamine, and 0.9% Pen-Strep. All plasticware should be free of estrogenic activity [see detailed protocol (7)].

Acceptability Criteria

11. Acceptance or rejection of a test is based on the evaluation of reference standard and control results from each experiment conducted on a 96-well plate. Each reference standard is tested in multiple concentrations and there are multiple samples of each reference and control concentration. Results are compared to quality controls (QC) for these parameters that were derived from the agonist and antagonist historical databases generated by each laboratory during the demonstration of proficiency. The historical databases are updated with reference standard and control values on a continuous basis. Changes in equipment or laboratory conditions may necessitate generation of updated historical databases.

Agonist Test

Range Finder Test

- Induction: Plate induction should be measured by dividing the average highest E2 reference standard relative light unit (RLU) value by the average DMSO control RLU value. Five-fold induction is usually achieved, but for purpose of acceptance, induction should be greater than or equal to four-fold.
- DMSO control results: Solvent control RLU values should be within 2.5 times the standard deviation of the historical solvent control mean RLU value.
- An experiment that fails either acceptance criterion should be discarded and repeated.

Comprehensive Test

It includes acceptability criteria from the agonist range finder test and the following:
- Reference standard results: The E2 reference standard concentration-response curve should be sigmoidal in shape and have at least three values within the linear portion of the concentration-response curve.
- Positive control results: Methoxychlor control RLU values should be greater than the DMSO mean plus three times the standard deviation from the DMSO mean.
- An experiment that fails any single acceptance criterion should be discarded and repeated.

Antagonist Test

Range Finder Test

- Reduction: Plate reduction is measured by dividing the average highest Ral/E2 reference standard RLU value by the average DMSO control RLU value. Five-fold reduction is usually achieved, but for the purposes of acceptance, reduction should be greater than or equal to three-fold.

- E2 control results: E2 control RLU values should be within 2.5 times the standard deviation of the historical E2 control mean RLU value.
- DMSO control results: DMSO control RLU values should be within 2.5 times the standard deviation of the historical solvent control mean RLU value.
- An experiment that fails any single acceptance criterion will be discarded and repeated.

Comprehensive Test

It includes acceptance criteria from the antagonist range finder test and the following:

- Reference standard results: The Ral/E2 reference standard concentration-response curve should be sigmoidal in shape and have at least three values within the linear portion of the concentration-response curve.
- Positive control results: Tamoxifen/E2 control RLU values should be less than the E2 control mean minus three times the standard deviation from the E2 control mean.
- An experiment that fails any single acceptance criterion will be discarded and repeated.

Reference Standards, Positive, and Vehicle Controls

Vehicle Control (Agonist and Antagonist Assays)

12. The vehicle that is used to dissolve the test chemicals should be tested as a vehicle control. The vehicle used during the validation of the VM7Luc ER TA assay was 1% (v/v) dimethylsulfoxide (DMSO, CASRN 67-68-5) (see paragraph 24). If a vehicle other than DMSO is used, all reference standards, controls, and test chemicals should be tested in the same vehicle, if appropriate.

Reference Standard (Agonist Range Finder)

13. The reference standard is E2 (CASRN 50-28-2). For range finder testing, the reference standard is comprised of a serial dilution of four concentrations of E2 (1.84×10^{-10}, 4.59×10^{-11}, 1.15×10^{-11} and 2.87×10^{-12} M), with each concentration tested in duplicate wells.

Reference Standard (Agonist Comprehensive)

14. E2 for comprehensive testing is comprised of a 1:2 serial dilution consisting of 11 concentrations (ranging from 3.67×10^{-10} to 3.59×10^{-13} M) of E2 in duplicate wells.

Reference Standard (Antagonist Range Finder)

15. The reference standard is a combination of Ral (CASRN 84449-90-1) and E2 (CASRN 50-28-2). Ral/E2 for range finder testing is comprised of a serial dilution of three concentrations of Ral (3.06×10^{-9}, 7.67×10^{-10}, and 1.92×10^{-10}M) plus a fixed concentration (9.18×10^{-11} M) of E2 in duplicate wells.

Reference Standard (Antagonist Comprehensive)

16. Ral/E2 for comprehensive testing is comprised of a 1:2 serial dilution of Ral (ranging from 2.45×10^{-8} to 9.57×10^{-11}M) plus a fixed concentration (9.18×10^{-11} M) of E2 consisting of nine concentrations of Ral/E2 in duplicate wells.

Weak Positive Control (Agonist)

17. The weak positive control is 9.06×10^{-6} M *p,p'*-methoxychlor (methoxychlor; CASRN 72-43-5) in EFM.

Weak Positive Control (Antagonist)

18. The weak positive control consists of tamoxifen (CASRN 10540-29-1) 3.36×10^{-6} M with 9.18×10^{-11} M E2 in EFM.

E2 Control (Antagonist Assay Only)

19. The E2 control is 9.18×10^{-11} M E2 in EFM and used as a base line negative control.

Fold-Induction (Agonist)

20. The induction of luciferase activity of the reference standard (E2) is measured by dividing the average highest E2 reference standard RLU value by the average DMSO control RLU value, and the result should be greater than four-fold.

Fold-Reduction (Antagonist)

21. The mean luciferase activity of the reference standard (Ral/E2) is measured by dividing the average highest Ral/E2 reference standard RLU value by the average DMSO control RLU value and should be greater than three-fold.

Demonstration of Laboratory Proficiency (see paragraph 14 and Tables 3 and 4 in "**ER TA TEST METHOD COMPONENTS**" of this Test Guideline (pages 8-15))

Vehicle

22. 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 DMSO are suitable vehicles. If DMSO is used, the level should not exceed 1% (v/v). For any vehicle, it should be demonstrated that the maximum volume used is not cytotoxic and does not interfere with the assay performance. Reference standards and controls are dissolved in 100% solvent and then diluted down to appropriate concentrations in EFM.

Preparation of Test chemicals

23. The test chemicals are dissolved in 100% DMSO (or appropriate solvent), and then diluted down to appropriate concentrations in EFM. All test chemicals should be allowed to equilibrate to room temperature before being dissolved and diluted. Test chemical solutions should be prepared fresh for each experiment. Solutions should not have noticeable precipitate or cloudiness. Reference standard and control stocks may be prepared in bulk; however, final reference standard, control dilutions and test chemicals should be freshly prepared for each experiment and used within 24 hours of preparation.

Solubility and Cytotoxicity: Considerations for Range Finding

24. Range finder testing consists of seven point - 1:10 serial dilutions run in duplicate. Initially, test chemicals are tested up to the maximum concentration of 1 mg/mL (~1 mM) for agonist testing and 20 µg/mL (~10 µM) for antagonist testing. Range finder experiments are used to determine the following:

- Test chemical starting concentrations to be used during comprehensive testing
- Test chemical dilutions (1:2 or 1:5) to be used during comprehensive testing

25. An assessment of cell viability/cytotoxicity is included in the agonist and antagonist test method protocols (7) and is incorporated into range finder and comprehensive testing. The cytotoxicity method that was used to assess cell viability during the validation of the VM7Luc ER TA (1) was a scaled qualitative visual observation method; however, a quantitative method for the determination of cytotoxicity can be used (see protocol (7)). Data from test chemical concentrations that cause more than 20% reduction in viability cannot be used.

Test chemical Exposure and Assay Plate Organisation

26. Cells are counted and plated into 96-well tissue culture plates (2 x 10^5 cells per well) in EFM and incubated for 24 hours to allow the cells to attach to the plate. The EFM is removed and replaced with test and reference chemicals in EFM and incubated for 19-24 hours. Special considerations will need to be applied to those substances that are highly volatile since nearby control wells may generate false positive results. In such cases, "plate sealers" may help to effectively isolate individual wells during testing, and are therefore recommended.

Range Finder Tests

27. Range finder testing uses all wells of the 96-well plate to test up to six test chemicals as seven point 1:10 serial dilutions in duplicate (see Figures 1 and 2).

- *Agonist* range finder testing uses four concentrations of E2 in duplicate as the reference standard and four replicate wells for the DMSO control.
- *Antagonist* range finder testing uses three concentrations of Ral/E2 with 9.18×10^{-11} M E2 in duplicate as the reference standard, with three replicate wells for the E2 and DMSO controls.

Figure 1: Agonist Range Finder Test 96-well Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	TC1-1	TC1-1	TC2-1	TC2-1	TC3-1	TC3-1	TC4-1	TC4-1	TC5-1	TC5-1	TC6-1	TC6-1
B	TC1-2	TC1-2	TC2-2	TC2-2	TC3-2	TC3-2	TC4-2	TC4-2	TC5-2	TC5-2	TC6-2	TC6-2
C	TC1-3	TC1-3	TC2-3	TC2-3	TC3-3	TC3-3	TC4-3	TC4-3	TC5-3	TC5-3	TC6-3	TC6-3
D	TC1-4	TC1-4	TC2-4	TC2-4	TC3-4	TC3-4	TC4-4	TC4-4	TC5-4	TC5-4	TC6-4	TC6-4
E	TC1-5	TC1-5	TC2-5	TC2-5	TC3-5	TC3-5	TC4-5	TC4-5	TC5-5	TC5-5	TC6-5	TC6-5
F	TC1-6	TC1-6	TC2-6	TC2-6	TC3-6	TC3-6	TC4-6	TC4-6	TC5-6	TC5-6	TC6-6	TC6-6
G	TC1-7	TC1-7	TC2-7	TC2-7	TC3-7	TC3-7	TC4-7	TC4-7	TC5-7	TC5-7	TC6-7	TC6-7
H	E2-1	E2-2	E2-3	E2-4	VC	VC	VC	VC	E2-1	E2-2	E2-3	E2-4

Abbreviations: E2-1 to E2-4 = concentrations of the E2 reference standard (from high to low); TC1-1 to TC1-7 = concentrations (from high to low) of test chemical 1 (TC1); TC2-1 to TC2-7 = concentrations (from high to low) of test chemical 2 (TC2); TC3-1 to TC3-7 = concentrations (from high to low) of test chemical 3 (TC3); TC4-1 to TC4-7 = concentrations (from high to low) of test chemical 4 (TC4); TC5-1 to TC5-7 = concentrations (from high to low) of test chemical 5 (TC5); TC6-1 to TC6-7 = concentrations (from high to low) of test chemical 6 (TC6); VC = vehicle control (DMSO [1% v/v EFM.]).

Figure 2: Antagonist Range Finder Test 96-well Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	TC1-1	TC1-1	TC2-1	TC2-1	TC3-1	TC3-1	TC4-1	TC4-1	TC5-1	TC5-1	TC6-1	TC6-1
B	TC1-2	TC1-2	TC2-2	TC2-2	TC3-2	TC3-2	TC4-2	TC4-2	TC5-2	TC5-2	TC6-2	TC6-2
C	TC1-3	TC1-3	TC2-3	TC2-3	TC3-3	TC3-3	TC4-3	TC4-3	TC5-3	TC5-3	TC6-3	TC6-3
D	TC1-4	TC1-4	TC2-4	TC2-4	TC3-4	TC3-4	TC4-4	TC4-4	TC5-4	TC5-4	TC6-4	TC6-4
E	TC1-5	TC1-5	TC2-5	TC2-5	TC3-5	TC3-5	TC4-5	TC4-5	TC5-5	TC5-5	TC6-5	TC6-5
F	TC1-6	TC1-6	TC2-6	TC2-6	TC3-6	TC3-6	TC4-6	TC4-6	TC5-6	TC5-6	TC6-6	TC6-6
G	TC1-7	TC1-7	TC2-7	TC2-7	TC3-7	TC3-7	TC4-7	TC4-7	TC5-7	TC5-7	TC6-7	TC6-7
H	Ral-1	Ral-2	Ral-3	VC	VC	VC	E2	E2	E2	Ral-1	Ral-2	Ral-3

Abbreviations: E2 = E2 control; Ral-1 to Ral-3 = concentrations of the Raloxifene/E2 reference standard (from high to low); TC1-1 to TC1-7 = concentrations (from high to low) of test chemical 1 (TC1); TC2-1 to TC2-7 = concentrations (from high to low) of test chemical 2 (TC2); TC3-1 to TC3-7 = concentrations (from high to low) of test chemical 3 (TC3); TC4-1 to TC4-7 = concentrations (from high to low) of test chemical 4 (TC4); TC5-1 to TC5-7 = concentrations (from high to low) of test chemical 5 (TC5); TC6-1 to TC6-7 = concentrations (from high to low) of test chemical 6 (TC6); VC = vehicle control (DMSO [1% v/v EFM.]).
Note: All test chemicals are tested in the presence of 9.18×10^{-11} M E2.

28. The recommended final volume of media required for each well is 200 µL. Only use test plates in which the cells in all wells give a viability of 80% and above.

29. Determination of starting concentrations for comprehensive ***agonist*** testing is described in depth in the agonist protocol (7). Briefly, the following criteria are used:

- If there are no points on the test chemical concentration curve that are greater than the mean plus three times the standard deviation of the DMSO control, comprehensive testing will be conducted using an 11-point 1:2 serial dilution starting at the maximum soluble concentration.

- If there are points on the test chemical concentration curve that are greater than the mean plus three times the standard deviation of the DMSO control, the starting concentration to be used for the 11-point dilution scheme in comprehensive testing should be one log higher than the concentration giving the highest adjusted RLU value in the range finder. The 11-point dilution scheme will be based on either 1:2 or 1:5 dilutions according to the following criteria:

  An 11-point 1:2 serial dilution should be used if the resulting concentration range will encompass the full range of responses based on the concentration response curve generated in the range finder test. Otherwise, use a 1:5 dilution.

- If a test chemical exhibits a biphasic concentration response curve in the range finder test, both phases should also be resolved in comprehensive testing.

30. Determination of starting concentrations for comprehensive ***antagonist*** testing is described in depth in the antagonist protocol (7). Briefly, the following criteria are used:

- If there are no points on the test chemical concentration curve that are less than the mean minus three times the standard deviation of the E2, control comprehensive testing will be conducted using an 11-point 1:2 serial dilution starting at the maximum soluble concentration.

- If there are points on the test chemical concentration curve that are less than the mean minus three times the standard deviation of the E2 control, the starting concentration to be used for the 11-point dilution scheme in comprehensive testing should be one of the following:
  - The concentration giving the lowest adjusted RLU value in the range finder
  - The maximum soluble concentration (See antagonist protocol (7), Figure 14-2)
  - The lowest cytotoxic concentration (See antagonist protocol (7), Figure 14-3 for a related example).

- The 11-point dilution scheme will be based on either a 1:2 or 1:5 serial or dilution according to the following criteria:

  An 11-point 1:2 serial dilution should be used if the resulting concentration range will encompass the full range of responses based on the concentration response curve generated in the range finder test. Otherwise a 1:5 dilution should be used.

Comprehensive Tests

31. Comprehensive testing consists of 11-point serial dilutions (either 1:2 or 1:5 serial dilutions based on the starting concentration for comprehensive testing criteria) with each concentration tested in triplicate wells of the 96-well plate (see Figures 3 and 4).

- *Agonist* comprehensive testing uses 11 concentrations of E2 in duplicate as the reference standard. Four replicate wells for the DMSO control and four replicate wells for the methoxychlor control (9.06×10^{-6} M) are included on each plate.

- *Antagonist* comprehensive testing uses nine concentrations of Ral/E2 with 9.18×10^{-11} M E2 in duplicate as the reference standard, with four replicate wells for the E2 9.18×10^{-11} M control, four replicate wells for DMSO controls, and four replicate wells for tamoxifen 3.36×10^{-6}M.

Figure 3: Agonist Comprehensive Test 96-well Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	TC1-9	TC1-10	TC1-11	VC
B	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	TC1-9	TC1-10	TC1-11	VC
C	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	TC1-9	TC1-10	TC1-11	VC
D	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	TC2-9	TC2-10	TC2-11	VC
E	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	TC2-9	TC2-10	TC2-11	Meth
F	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	TC2-9	TC2-10	TC2-11	Meth
G	E2-1	E2-2	E2-3	E2-4	E2-5	E2-6	E2-7	E2-8	E2-9	E2-10	E2-11	Meth
H	E2-1	E2-2	E2-3	E2-4	E2-5	E2-6	E2-7	E2-8	E2-9	E2-10	E2-11	Meth

Abbreviations: TC11-1 to TC1-11 = concentrations (from high to low) of test chemical 1; TC2-1 to TC2-11 = concentrations (from high to low) of test chemical 2; E2-1 to E2-11 = concentrations of the E2 reference standard (from high to low); Meth = p,p' methoxychlor weak positive control; VC = DMSO (1% v/v) EFM vehicle control

Figure 4: Antagonist Comprehensive Test 96-well Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	TC1-9	TC1-10	TC1-11	VC
B	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	TC1-9	TC1-10	TC1-11	VC
C	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	TC1-9	TC1-10	TC1-11	VC
D	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	TC2-9	TC2-10	TC2-11	VC
E	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	TC2-9	TC2-10	TC2-11	Tam
F	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	TC2-9	TC2-10	TC2-11	Tam
G	Ral-1	Ral-2	Ral-3	Ral-4	Ral-5	Ral-6	Ral-7	Ral-8	Ral-9	E2	E2	Tam
H	Ral-1	Ral-2	Ral-3	Ral-4	Ral-5	Ral-6	Ral-7	Ral-8	Ral-9	E2	E2	Tam

Abbreviations: E2 = E2 control; Ral-1 to Ral-9 = concentrations of the Raloxifene/E2 reference standard (from high to low); Tam = Tamoxifen/E2 weak positive control; TC1-1 to TC1-11 = concentrations (from high to low) of test chemical 1 (TC1); TC2-1 to TC2-11 = concentrations (from high to low) of test chemical 2 (TC2); VC = vehicle control (DMSO [1% v/v EFM.]).
Note: As noted, all reference and test wells contain a fixed concentration of E2 (9.18×10^{-11}M)

32. Repeat comprehensive tests for the same chemical should be conducted on different days, to ensure independence. At least two comprehensive tests should be conducted. If the results of the tests contradict each other (e.g. one test is positive, the other negative), or if one of the tests is inadequate, a third additional test should be conducted.

Measure of Luminescence

33. Luminescence is measured in the range of 300 to 650 nm, using an injecting luminometer and with software that controls the injection volume and measurement interval (7). Light emission from each well is expressed as RLU per well.

ANALYSIS OF DATA

EC_{50} /IC_{50} determination

34. The EC_{50} value (half maximal effective concentration of a test chemical [agonists]) and the IC_{50} value (half maximal inhibitory concentration of a test chemical [antagonists]) are determined from the concentration-response data. For test chemicals that are positive at one or more concentrations, the concentration of test chemical that causes a half-maximal response (IC_{50} or EC_{50}) is calculated using a Hill function analysis or an appropriate alternative. The Hill function is a four-parameter logistic mathematical model relating the test chemical concentration to the response (typically following a sigmoidal curve) using the equation below:

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(\lg EC_{50} - X)\text{HillSlope}}}$$

where Y = response (i.e. RLUs); X = the logarithm of concentration; Bottom = the minimum response; Top = the maximum response; lg EC_{50} (or lg IC_{50}) = the logarithm of X as the response midway between Top and Bottom; and Hillslope describes the steepness of the curve. The model calculates the best fit for the Top, Bottom, Hillslope, and IC_{50} and EC_{50} parameters. For the calculation of EC_{50} and IC_{50} values, appropriate statistical software should be used (e.g. Graphpad Prism^{R} statistical software).

Determination of Outliers

35. Good statistical judgment could be facilitated by including (but not limited to) the Q-test (see agonist and antagonist protocols (7) for determining "unusable" wells that will be excluded from the data analysis.

36. For E2 reference standard replicates (sample size of two), any adjusted RLU value for a replicate at a given concentration of E2 is considered an outlier if its value is more than 20% above or below the adjusted RLU value for that concentration in the historical database.

Collection and Adjustment of Luminometer Data for Range Finder Testing

37. Raw data from the luminometer should be transferred to a spreadsheet template designed for the test method. It should be determined whether there are outlier data points that need to be removed. (See Test Acceptance Criteria for parameters that are determined in the analyses.) The following calculations should be performed:

Agonist

Step 1 Calculate the mean value for the DMSO vehicle control (VC).
Step 2 Subtract the mean value of the DMSO VC from each well value to normalise the data.
Step 3 Calculate the mean fold induction for the reference standard (E2).
Step 4 Calculate the mean EC_{50} value for the test chemicals.

Antagonist

Step 1 Calculate the mean value for the DMSO VC.
Step 2 Subtract the mean value of the DMSO VC from each well value to normalise the data.
Step 3 Calculate the mean fold reduction for the reference standard (Ral/E2).
Step 4 Calculate the mean value for the E2 reference standard.
Step 5 Calculate the mean IC_{50} value for the test chemicals.

Collection and Adjustment of Luminometer Data for Comprehensive Testing

38. Raw data from the luminometer should be transferred to a spreadsheet template designed for the test method. It should be determined whether there are outlier data points that need to be removed. (See Test Acceptance Criteria for parameters that are determined in the analyses.) The following calculations are performed:

Agonist

Step 1 Calculate the mean value for the DMSO VC.
Step 2 Subtract the mean value of the DMSO VC from each well value to normalise the data.
Step 3 Calculate the mean fold induction for the reference standard (E2).
Step 4 Calculate the mean EC_{50} value for E2 and the test chemicals.
Step 5 Calculate the mean adjusted RLU value for methoxychlor.

Antagonist

Step 1 Calculate the mean value for the DMSO VC.
Step 2 Subtract the mean value of the DMSO VC from each well value to normalise the data.
Step 3 Calculate the mean fold induction for the reference standard (Ral/E2).
Step 4 Calculate the mean IC_{50} value for Ral/E2 and the test chemicals.
Step 5 Calculate the mean adjusted RLU value for tamoxifen.
Step 6 Calculate the mean value for the E2 reference standard.

Data Interpretation Criteria

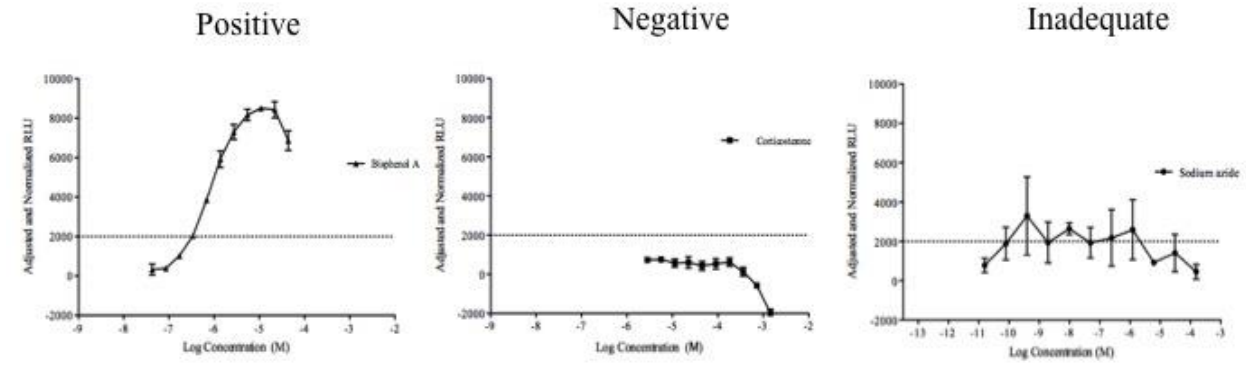
39. The VM7Luc ER TA is intended as part of a weight of evidence approach to help prioritise substances for ED testing *in vivo*. Part of this prioritisation procedure will be the classification of the test chemical as positive or negative for either ER agonist or antagonist activity. The positive and negative decision criteria used in the VM7Luc ER TA validation study are described in Table 1.

Table 1: Positive and Negative Decision Criteria

AGONIST ACTIVITY	
Positive	• All test chemicals classified as *positive for* ER agonist activity should have a concentration–response curve consisting of a baseline, followed by a positive slope, and concluding in a plateau or peak. In some cases, only two of these characteristics (baseline–slope or slope–peak) may be defined. • The line defining the positive slope should contain at least three points with non-overlapping error bars (mean ± SD). Points forming the baseline are excluded, but the linear portion of the curve may include the peak or first point of the plateau. • A positive classification requires a response amplitude, the difference between baseline and peak, of at least 20% of the maximal value for the reference standard, E2 (i.e. 2000 RLUs or more when the maximal response value of the reference standards [E2] is adjusted to 10,000 RLUs). • If possible, an EC_{50} value should be calculated for each positive test chemical.
Negative	The average adjusted RLU for a given concentration is at or below the mean DMSO control RLU value plus three times the standard deviation of the DMSO RLU.
Inadequate	Data that cannot be interpreted as valid for showing either the presence or absence of activity because of major qualitative or quantitative limitations are considered inadequate and cannot be used to determine whether the test chemical is positive or negative. Chemicals should be retested.
ANTAGONIST ACTIVITY	
Positive	• Test chemical data produce a concentration-response curve consisting of a baseline, which is followed by a negative slope. • The line defining the negative slope should contain at least three points with non-overlapping error bars; points forming the baseline are excluded but the linear portion of the curve may include the first point of the plateau. • There should be at least a 20% reduction in activity from the maximal value for the reference standard, Ral/E2 (i.e. 8000 RLU or less when the maximal response value of the reference standard [Ral/E2] is adjusted to 10,000 RLUs). • The highest non-cytotoxic concentrations of the test chemical should be less than or equal to $1x10^{-5}$ M. • If possible, an IC_{50} value should be calculated for each positive test chemical.
Negative	All data points are above the ED_{80} value (80% of the E2 response, or 8000 RLUs), at concentrations less than 1.0×10^{-5} M.
Inadequate	Data that cannot be interpreted as valid for showing either the presence or absence of activity because of major qualitative or quantitative limitations are considered inadequate and cannot be used to determine whether the test chemical is positive or negative. Chemical should be retested.

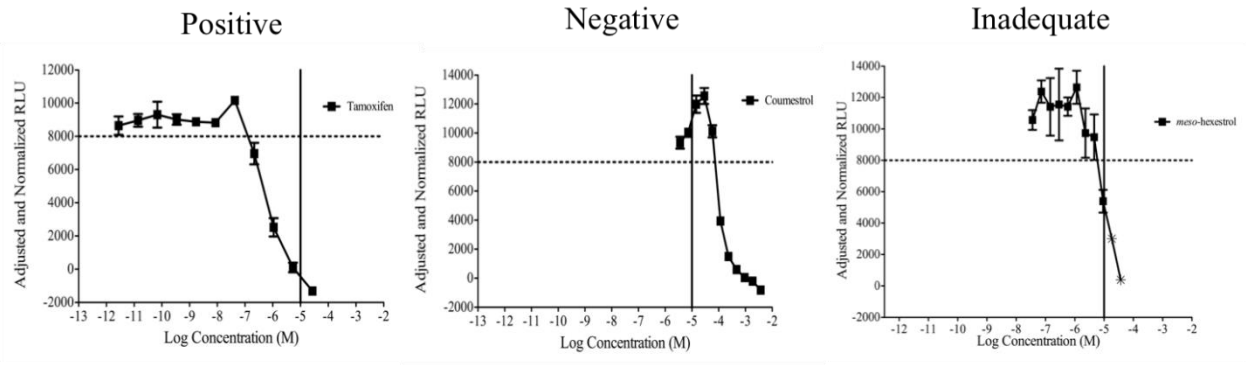
40. Positive results will be characterised by both the magnitude of the effect and the concentration at which the effect occurs, where possible. Examples of positive, negative and inadequate data are shown in Figures 5 and 6.

Figure 5: Agonist Examples: Positive, Negative and Inadequate Data



Dashed line indicates 20% of E2 response, 2000 adjusted and normalised RLUs.

Figure 6: Antagonist Examples: Positive, Negative, and Inadequate Data



Dashed line indicates 80% of Ral/E2 response, 8000 adjusted and normalised RLUs.

Solid line indicates 1.00×10^{-5} M. For a response to be considered positive, it should be below the 8000 RLU line, and at concentrations less than 1.00×10^{-5}M.

Asterisked concentrations in the meso-hexestrol graph indicate viability scores of "2" or greater.

The test results for *meso*-hexestrol are considered inadequate data because the only response that is below 8,000 RLU occurs at 1.00×10^{-5}M.

41. The calculations of EC_{50} and IC_{50} can be made using a four-parameter Hill Function (see agonist protocol and antagonist protocol for more details (7)). Meeting the acceptability criteria indicates the 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 (see paragraph 19 of "ER TA TEST METHOD COMPONENTS" - Page 15 of this Test Guideline).

TEST REPORT

44. See paragraph 20 of "**ER TA TEST METHOD COMPONENTS**" (Page 8-15 of this Test Guideline).

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ANNEX 4

Stably Transfected Human Estrogen Receptor-α Transactivation Assay for Detection of Estrogenic Agonist and Antagonist Activity of Chemicals using the ERα CALUX cell line

INITIAL CONSIDERATIONS AND LIMITATIONS (See also **GENERAL INTRODUCTION**, page 1**)**

1. The ERα CALUX transactivation assay uses the human U2OS cell line to detect estrogenic agonist and antagonist activity mediated through human estrogen receptor alpha (hERα). The validation study of the stably transfected ERα CALUX bioassay by BioDetection Systems BV (Amsterdam, the Netherlands) demonstrated the relevance and reliability of the assay for its intended purpose (1). The ERα CALUX cell line expresses stably transfected human ERα only (2) (3).

2. This test method is specifically designed to detect hERα-mediated transactivation by measuring bioluminescence as the endpoint. The use of bioluminescence is commonly used in bioassays because of the high signal-to-noise ratio (4).

3. Phytoestrogen concentrations higher than 1 µM have been reported to over-activate the luciferase reporter gene, resulting in non-receptor-mediated luminescence (5) (6) (7). Therefore, higher concentrations of phytoestrogens or other similar compounds that can over activate the luciferase expression, have to be examined carefully in stably transfected ER transactivation assays (see Annex 2).

4. The "**GENERAL INTRODUCTION**" and "**ER TA TEST METHOD COMPONENTS"** (pages 1-15) should be read before using this test method for regulatory purposes. Definitions and abbreviations used in this TG are described in Annex 1.

PRINCIPLE OF THE TEST METHOD (See also **GENERAL INTRODUCTION**, page 1)

5. The bioassay is used to assess ER ligand binding and subsequent translocation of the receptor-ligand complex to the nucleus. In the nucleus, the receptor-ligand complex binds specific DNA response elements and transactivates a firefly luciferase reporter gene, resulting in increased cellular expression of the luciferase enzyme. Following the addition of the luciferase substrate luciferine, the luciferine is transformed into a bioluminescent product. The light produced can easily be detected and quantified using a luminometer.

6. The test system utilises stably transfected ERα CALUX cells. ERα CALUX cells originated from the human osteoblastic osteosarcoma U2OS cell line. Human U2OS cells were stably transfected with 3xHRE-TATA-Luc and pSG5-neo-hERα using the calcium phosphate co-precipitation method. The U2OS cell line was selected as the best candidate to serve as the estrogen- (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 (2) (3) (8).

7. Testing chemicals for estrogenic or anti-estrogenic activity using the ERα CALUX cell line include a prescreen run and comprehensive runs. During the prescreen run, the solubility, cytotoxicity and a refined concentration-range of test chemicals for comprehensive testing are determined. During the comprehensive

runs, the refined concentration-ranges of test chemicals are tested in the ERα CALUX bioassays followed by the classification of the test chemicals for agonism or antagonism.

8. Criteria for data interpretation are described in detail in paragraph 59. 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 17β-estradiol (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 tamoxifen (PC_{80}).

PROCEDURE

Cell lines

9. The stably transfected U2OS ERα CALUX cell line should be used for the assay. The cell line can be obtained from BioDetection Systems BV, Amsterdam, the Netherlands with a technical licensing agreement.

10. 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. RT-PCR (Real Time Polymerase Chain Reaction) should be used for sensitive detection of mycoplasma infection (9).

Stability of the cell line

11. To maintain the stability and integrity of the CALUX cells, the cells should be stored in liquid nitrogen (-80^0C). Following thawing of cells to start a new culture, cells should be sub-cultured at least twice before being used to assess the estrogenic agonist and antagonist activity of chemicals. Cells should not be sub-cultured for more than 30 passages.

12. To monitor the stability of the cell line over time, the responsiveness of the agonistic and antagonistic test system should be verified by evaluating the EC_{50} or IC_{50} of the reference standard. In addition, the relative induction of the positive control sample (PC) and the negative control sample (NC) should be monitored. The results should be in agreement with the acceptance criteria for the agonistic (Table 3C) or antagonistic ERα CALUX bioassay (Table 4C). The reference standards, positive and negative controls are given in Table 1 and Table 2 for the agonistic and antagonistic mode respectively.

Cell Culture and plating conditions

13. The U2OS cells should be cultured in growth medium (DMEM/F12 (1:1) medium with phenol red as pH indicator, supplemented with fetal bovine serum (7.5%), non-essential amino acids (1%), 10 Units/mL of penicillin, streptomycin and geneticin (G-418) as selection marker). Cells should be placed in a CO_2 incubator (5% CO_2) at 37^0C and 100% humidity. When cells reach an 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 estrogen free assay medium (DMEM/F12 (1:1) medium without phenol red, supplemented with Dextran-Coated Charcoal treated fetal bovine serum (5% v/v),

non-essential amino acids (1% v/v), 10 Units/mL of penicillin and streptomycin) and plated into the wells of the 96-well microtiterplates (100 µl of homogenised cell suspension). Cells should be pre-incubated in a CO_2 incubator (5% CO_2, 37^0C, 100% humidity) for 24 hours prior to exposure. Plastic ware should be estrogen free.

Acceptability criteria

14. Agonistic and antagonistic activities of the test chemical(s) are tested in test series. A test series consists of a maximum of 6 microtiter plates. Each test series contains at least 1 full series of dilutions of a reference standard, a positive control sample, a negative control sample and solvent controls. Figures 1 and 2 give the plate setup for agonistic and antagonistic tests series.

15. Each dilution of the reference standards, test chemicals, all solvent controls, and positive and negative controls should be analysed in triplicate. Each of the triplicate analyses should fulfil the requirements given in Table 3A and Table 4A.

16. A complete series of dilutions of the reference standard (17β-estradiol for agonism; tamoxifen for antagonism) is measured on the first plate in each test series. To be able to compare the analysis results of the remaining 5 microtiter plates with the first microtiter plate containing the complete concentration-response curve of the reference standard, all plates should contain 3 control samples: solvent control, the highest concentration of the reference standard tested, and the approximate EC_{50} (agonism) or IC_{50} (antagonism) concentration of the reference standard. The ratio of the average control samples on the first plate and the remaining 5 plates should fulfil the requirements as given in Table 3C (agonism) or Table 4C (antagonism).

17. For each of the microtiter plates within a test series, the z-factor is calculated (10). The z-factor should be calculated using the responses at the highest and lowest concentration of the reference standard. A microtiter plate is considered valid in case it fulfils the requirements as stated in Table 3C (agonism) or Table 4C (antagonism).

18. The reference standard 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 standard, should fulfil the requirements as indicated in Table 3C (agonism) or Table 4C (antagonism).

19. Each test series should contain a positive control and negative control sample. The calculated relative induction of both the positive and negative control sample should fulfil the requirements as indicated in Table 3C (agonism) or Table 4C (antagonism).

20. During all measurements, the induction factor of the highest concentration of the reference standard should be measured by dividing the average highest 17β-estradiol reference standard relative light unit (RLU) response by the average reference solvent control RLU response. This induction factor should fulfil the minimum requirements for the fold induction as indicated in Table 3C (agonism) or Table 4C (antagonism).

21. Only microtiter plates that fulfil all above mentioned acceptance criteria are considered valid and can be used to evaluate the response of test chemicals.

22. The acceptance criteria are applicable to both prescreen and comprehensive runs.

Table 1 Concentrations of reference standard, positive control (PC) and negative control (NC) for the agonistic CALUX bioassay

	Substance	CAS RN	Test range (M)
Reference standard	17β-estradiol	50-28-2	$1.0*10^{-13}$ - $1.0*10^{-10}$
Positive control (PC)	17α-methyltestosterone	58-18-4	$3.0*10^{-06}$
Negative control (NC)	corticosterone	50-22-6	$1.0*10^{-08}$

Table 2 Concentrations of reference standard, positive control (PC) and negative control (NC) for the antagonistic CALUX bioassay

	Substance	CAS RN	Test range (M)
Reference standard	tamoxifen	10540-29-1	$3.0*10^{-09}$ - $1.0*10^{-05}$
Positive control (PC)	4-hydroxytamoxifen	68047-06-3	$1.0*10^{-09}$
Negative control (NC)	resveratrol	501-36-0	$1.0*10^{-05}$

Table 3 Acceptance criteria for the agonistic ERα CALUX bioassay.

	A - individual samples on a plate	**Criterium**
1	Maximum %SD of triplicate wells (for NC, PC, each dilution of the test chemical and the reference standard, except C0)	< 15%
2	Maximum %SD of triplicate wells (for reference standard and test chemical solvent controls (C0, SC))	< 30%
3	Maximum LDH leakage, as a measure of cytotoxicity.	< 120%
	B - within a single microtiter plate	
4	Ratio of the reference standard solvent control (C0; plate 1) and test chemical solvent control (SC; plates 2 to x)	0.5 to 2.0
5	Ratio of the appr. EC_{50} and highest reference standard concentrations on plate 1 and the appr. EC_{50} and highest reference standard concentrations on plates 2 to x (C4, C8)	0.70 to 1.30
6	Z-factor for each plate	>0.6
	C - within a single series of analyses (all plates within one series)	
7	Sigmoidal curve of reference standard	Yes (17ß-estradiol)
8	EC_{50} range reference standard 17ß-estradiol	$4*10^{-12}$ – $4*10^{-11}$ M
9	Minimum fold induction of the highest 17ß-estradiol concentration, with respect to the reference standard solvent control.	5
10	Relative induction (%) PC.	> 30%
11	Relative induction (%) NC	<10%

Appr.: approximative; PC: positive control; NC: negative control; SC: test chemical solvent control; C0: reference standard solvent control; SD: standard deviation; LDH: lactate dehydrogenase

Table 4 Acceptance criteria for the antagonistic ERα CALUX bioassay.

	A - individual samples on a plate	**Criterium**
1	Maximum %SD of triplicate wells (for NC, PC, each dilution of the test chemical and the reference standard, solvent control (C0))	< 15%
2	Maximum %SD of triplicate wells (for vehicle control (VC) and highest reference standard concentration (C8))	< 30%
3	Maximum LDH leakage, as a measure of cytotoxicity.	< 120%
	B - within a single microtiter plate	
4	Ratio of the reference standard solvent control (C0; plate 1) and test chemical solvent control (SC; plates 2 to x)	0.70 to 1.30
5	Ratio of the appr. IC_{50} reference standard concentrations on plate 1 and the appr. IC_{50} reference standard concentrations on plates 2 to x (C4)	0.70 to 1.30
6	Ratio of the highest reference standard concentrations on plate 1 and the highest reference standard concentrations on plates 2 to x (C8)	0.50 to 2.0
7	Z-factor for each plate	>0.6
	C - within a single series of analyses (all plates within one series)	
8	Sigmoidal curve of reference standard	Yes (Tamoxifen)
9	IC_{50} range reference standard (Tamoxifen)	$1*10^{-8}$ - $1*10^{-7}$ M
10	Minimum fold induction of the reference standard solvent control, with respect to the highest Tamoxifen concentration.	2.5
11	Relative induction (%) PC.	<70%
12	Relative induction (%) NC	>85%

Appr.: approximative; PC: positive control; NC: negative control; VC: vehicle control (solvent control without fixed concentration of agonist reference standard); SC: test chemical solvent control; C0: reference standard solvent control; SD: standard deviation; LDH: lactate dehydrogenase

Solvent/vehicle control, reference standards, positive controls, negative controls

23. For both the prescreen run and comprehensive runs, the same solvent/vehicle control, reference standards, positive controls and negative controls should be used. In addition, the concentration of reference standards, positive controls and negative controls should be the same.

Solvent control

24. The solvent used to dissolve the test chemicals should be tested as a solvent control. Dimethylsulfoxide (DMSO, 1% (v/v); CASRN 67-68-5) was used as vehicle during the validation of the ERα CALUX bioassay. If a solvent other than DMSO is used, all reference standards, controls, and test chemicals should be tested in the same vehicle. Please note that the solvent control for antagonistic studies contains a fixed concentration of the agonist reference standard 17β-estradiol (approximately EC_{50} concentration). To test the solvent used for antagonistic studies, a vehicle control should be prepared and tested.

Vehicle control (antagonism)

25. For testing antagonism, the assay medium is supplemented with a fixed concentration of the agonist reference standard 17β-estradiol (approximately EC_{50} concentration). To test the solvent used to dissolve the test chemicals for antagonism, an assay medium without a fixed concentration of the agonist reference standard 17β-estradiol should be prepared. This control sample is indicated as the vehicle control. Dimethylsulfoxide (DMSO, 1% (v/v); CASRN 67-68-5) was used as vehicle during the validation of the ERα CALUX bioassay. If a solvent other than DMSO is used, all reference standards, controls, and test chemicals should be tested in the same vehicle.

Reference standards

26. The agonistic reference standard is 17β-estradiol (Table 1). The reference standards comprise a series of dilutions of eight concentrations of 17β-estradiol ($1.0*10^{-13}$, $3.0*10^{-13}$, $1.0*10^{-12}$, $3.0*10^{-12}$, $6.0*10^{-12}$, $1.0*10^{-11}$, $3.0*10^{-11}$, $1.0*10^{-10}$ M).

27. The antagonistic reference standard is tamoxifen (Table 2). The reference standards comprise a series of dilutions of eight concentrations of tamoxifen ($3.0*10^{-09}$, $1.0*10^{-08}$, $3.0*10^{-08}$, $1.0*10^{-07}$, $3.0*10^{-07}$, $1.0*10^{-06}$, $3.0*10^{-06}$, $1.0*10^{-05}$ M). Each of the concentrations of the antagonistic reference standard is co-incubated with a fixed concentration of the agonistic reference standard 17β-estradiol ($3.0*10^{-12}$ M).

Positive control

28. The positive control for agonistic studies is 17α-methyltestosterone (Table 1).

29. The positive control for antagonistic studies is 4-hydroxytamoxifen (Table 2). The antagonistic positive control is co-incubated with a fixed concentration of the agonistic reference standard 17β-estradiol ($3.0*10^{-12}$ M).

Negative control

30. The negative control for agonistic studies is corticosterone (Table 1).

31. The negative control for antagonistic studies is resveratrol (Table 2). The antagonistic negative control is co-incubated with a fixed concentration of the agonistic reference standard 17β-estradiol ($3.0*10^{-12}$ M).

Demonstration of laboratory proficiency (see paragraph 14 and Tables 3 and 4 in **« ER TA TEST METHOD COMPONENTS»** of this Test Guideline (pages 8-15))

Vehicle

32. The solvent used to dissolve test chemicals should solubilise the test chemical completely and should be miscible with the cell medium. DMSO, water and ethanol (95% to 100% purity) are suitable solvents. In case DMSO is used as solvent, the maximum concentration of DMSO during incubation should not exceed 1% (v/v). Prior to use, the solvent should be tested for absence of cytotoxicity and interference with the assays performance.

Preparation of reference standards, positive controls, negative controls and test chemicals

33. Reference standards, 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. Before being dissolved, all substances should be allowed to equilibrate to room temperature. Freshly prepared stock solutions of reference standards, positive controls, negative controls and test chemicals should not have noticeable precipitate or cloudiness. Reference standard and control stocks may be prepared in bulk. Stock solutions of test chemicals should be prepared fresh before each experiment. Final dilutions of reference standards, positive controls, negative controls and test chemicals should be prepared for each experiment fresh and used within 24 hours of preparation.

Solubility, cytotoxicity and range finding.

34. During the prescreen run, the solubility of the test chemicals in the solvent of choice is determined. A maximum stock concentration of 0.1 M is prepared. In case this concentration shows solubility problems, lower stock solutions should be prepared until test chemicals are fully solubilised. During the prescreen run, 1:10 serial dilutions of test chemical are tested. The maximum assay concentration for agonist or antagonist testing is 1 mM. Following prescreening, an appropriate refined concentration range for test chemicals is derived that should be tested during the comprehensive runs. The dilutions used for comprehensive testing should be 1x, 3x, 10x, 30x, 100x, 300x, 1000x and 3000x.

35. Cytotoxicity testing is included in the agonist and antagonist test method protocol (11). Cytotoxicity testing is incorporated in both the prescreen run and comprehensive runs. The method used to assess cytotoxicity during the validation of the ERα CALUX bioassay was the lactate dehydrogenase (LDH) leakage test in combination with qualitative visual inspection of cells (see Appendix 1) following exposure to test chemicals. However, other quantitative methods for the determination of cytotoxicity (e.g. tetrazolium-based colorimetric (MTT) assay or cytotoxicity CALUX bioassay) can be used. In general, test chemical concentrations that show more than 20% reduction of cell viability are considered cytotoxic and therefore cannot be used for data evaluation. With respect to the LDH leakage assay, the concentration of the test chemical is regarded cytotoxic when the percentage LDH leakage is higher than 120%.

Test chemical exposure and assay plate organisation

36. Following trypsination of a confluent flask of cultured cells, cells are re-suspended at $1x10^5$ cells/mL in estrogen free assay medium. Hundred µl of re-suspended cells are plated in the inner-wells of a 96-well microtiter plate. The outer wells are filled with 200 µl of Phosphate Buffered Saline (PBS) (see Figures 1 and 2). The plated cells are pre-incubated for 24 hours in a CO_2 incubator (5% CO_2, 37^0C, 100% humidity).

37. After pre-incubation, the plates are inspected for visual cytotoxicity (see Appendix 1), contamination and confluence. Only plates that show no visual cytotoxicity, contamination and have a minimum of 85% confluence are used for testing. The medium from the inner wells is carefully removed and replaced by 200 µl of estrogen free assay medium containing appropriate dilutions series of reference standards, test chemicals, positive controls, negative controls and solvent controls (Table 5: agonist studies; Table 6: antagonist studies). All reference standards, test chemicals, positive controls, negative controls and solvent controls are tested in triplicate. In Figure 1, the plate layout for agonist testing is given. In Figure 2, the plate layout for antagonist testing is given. The plate layout for prescreen testing and comprehensive testing is identical. For antagonist testing, all inner-wells, except for the vehicle control wells (VC), also contain a fixed concentration of agonist reference standard 17β-estradiol ($3.0*10^{-12}$ M). Note that reference standards C8 and C4 should be added to each TC plate.

38. Following exposure of the cells to all chemicals, the 96-well microtiter plates should be incubated for another 24 hours in a CO_2 incubator (5% CO_2, 37^0C, 100% humidity).

Figure 1 Plate layout of the 96-well microtiter plates for prescreening and assessment of agonistic effect.

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		C0	C1	C2	C3	C4	C5	C6	C7	C8	PC	
C		C0	C1	C2	C3	C4	C5	C6	C7	C8	PC	
D		C0	C1	C2	C3	C4	C5	C6	C7	C8	PC	
E		SC	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	NC	
F		SC	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	NC	
G		SC	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	NC	
H												

Subsequent plates

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		SC	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	C8 (max)	
C		SC	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	C8 (max)	
D		SC	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	C8 (max)	
E		SC	TCx-1	TCx-2	TCx-3	TCx-4	TCx-5	TCx-6	TCx-7	TCx-8	C4 (EC50)	
F		SC	TCx-1	TCx-2	TCx-3	TCx-4	TCx-5	TCx-6	TCx-7	TCx-8	C4 (EC50)	
G		SC	TCx-1	TCx-2	TCx-3	TCx-4	TCx-5	TCx-6	TCx-7	TCx-8	C4 (EC50)	
H												

C0 = reference standard solvent.
C(1-8) = series of dilutions (1-8, low-to-high concentrations) of reference standard.
PC = positive control.
NC = negative control.
TCx-(1-8) = dilutions (1-8, low-to-high concentrations) of test chemical for the prescreen run and assessment of agonistic effect of test chemical x.
SC = solvent control of the test chemical (optimally the same solvent as in C0, but possibly from another batch).
Grey cells: = Outer wells, filled up with 200 µl of PBS.

Figure 2 Plate layout of the 96-well microtiter plates for antagonistic prescreening and assessment of antagonistic effect.

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		C0	C1	C2	C3	C4	C5	C6	C7	C8	VC	
C		C0	C1	C2	C3	C4	C5	C6	C7	C8	VC	
D		C0	C1	C2	C3	C4	C5	C6	C7	C8	VC	
E		NC	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	PC	
F		NC	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	PC	
G		NC	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	PC	
H												

Subsequent plates

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		SC	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	C8 (max)	
C		SC	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	C8 (max)	
D		SC	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	C8 (max)	
E		C4 (IC50)	TCx-1	TCx-2	TCx-3	TCx-4	TCx-5	TCx-6	TCx-7	TCx-8	C8 (max)	
F		C4 (IC50)	TCx-1	TCx-2	TCx-3	TCx-4	TCx-5	TCx-6	TCx-7	TCx-8	C8 (max)	
G		C4 (IC50)	TCx-1	TCx-2	TCx-3	TCx-4	TCx-5	TCx-6	TCx-7	TCx-8	C8 (max)	
H												

C0 = reference standard solvent.
C(1-8) = series of dilutions (1-8, low-to-high concentrations) of reference standard.
NC = negative control.
PC = positive control.
TCx-(1-8) = dilutions (1-8, low-to-high concentrations) of test chemical for the prescreen run and assessment of agonistic effect of test chemical x.
SC = solvent control of the test chemical (optimally the same solvent as in C0, but possibly from another batch).
VC = vehicle control (solvent control without fixed concentration of agonist reference standard 17β-estradiol).
Grey cells: = Outer wells, filled up with 200 µl of PBS.

Note: all inner-wells, except for the vehicle control wells (VC), also contain a fixed concentration of agonist reference standard 17β-estradiol ($3.0*10^{-12}$ M)

Measurement of luminescence

39. The measurement of luminescence is described in detail in the agonist and antagonist test method protocol (10). The medium from the wells should be removed and the cells should be lysed following 24 hours of incubation in order to open up the cell membrane and allow measurement of luciferase activity.

40. For measuring the luminescence, this procedure requires a luminometer equipped with 2 injectors. The luciferase reaction is started by injection of the substrate luciferin. The reaction is stopped by addition of 0.2 M NaOH. The reaction is stopped to prevent carry over of luminescence from one well to the other.

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

Prescreen run

42. The prescreen analysis results are used to determine a refined concentration-range of test chemicals for comprehensive testing. Evaluation of prescreen 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 (10). Here, a brief summary of the procedures for determining the concentration range of test chemicals for agonist and antagonist testing, is given. See Tables 5 and 6 for guidance of serial dilution design.

Selection of concentrations for assessment of agonistic effects

43. During the prescreen run, test chemicals should be tested using the series of dilutions as indicated in Tables 5 (agonism) and 6 (antagonism). All concentrations should be tested in triplicate wells according to the plate layout as indicated in Figure 1 (agonism) or 2 (antagonism).

44. Only analysis results that fulfil the acceptance criteria (Table 3) are considered valid and can be used to evaluate the response of test chemicals. In case one or more microtiter plates in an analysis series fail to fulfil the acceptance criteria, the respective microtiterplates should be re-analysed. In case the first plate containing the complete series of dilutions of the reference standard fails the acceptance criteria, the complete test series (6 plates) have to be re-analysed.

45. Initial concentration ranges of test chemicals should be adjusted and the prescreen run should be repeated in case:

- cytotoxicity is observed. The prescreen procedure should be repeated with lower non-cytotoxic concentrations of the test chemical.
- the prescreen of the test chemical does not show a full dose-response curve because the concentrations tested generate maximum induction. The prescreen run should be repeated using lower concentrations of the test chemical.

46. When a valid dose-related response is observed, the (lowest) concentration at which maximum induction is observed and does not show cytotoxicity, should be selected. The highest concentration of the test chemical to be tested in the comprehensive runs, should be 3-times this selected concentration.

47. A complete refined dilution series of the test chemical should be prepared with dilutions steps as indicated in Table 5, starting with the highest concentration as determined above.

48. A test chemical that does not elicit any agonistic effect, should be tested in the comprehensive runs starting with the highest, non-cytotoxic concentration identified during prescreening.

Selection of concentrations for assessment of antagonistic effects

49. Only analysis results that fulfil the acceptance criteria (Table 4) are considered valid and can be used to evaluate the response of test chemicals. In case one or more microtiter plates in an analysis series fail to fulfil the acceptance criteria, the respective microtiterplates should be re-analysed. In case the first plate containing the complete series of dilutions of the reference standard fails the acceptance criteria, the complete test series (6 plates) have to be re-analysed.

50. Initial concentration ranges of test chemicals should be adjusted and the prescreen run should be repeated in case:

- cytotoxicity is observed. The prescreen procedure should be repeated with lower non-cytotoxic concentrations of the test chemical.
- the prescreen of the test chemical does not show a full dose-response curve because the concentrations tested generate maximum inhibition. The prescreen should be repeated using lower concentrations of the test chemical.

51. When a valid dose-related response is found, the (lowest) concentration at which maximum inhibition is observed and does not show cytotoxicity, should be selected. The highest concentration of the test chemical to be tested in the comprehensive runs, should be 3-times this selected concentration.

52. A complete refined dilution series of the test chemical should be prepared with the dilutions steps as indicated in Table 6, starting with the highest concentration as determined above.

53. Test chemicals that do not elicit any antagonistic effects, should be tested in the comprehensive runs starting with the highest, non-cytotoxic concentration tested during prescreening.

Comprehensive runs

54. Following the selection of the refined concentration ranges, test chemicals should be tested comprehensively using the series of dilutions as indicated in Tables 5 (agonism) and 6 (antagonism). All concentrations should be tested in triplicate wells according to the plate layout as indicated in Figure 1 (agonism) or 2 (antagonism).

55. Only analysis results that fulfil the acceptance criteria (Table 3 and 4) are considered valid and can be used to evaluate the response of test chemicals. In case one or more microtiter plates in an analysis series fail to fulfil the acceptance criteria, the respective microtiterplates should be re-analysed. In case the first plate containing the complete series of dilutions of the reference standard fails the acceptance criteria, the complete test series (6 plates) have to be re-analysed.

Table 5 Concentration and dilutions of reference standards, controls and test chemicals used for agonist testing

Reference 17β-estradiol conc. (M)		TCx - prescreen run dilution		TCx - comprehensive run dilution		Controls conc. (M)	
C0	0	TCx-1	10,000,000 x	TCx-1	3,000 x	PC	$3.0*10^{-06}$
C1	$1.0*10^{-13}$	TCx-2	1,000,000 x	TCx-2	1,000 x	NC	$1.0*10^{-08}$
C2	$3.0*10^{-13}$	TCx-3	100,000 x	TCx-3	300 x	C0	0
C3	$1.0*10^{-12}$	TCx-4	10,000 x	TCx-4	100 x	SC	0
C4	$3.0*10^{-12}$	TCx-5	1,000 x	TCx-5	30 x		
C5	$6.0*10^{-12}$	TCx-6	100 x	TCx-6	10 x		
C6	$1.0*10^{-11}$	TCx-7	10 x	TCx-7	3 x		
C7	$3.0*10^{-11}$	TCx-8	1 x	TCx-8	1 x		
C8	$1.0*10^{-10}$						

TCx - test chemical x
PC - positive control (17α-methyltestosterone)
NC - negative control (corticosterone)
C0 - reference standard solvent control
SC - test chemical solvent control

Table 6 Concentration and dilutions of reference standards, controls and test chemicals used for antagonist testing

Reference tamoxifen conc. (M)		TCx - prescreen run dilution		TCx - comprehensive run dilution		Controls conc. (M)	
C0	0	TCx-1	10,000,000 x	TCx-1	3,000 x	PC	$1.0*10^{-09}$
C1	$3.0*10^{-09}$	TCx-2	1,000,000 x	TCx-2	1,000 x	NC	$1.0*10^{-05}$
C2	$1.0*10^{-08}$	TCx-3	100,000 x	TCx-3	300 x	C0	0
C3	$3.0*10^{-08}$	TCx-4	10,000 x	TCx-4	100 x	SC	0
C4	$1.0*10^{-07}$	TCx-5	1,000 x	TCx-5	30 x		
C5	$3.0*10^{-07}$	TCx-6	100 x	TCx-6	10 x	**Supplemented agonist conc. (M)**	
C6	$1.0*10^{-06}$	TCx-7	10 x	TCx-7	3 x		
C7	$3.0*10^{-06}$	TCx-8	1 x	TCx-8	1 x	17β-estradiol	$3.0*10^{-12}$
C8	$1.0*10^{-05}$						

TCx - test chemical x
PC - positive control (4-hydroxytamoxifen)
NC - negative control (resveratrol)
C0 - reference standard solvent control
SC - test chemical solvent control
VC - vehicle control (does not contain fixed concentration of the agonistic reference standard 17β-estradiol ($3.0*10^{-12}$ M)

Collection of data and data analysis

56. Following the prescreen and comprehensive runs, the EC_{10}, EC_{50}, PC_{10}, PC_{50} and maximum induction (TCx_{max}) of a test chemical should be determined for agonistic testing. For antagonistic testing, the IC_{20}, IC_{50}, PC_{80}, PC_{50} and minimum induction (TCx_{min}) should be calculated. In Figure 3 (agonism) and 4 (antagonism), a graphical representation of these parameters are given. The required parameters are calculated based on the relative induction of each test chemical (relative to the maximum induction of the reference standard (=100%)). Non-linear regression (variable slope, 4 parameters) should be used for evaluation of data according to 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

57. Raw data from the luminometer, expressed as Relative Light Units (RLUs), should be transferred to the data analysis spreadsheet designed for the prescreen and comprehensive runs. Raw data should meet the acceptance criteria as indicated in Table 3A and 3B (agonism) or 4A and 4B (antagonism). In case the raw data meet the acceptance criteria, the following calculation steps are performed to determine the required parameters:

Agonism

- Subtract the average RLU of the reference standard solvent control from each of the raw analysis data of the reference standards.
- Subtract the average RLU for the test chemical solvent control from each of the raw analysis data of the test chemicals.
- Calculate the relative induction of each concentration of the reference standard. Set the induction of the highest concentration of the reference standard at 100%.

- Calculate the relative induction of each concentration of test chemical compared to the highest concentration of the reference standard as 100%.
- Evaluate the analysis results following non-linear regression (variable slope, 4 parameters).
- Determine the EC_{50} and EC_{10} of the reference standard.
- Determine the EC_{50} and EC_{10} of the test chemicals.
- Determine the maximum relative induction of the test chemical (TC_{max}).
- Determine the PC_{10} and PC_{50} of the test chemicals.

For test chemicals, a full dose-response curve may not always be achieved due to e.g. cytotoxicity or solubility problems. Hence, the EC_{50}, EC_{10} and PC_{50} cannot be determined. In such case, only the PC_{10} and TC_{max} can be determined.

Antagonism

- Subtract the average RLU of the highest reference standard concentration from each of the raw analysis data of the reference standard s.
- Subtract the average RLU of the highest reference standard concentration from each of the raw analysis data of the test chemicals.
- Calculate the relative induction of each concentration of the reference standard. Set the induction of the lowest concentration of the reference standard at 100%.
- Calculate the relative induction of each concentration of test chemical compared to the lowest concentration of the reference standard as 100%.
- Evaluate the analysis results following non-linear regression (variable slope, 4 parameters).
- Determine the IC_{50} and IC_{20} of the reference standard.
- Determine the IC_{50} and IC_{20} of the test chemicals.
- Determine the minimum relative induction of the test chemical (TC_{min}).
- Determine the PC_{80} and PC_{50} of the test chemicals.

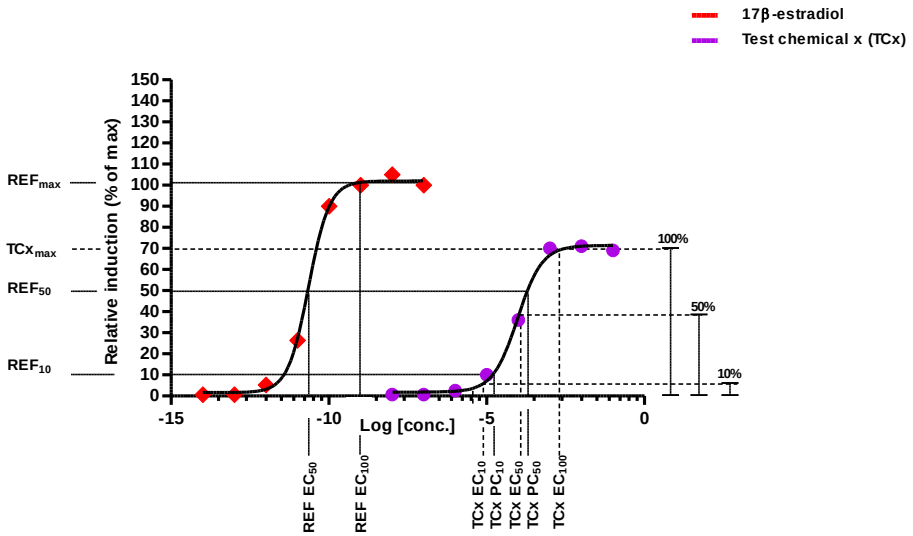

Figure 3 Overview of parameters determined in the agonist assay.

EC_{10}	=	concentration of a substance at which 10% of its maximum response is observed.
EC_{50}	=	concentration of a substance 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 standard.
PC_{50}	=	concentration of a test chemical at which its response is equal to the EC_{50} of the reference standard.
TCx_{max}	=	maximum relative induction of test chemical.

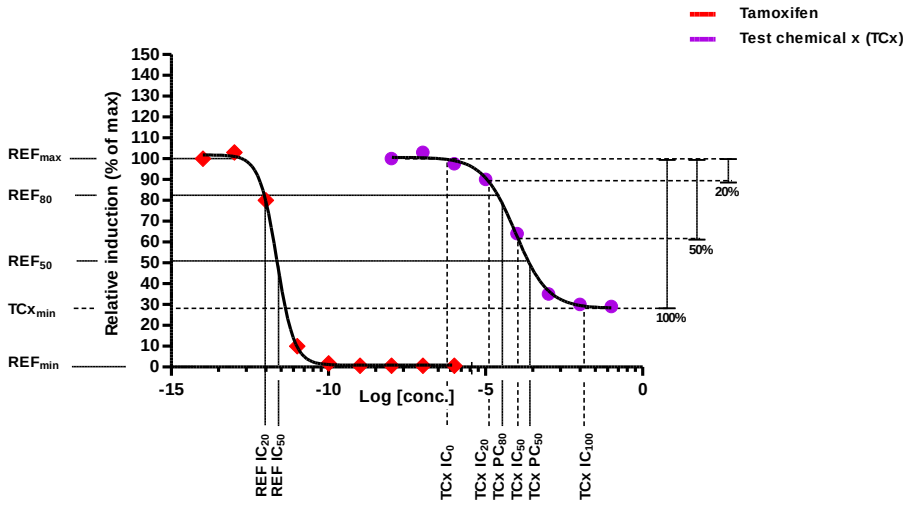

Figure 4 Overview of parameters determined in the antagonist assay.

IC_{20}	=	concentration of a substance at which 80% of its maximum response is observed (20% inhibition).
IC_{50}	=	concentration of a substance 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 standard.
PC_{50}	=	concentration of a test chemical at which its response is equal to the IC_{50} of the reference standard.
TCx_{min}	=	minimum relative induction of test chemical.

For test chemicals, a full dose-response curve may not always be achieved due to e.g. cytotoxicity or solubility problems. Hence, the IC_{50}, IC_{20} and PC_{50} cannot be determined. In such case, only the PC_{20} and TC_{min} can be determined.

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

- Meet the acceptability criteria (see Acceptability criteria paragraphs 14-22),
- Be reproducible.

Data interpretation criteria

59. For the interpretation of data and the decision whether a test chemical is considered positive or negative, the following criteria are to be used:

Agonism

For each comprehensive run, a test chemical is considered **positive** in case:

1 The TC_{max} is equal or exceeds 10% of the maximum response of the reference standard (REF_{10}).
2 At least 2 consecutive concentrations of the test chemical are equal to or exceed the REF_{10}.

For each comprehensive run, a test chemical is considered **negative** in case:
1 The TC_{max} does not exceed 10% of the maximum response of the reference standard (REF_{10}).
2 Less than 2 concentrations of the test chemical are equal to or exceed the REF_{10}.

Antagonism
For each comprehensive run, a test chemical is considered **positive** in case:
1 The TC_{min} is equal or lower than 80% of the maximum response of the reference standard (REF_{80} = 20% inhibition).
2 At least 2 consecutive concentrations of the test chemical are equal to or lower than the REF_{80}.

For each comprehensive run, a test chemical is considered **negative** in case:
1 The TC_{min} exceeds 80% of the maximum response of the reference standard (REF_{80} = 20% inhibition).
2 Less than 2 concentrations of the test chemical are equal to or lower than the REF_{80}.

60. To characterise the potency of the positive response of a test chemical, the magnitude of the effect (agonism: TC_{max}; antagonism: TC_{min}) and the concentration at which the effect occurs (agonism: EC_{10}, EC_{50}, PC_{10}, PC_{50}; antagonism: IC_{20}, IC_{50}, PC_{80}, PC_{50}) should be reported.

TEST REPORT

61. See paragraph 20 of "**ER TA TEST METHOD COMPONENTS**" (Pages 8-14 of this Test Guideline)

LITERATURE (4)

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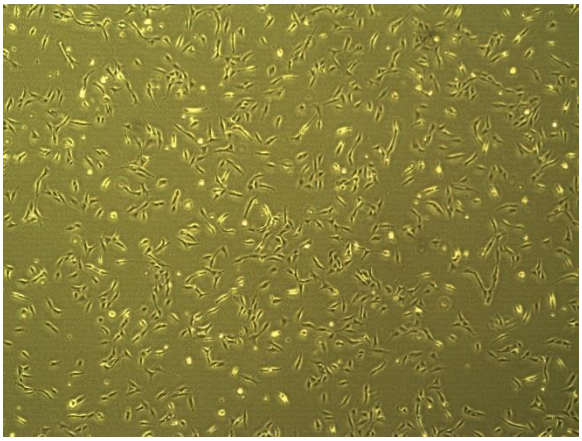
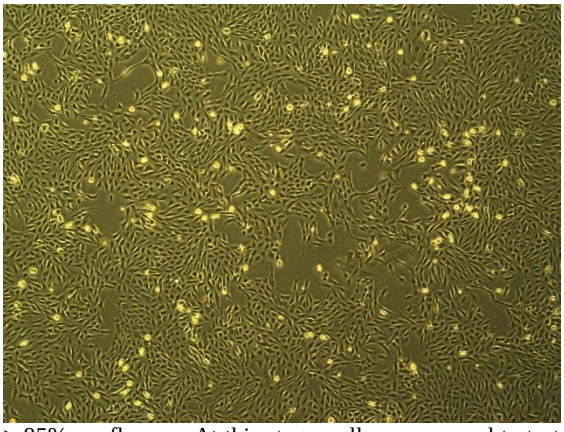
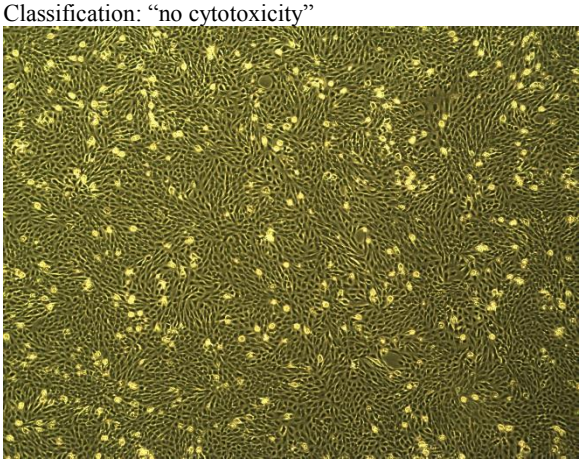
Appendix 1: Visual inspection of cell viability



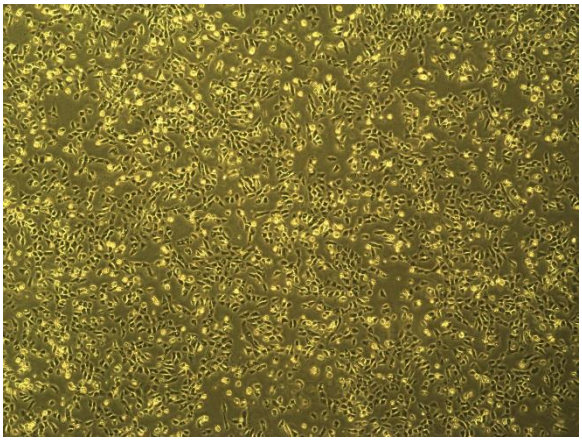
<5% confluency. Cells have just been seeded. 100% cell viability.
Classification: “no cytotoxicity”



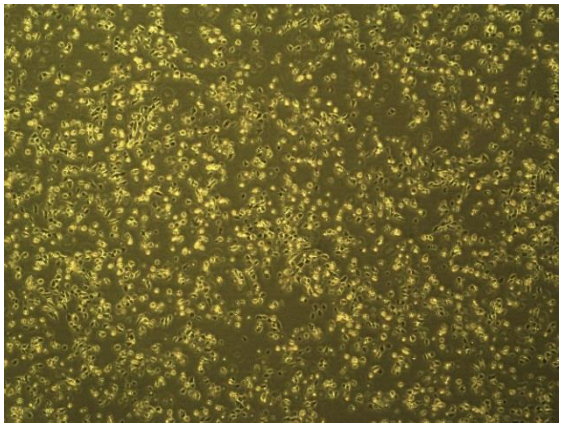
> 85% confluency. At this stage, cells are exposed to test chemicals. > 95% cell viability.
Classification: “no cytotoxicity”



> 95% confluency. Cells are densely packed and start to overgrow. > 95% cell viability.
Classification: “no cytotoxicity”



< 25% cell viability. Cells become detached and contact between cells decreases. Cells are rounded.
Classification: “cytotoxicity



< 5% cell viability. Cells are fully detached and contact between cells is broken. Cells are rounded. Classification: “cytotoxicity”