

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT PROPOSAL FOR AN UPDATED TG 455

Performance-Based Test Guideline for Stably Transfected Transcriptional Activation *In Vitro* Assays to Detect Estrogenic Agonist Activity of Chemicals

GENERAL INTRODUCTION

Performance-Based Test Guideline

1. This Performance-Based Test Guideline (PBTG) comprises several mechanistic and functionally similar test methods for the same hazard, and allows for development of new 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 PBTG provides the framework for testing and test method acceptability criteria, and describes potential limitations of the individual test methods. The primary purpose of the performance standards (2) developed to support this PBTG is to facilitate the validation of similar test methods for the same hazard endpoint and allow for timely amendment of this PBTG so that new similar test methods can be added to an updated PBTG if performance standards are met. The test methods currently covered by the PBTG are included as [Annex 2](#) and [Annex 3](#).

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 2011. The original and revised CFs are included as Annexes in the Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption (3). The revised CF comprises five levels, each level corresponding to a difference level of biological complexity. The Transcriptional Activation (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* Transcriptional Activation (TA) test methods designed to identify oestrogen receptor (ER) agonists.

3. The interaction of estrogens with ERs can affect transcription of estrogen-controlled genes, which could lead to the initiation or inhibition of cellular processes, including those necessary for cell proliferation, normal fetal development, and reproductive function, (4) (5) (6). Perturbation of normal estrogenic systems may have the potential to trigger adverse health effects.

4. *In vitro* TA assays are based upon the production of a reporter gene product induced by a chemical, following binding of the chemical to a specific receptor and subsequent downstream transcriptional activation. Such assays have been used extensively to evaluate gene expression regulated by specific nuclear receptors, such as ERs (7) (8) (9) (10) (11). They have been proposed for the detection of oestrogenic transactivation regulated by the ER (12) (13) (14). There are at least two subtypes of nuclear ERs, termed α and β , which are encoded by distinct genes and with different tissue distributions,

relative ligand binding affinities and biological functions (15) (16) (17) (18) (19) (20) (21). Nuclear ER α mediates the classic estrogenic response (22) (23) (24) (25), and therefore most models currently being developed to measure ER activation are specific to ER α . The assays are used to identify chemicals that activate 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, luciferin is the substrate that is transformed by the luciferase enzyme to a bioluminescent product that can be quantitatively measured with a luminometer. Other examples of common reporters are green fluorescent protein and the LacZ gene, which encodes β -galactosidase. These reporters can be evaluated quickly and inexpensively with commercially available test kits.

5. The reference test methods that provide the basis for this PBTG are the Stably Transfected TA assay (STTA) using the human (h) ER α -*HeLa*-9903 cell line (26) and the BG1Luc ER TA assay (27) using the BG1Luc-4E2 cell line which predominately expresses hER α with some contribution from hER β (28) (29). Validation studies of the STTA and the BG1Luc TA assays have demonstrated their relevance and reliability for their intended purpose (27) (28) (29) (30). Performance standards for luminescence-based ER TA assays using ovarian cells lines are included in ICCVAM Test Method Evaluation Report The LUMI-CELL[®] ER (BG1Luc ER TA) Test Method: An In Vitro Assay for Identifying Human Estrogen Receptor Agonist and Antagonist Activity of Chemicals (8). These performance standards have been modified to be applicable to both the STTA and BG1 methods (2).

Scope and limitations related to the TA assays

6. 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*.

7. 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 of other receptors and enzymatic systems with the endocrine system, (ii) metabolic activation and/or inactivation of hormones, (iii) distribution of hormones to tissues, and (iv) clearance of hormones from the body. None of the test methods under this PBTG addresses these modes of action.

8. This PBTG addresses the ability of chemicals to activate (*i.e.* act as agonists) but not to suppress ligand-activation ER (*i.e.* act as antagonists). Therefore, chemicals that are negative in these test methods should be evaluated in an ER binding assay or in an assay known to detect ER antagonists before concluding that the chemical does not bind to the receptor.

Definitions

9. Definitions and abbreviations used in this Test Guideline are described in [Annex 1](#).

PERFORMANCE RESULTS FROM THE VALIDATION STUDIES OF THE TA ASSAYS

10. The performance of TA assays covered by this PBTG have been assessed by validation studies and the results from these are presented in [Table 1](#).

Table 1. Performance results from the validation studies of the TA assays.

	STTA	BG1Luc (Agonist)
No. of chemicals tested	34¹	34¹
Sensitivity	95%²	100%²
Specificity	100%²	100%²
Accuracy	96%²	100%²
Within-laboratory repeatability		100%
Between-laboratory repeatability	%	78 - 82%

¹34 substances were used during the evaluation of ER agonist accuracy in the performance standards section of the ICCVAM Test Method Evaluation Report: The LUMI-CELL[®] ER (BG1Luc ER TA) Test Method, An In Vitro Assay for Identifying Human Estrogen Receptor Agonist and Antagonist Activity of Chemicals. (8)

²Compared with ICCVAM reference classifications (8)

COMMON ELEMENTS FOR ALL TEST METHODS

Essential Invariable Test Method Components

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

Reference/Control substances

12. The basis for proposed concurrent controls 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 bases for experiment-to-experiment comparisons; they are usually part of the acceptance criteria for a given experiment (1).

Standard Quality Control Procedures

13. Standard quality control procedures should be established to ensure the stability of the cell line through multiple passages, maintain mycoplasma-free, and retain the ability to provide the expected ER-mediated responses over time.

Demonstration of Laboratory Proficiency

14. Prior to testing unknown chemicals with any of the assays under this PBTG, the responsiveness of the test system should be confirmed by each laboratory by independent testing of the proficiency chemicals listed in Table 2. This list is a subset of the Reference Substances listed in the Performance Standards for the ER TA. Testing of these chemicals should be replicated at least twice, on different days. Proficiency is demonstrated by correct classification of each proficiency substance.

Table 2. List of Proficiency Chemicals

Substance Name	CASRN	Expected Response ¹	STTA PC ₅₀ Value (M) ²	Bg1Luc EC ₅₀ Value (M) ³	MeSH Chemical Class ⁴	Product Class ⁵
Ethyl paraben	120-47-8	POS	Not reported	2.48×10^{-5}	Carboxylic Acid, Phenol	Pharmaceutical, Preservative
Kaempferol	520-18-3	POS	Not reported	3.99×10^{-6}	Flavonoid, Heterocyclic Compound	Natural Product
Butylbenzyl phthalate	85-68-7	POS	Not reported	1.98×10^{-6}	Carboxylic Acid, Ester, Phthalic Acid	Plasticizer, Industrial Chemical
Apigenin	520-36-5	POS	Not reported	1.85×10^{-6}	Heterocyclic Compound	Dye, Natural Product, Pharmaceutical Intermediate
Daidzein	486-66-8	POS	1.51×10^{-7}	8.71×10^{-7}	Flavonoid, Heterocyclic Compound	Natural Product
Bisphenol A	80-05-7	POS	1.52×10^{-7}	5.33×10^{-7}	Phenol	Chemical Intermediate, Flame Retardant, Fungicide
Genistein	446-72-0	POS	2.45×10^{-8}	2.71×10^{-7}	Flavonoid, Heterocyclic Compound	Natural Product, Pharmaceutical
Coumestrol	479-13-0	POS	Not reported	8.77×10^{-8}	Heterocyclic Compound	Natural Product
17 α -Estradiol	57-91-0	POS	6.44×10^{-10}	1.54×10^{-9}	Steroid	Pharmaceutical, Veterinary Agent
Estrone	53-16-7	POS	5.88×10^{-10}	2.57×10^{-10}	Steroid	Pharmaceutical, Veterinary Agent
Diethylstilbestrol	56-53-1	POS	Not reported	3.34×10^{-11}	Hydrocarbon (Cyclic)	Pharmaceutical, Veterinary Agent
17 α -Ethinyl estradiol	57-63-6	POS	$>1.0 \times 10^{-11}$	7.31×10^{-12}	Steroid	Pharmaceutical, Veterinary Agent
Atrazine	1912-24-9	NEG	-	-	Heterocyclic Compound	Herbicide
Corticosterone	50-22-6	NEG	-	-	Steroid	Pharmaceutical
Linuron	330-55-2	NEG	-	-	Urea	Herbicide
Spironolactone	52-01-7	NEG	-	-	Lactone, Steroid	Pharmaceutical

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; EC₅₀ – half maximal effective concentration; NEG = negative; PC₅₀ - the concentration of a test chemical which induces a response which is 50% of the maximal positive control response; POS = positive.

¹ICCVAM Test Method Evaluation Report on the LUMI-CELL[®] ER (BG1Luc ER TA) Test Method An In Vitro Method for Identifying ER Agonists and Antagonists (27)

²PC₁₀ 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)

³Mean EC₅₀ values were calculated with values reported by the laboratories of the BG1Luc ER TA validation study (XDS, ECVAM, and Hiyoshi).

⁴Substances were assigned into one or more chemical classes using the U.S. National Library of Medicine's Medical Subject Headings (MeSH), an internationally recognized standardized classification scheme (available at: <http://www.nlm.nih.gov/mesh>).

⁵Substances 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>)

Acceptability Criteria

15. To be acceptable, the following acceptability criteria should be met in all testing:

- The mean reporter activity of the vehicle control should be within 2.5 SD of the mean activity of the historical vehicle control.
- The mean reporter activity of the reference concentration of the E2 should be at least the minimum specified in the test method relative to that of the vehicle control to ensure adequate sensitivity. For the STTA and the BG1Luc test methods, this is 4 times and 3 times, respectively, that of the mean vehicle control on each plate.
- The results of reference substances and controls should be within the acceptable range for the test method.

Analysis of data

16. Each test method should establish a well-defined method for classifying a positive and negative response.

17. Meeting the acceptability criteria (paragraph 15) indicates the assay system is operating properly, but it does not ensure that any particular test will produce accurate data. Replicating the results of the first test is the best indication that accurate data were produced. If two tests give reproducible results (e.g., both test results indicate a substance is positive), it is not necessary to conduct a third test.

18. If two results do not give reproducible results (e.g., a substance is positive in one test and negative in the other test), or if a higher degree of certainty is required regarding the outcome of this assay, at least three independent tests should be conducted.

General Data Interpretation Criteria

19. Where possible, positive results should be characterised by both the magnitude of the effect as compared to the solvent control or reference estrogen and the concentration at which the effect occurs (e.g., an EC₅₀, PC₅₀, % E₂max, etc.).

Test Report

20. The test report should include the following information:

Test method:

- Test method used;

Test substance:

- identification data and Chemical Abstracts Service Registry Number (CAS RN), if known;
- physical nature and purity;
- physicochemical properties relevant to the conduct of the study;
- stability of the test substance.

Solvent/Vehicle:

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

Cells:

- type and source of cells:
 - Is ER endogenously expressed? If not, which receptor(s) were transfected in
 - Reporter construct(s) used
 - Transfection method
 - Selection method for maintenance of stable transfection (where applicable)
- number of cell passages;
- methods for maintenance of cell cultures.

Test conditions:

- solubility limitations
- description of the cytotoxicity assay applied
- composition of media, CO₂ concentration;
- concentration of test substance;
- volume of vehicle and test substance added;
- incubation temperature and humidity;
- duration of treatment;
- cell density at the start of - and during treatment;
- positive and negative reference chemicals;
- duration of treatment period;
- reporter reagents (Product name, supplier and lot);
- criteria for considering tests as positive, negative or equivocal.

Reliability check:

- fold inductions for each assay plate and whether they meet the minimum required by the test method based on historical controls.
- actual log₁₀EC50, log₁₀PC50, and Hillslope values for concurrent positive controls/reference substances;

Results:

- raw and normalised data

- the maximum fold induction level
- cytotoxicity data
- if it exists, the lowest effective concentration (LEC);
- PRCMax, PCMax, PC50 and/or EC50 values, as appropriate;
- concentration-response relationship, where possible;
- statistical analyses, if any, together with a measure of error (*e.g.* SEM, SD, CV or 95% CI) and a description of how these values were obtained.

Discussion of the results

Conclusion

LITERATURE

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ANNEX 1

Definitions and Abbreviations

Acceptance criteria: Minimum standards for the performance of experimental controls and reference standards. All acceptance criteria must be met for an experiment to be considered valid.

Accuracy: (a) The closeness of agreement between a test method result and an accepted reference value.
(b) The proportion of correct outcomes of a test method.

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 17 β -estradiol mediated through estrogen receptors.

BG-1: An immortalized adenocarcinoma cells that endogenously express estrogen receptor.

BG-1Luc4E2: The BG-1Luc4E2 cell line was derived from BG-1 immortalized adenocarcinoma cells that endogenously express both forms of the estrogen receptor (ER α and ER β) 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.

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 cellular morphology.

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

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: 17 β -estradiol

EC₅₀: The half maximal effective concentration of a test substance.

EE: 17 α -ethynyl estradiol

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

hER α : Human estrogen receptor alpha

hER β : Human estrogen receptor beta

ERE: Estrogen response element

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

FBS: Fetal bovine serum

HeLa: An immortal human cervical cell line

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

hER α : Human estrogen receptor alpha

hER β : Human estrogen receptor beta

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

Interlaboratory 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 transferred successfully among laboratories.

Intralaboratory repeatability: The closeness of agreement between test results obtained within a single laboratory when the procedure is performed on the same substance under identical conditions within a given time period.

MT: Metallothionein

MMTV: Mouse Mammary Tumor Virus

OHT: 4-Hydroxytamoxifen

PBTG: Performance-Based Test Guideline.

PC: Positive control (1 nM of E2)

PC10: 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.

PC50: 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.

PCMax: the concentration of a test chemical inducing the RPCMax

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 substances 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.

Proficiency chemicals: A subset of 8 Reference Substances (RS), chosen by the test method developer, to be used to demonstrate the ability of a laboratory to conduct the assay for the first time. This same set of chemicals would be used to develop reliability data for consideration of a new method for acceptance under this PBTG.

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

Reference standard: a reference substance used to demonstrate the adequacy of a test method. 17 β -estradiol is the estrogenic reference standard for the BG1Luc ER TA.

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

Reliability: A measure of the degree to which a test method can be performed reproducibly within and among laboratories

RLU: Relative Light Units

RNA: Ribonucleic Acid

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

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

RT PCR: Real Time polymerase chain reaction

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.

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 attest method.

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: Stably Transfected Transcriptional Activation Assay, the ER α transcriptional activation assay using the HeLA 9903 Cell Line.

TA: Transcriptional activation.

Threshold response: The lowest level of reporter response that is statistically different from that of the concurrent vehicle control (*i.e.* the response that corresponds to the LEC).

Transcription: mRNA synthesis

Transcriptional activation: The initiation of mRNA synthesis in response to a specific chemical signal, such as a binding of an estrogen to the estrogen receptor.

Validated test method: An accepted test method for which validation studies have been completed to determine the accuracy and reliability of the method for a specific proposed use.

Validation, a process based on scientifically sound principles by which the reliability and relevance of a particular test, approach, method, or process are established for a specific purpose. Reliability is defined as the extent of reproducibility of results from a test within and among laboratories over time, when performed using the same standardised protocol. The relevance of a test method describes the relationship between the test and the effect in the target species and whether the test method is meaningful and useful for a defined purpose, with the limitations identified. In brief, it is the extent to which the test method correctly measures or predicts the (biological) effect of interest, as appropriate (16).

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

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

Annex 2

Stably Transfected Human Estrogen Receptor- α Transcriptional Activation Assay for Detection of Estrogenic Agonist-Activity of Chemicals using the hER α -HeLa-9903 cell line

INITIAL CONSIDERATIONS AND LIMITATIONS (See also General Introduction)

1. This transcriptional activation (TA) assay uses the hER α -HeLa-9903 cell line to detect estrogenic agonist activity mediated through human estrogen receptor alpha (hER α). The validation study of the Stably Transfected Transactivation Assay (STTA) by the Japanese Chemicals Evaluation and Research Institute (CERI) using the hER α -HeLa-9903 cell line to detect estrogenic agonist activity mediated through human estrogen receptor alpha (hER α) demonstrated the relevance and reliability of the assay for its intended purpose (1).
2. This test method is specifically designed to detect hER α -mediated TA by measuring chemiluminescence as the endpoint. However, non-receptor-mediated luminescence signals have been reported at phytoestrogen concentrations higher than 1 μ M 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, performance results from the validation of the TA assays and the common elements for all test methods 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)

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 hER α -HeLa-9903 cell line, which is derived from a human cervical tumor, with two stably inserted constructs: (i) the hER α 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 hER α -HeLa-9903 cell line can measure the ability of a test chemical to induce hER α -mediated transactivation of luciferase gene expression.
6. Data interpretation for this assay is based upon whether or not the maximum response level induced by a test chemical equals or exceeds an agonist response equal to 10% of that induced by a

maximally inducing (1 nM) concentration of the positive control (PC) 17 β estradiol (E2) (*i.e.* the PC10). Data analysis and interpretation are discussed in greater detail in paragraphs 30- 40.

PROCEDURE

Cell Lines

7. The stably transfected hER α -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 Bank¹, 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, 17 α -estradiol, 17 α -methyltestosterone, and corticosterone should be used as the reference chemicals and a complete concentration-response curve in the test concentration range provided in Table 1 should be measured at least once each time the assay is performed, and the results should be in agreement with the results provided in Table 1.

Cell Culture and Plating Conditions

10. 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 serum (DCC-FBS), in a CO₂ incubator (5% CO₂) at 37 \pm 1 $^{\circ}$ C. Upon reaching 75-90% confluency, cells can be subcultured at 10 mL of 0.4 x 10⁵ – 1 x 10⁵ 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 10⁴ cells/100 μ L/well. Next, the cells should be pre-incubated in a 5% CO₂ incubator at 37 \pm 1 $^{\circ}$ C for 3 hours before the chemical exposure. The plastic-ware should be free of estrogenic activity.

11. 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 hER α -HeLa-9903 cell line, this will be less than three months.

12. The DCC-FBS can be prepared as described in [Appendix 2](#), or obtained from commercial sources.

Acceptability Criteria

Positive and Negative Reference Chemicals

13. 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 (17 α -estradiol), a very weak agonist (17 α -methyltestosterone) and a negative compound (corticosterone). Acceptable range values derived from the validation study are given in Table 1 (1). These 4 concurrent reference chemicals should be

¹ JCRB Cell Bank : National Institute of Biomedical Innovation, 7-6-8 Asagi Saito, Ibaraki-shi, Osaka 567-0085, Japan Fax: +81-72-641-9812

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 EC50, PC50 and PC10 values, consistent use of materials for cell culturing is essential. The four concurrent reference chemicals, which should be included in each experiment (conducted under the same conditions including the materials, passage level of cells and technicians), can ensure the sensitivity of the assay because the PC10s of the three positive reference chemicals should fall within the acceptable range, as should the PC50s and EC50s where they can be calculated (see Table 1).

Table 1. Acceptable range values of the 4 reference chemicals for the STTA assay (means \pm 2 standard deviations)(SD).

Name	logPC50	logPC10	logEC50	Hill slope	Test range
17 β -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
17 α -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
17 α -Methyltestosterone CAS No: 58-18-4	-6.0 ~ -5.1	-8.0 ~ -6.2	–	–	10 ⁻¹¹ ~ 10 ⁻⁵ M

Positive and Vehicle Controls

14. The positive control (PC) (1 nM of E2) 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.

Fold-induction

15. The mean luciferase activity of the PC (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).

16. With respect to the quality control of the assay, the fold-induction corresponding to the PC10 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 PC10 value can be useful to simplify the data analysis required compared to a statistical analysis. Although a statistical analysis provides information on significance, such an analysis is not a quantitative parameter with respect to concentration-based potential, and so is less useful for prioritisation purposes.

Chemicals to Demonstrate Laboratory Proficiency

Vehicle

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

Preparation of Test Chemicals

18. 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.

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

20. For ER agonists, the presence of increasing levels of cytotoxicity can significantly alter or eliminate the typical sigmoidal response and should be considered when interpreting the data. Cytotoxicity testing methods that can provide information regarding 80% cell viability should be used, utilising an appropriate assay based upon laboratory experience.

21. Should the results of the cytotoxicity test show that the concentration of the test substance has reduced the cell number by 20% or more, this concentration is regarded as cytotoxic, and the concentrations at or above the cytotoxic concentration should be excluded from the evaluation.

Chemical Exposure and Assay Plate Organisation

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

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

Step-2: Chemical dilution: First dilute 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 chemicals can be assigned as shown in Table 3.

Table 3.: Example of plate concentration assignment of the reference chemicals in the assay plate

Row	17 α -Methyltestosterone			Corticosterone			17 α -Estradiol			E2		
	1	2	3	4	5	6	7	8	9	10	11	12
A	conc 1 (10 μ M)	→	→	100 μ M	→	→	1 μ M	→	→	10 nM	→	→
B	conc 2 (1 μ M)	→	→	10 μ M	→	→	100 nM	→	→	1 nM	→	→
C	conc 3 (100 nM)	→	→	1 μ M	→	→	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	→	→	→	→	→

Plate controls = VC: Vehicle control (DMSO); PC: Positive control (1 nM E2)

23. The reference chemicals (E2, 17 α -Estradiol, 17 α -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 chemicals should be tested for each cell source.

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

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 μ M)	→	→	1 mM	→	→	1 μ M	→	→	10 nM	→	→
B	conc 2 (1 μ M)	→	→	100 μ M	→	→	100 nM	→	→	1 nM	→	→
C	conc 3 (100 nM)	→	→	10 μ M	→	→	10 nM	→	→	100 pM	→	→
D	conc 4 (10 nM)	→	→	1 μ M	→	→	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	→	→	→	→	→

24. 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.

25. After adding the chemicals, the assay plates should be incubated in a 5% CO₂ incubator at 37 \pm 1°C for 20-24 hours to induce the reporter gene products.

26. 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.

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

Luciferase assay

28. 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 is met. The assay reagents should be selected based on the sensitivity of the luminometer to be used. When using the standard luciferase assay system, Cell Culture Lysis Reagent (Promega, E1531, or equivalents) should be used before adding the substrate. The luciferase reagent should be applied following the manufacturers' instructions.

ANALYSIS OF DATA

29. 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 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).

EC50, PC50 and PC10 induction considerations

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

31. If the Hill's logistic equation is applicable to the concentration response data, the EC50 should be calculated by the following equation (7):

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{\exp((\log \text{EC50} - X) \times \text{Hill slope}))}$$

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.

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

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

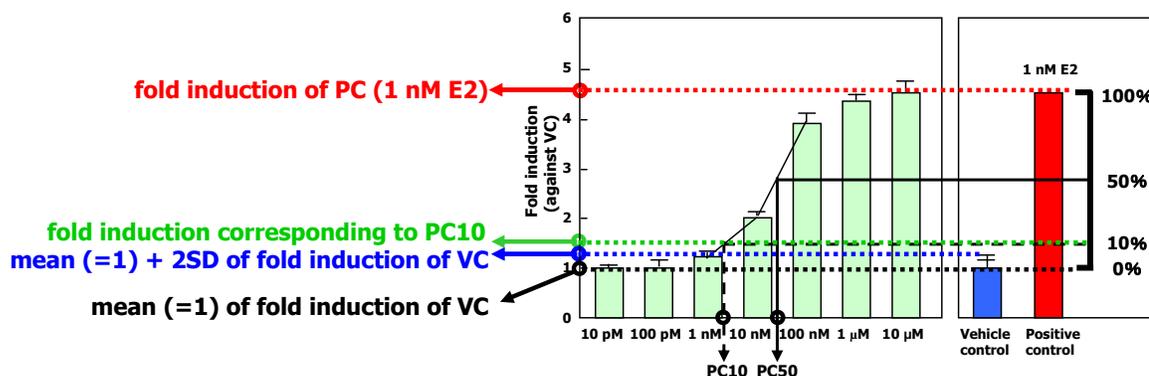
(ii) For positive chemicals, the concentrations that induce the PC10 and, if appropriate, the PC50.

33. 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:

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

34. 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



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

- Meet the performance standard requirements:
 - The mean luciferase activity of the PC (1 nM E2) should be at least 4-fold that of the mean VC on each plate
 - The fold induction corresponding to the PC10 value of the concurrent PC (1 nM E2) should be greater than 1+2SD of the fold induction value (=1) of the VC.
 - The results of 4 reference chemicals should be within the acceptable range (Table 1).
- Be reproducible.

Data Interpretation Criteria

Table 5. : Positive and negative decision criteria

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

36. Data interpretation criteria are shown in Table 5. 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 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.

37. The calculations of PC10, PC50 and PCMax can be made by using a spreadsheet available with the Test Guideline on the OECD public website².

38. It should be sufficient to obtain PC10 or PC50 values at least twice. However, should the resulting base-line for data in the same concentration range show variability with an unacceptably high coefficient of variation (CV; %) the data may not be considered reliable and the source of the high variability should be identified. The CV of the raw data triplicates (*i.e.* luminescence intensity data) of the data points that are used for the calculation of PC10 should be less than 20%.

39. Meeting the acceptability criteria indicates the assay system is operating properly, but it does not ensure that any particular run will produce accurate data. Duplicating the results of the first run is the best insurance that accurate data were produced, see paragraphs 41 and 42.

40. Where more information is required in addition to the screening and prioritisation purposes of this TG for positive test compounds, 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 ER α -specific response, using an ER α antagonist (see Appendix 1).

TEST REPORT

41. See paragraph 19 of the Common Elements to all methods.

² [<http://www.oecd.org/env/testguidelines>]

LITERATURE

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Appendix 1

False positives: Assessment of non-receptor mediated luminescence signals

1. False positives 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 128780 may not be suitable for this purpose as a sufficient concentration of ICI 128780 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 μ M 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, PC10 response the unknown chemical is inhibited equal to or exceeding PC10 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.

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 ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$)
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

BG1Luc Estrogen Receptor Transcriptional Activation Test Method for Identifying Estrogen Receptor Agonists and Antagonists

INITIAL CONSIDERATIONS AND LIMITATIONS (See also General Introduction)

1. This assay uses the BG1Luc4E2 cell line. 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 BG1Luc Estrogen Receptor Transcriptional Activation (ER TA) test method offers the advantage of evaluating both ER α and ER β ligand mediated responses.
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. The demonstrated performance of the BG1Luc ER TA agonist test method suggests that data generated with this test method could be routinely considered for prioritization of substances for further testing.
3. The general introduction, performance results from the validation of the TA assays and the common elements for all test methods 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)

4. 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*) production of luciferase, which can be quantified using a luminometer. Luciferase activity can be quickly and inexpensively evaluated with a number of commercially available kits. The BG1Luc ER TA utilizes an ER responsive human ovarian adenocarcinoma cell line, BG-1, which has been stably transfected with a firefly *luc* reporter construct under control of four estrogen response elements placed upstream of the mouse mammary tumor virus promoter, to detect substances with *in vitro* ER agonist activity. Criteria for data interpretation are described in detail in paragraph 52. Briefly, a positive response is identified by a concentration-response curve containing at least three points with nonoverlapping error bars (mean \pm SD), as well as a change in amplitude of at least 20% of the maximal value for the reference substance (17 β -estradiol [E2; CASRN 50-28-2]).

PROCEDURE

Cell Line

5. BG1Luc4E2 Cells: Human ovarian cancer cell line stably transfected with a plasmid containing an estrogen response element, pGudLuc7.0. The cell line is available with a technical licensing agreement from the University of California, Davis, California, USA, and from Xenobiotic Detection Systems Inc., Durham, North Carolina, USA.

Stability of the Cell Line

6. To maintain the stability and integrity of the cell line, cells should not be cultured for more than 30 passages. For the BG1Luc4E2 cell line, 30 passages will be approximately three months.

Cell Culture and Plating Conditions

7. BG1Luc4E2 cells are based on a continuous ovarian carcinoma cell line (BG-1 cells) that endogenously express ER α and ER β and have been stably transfected with an ER responsive reporter gene (*luc*). Procedures specified in the Guidance on Good Cell Culture Practice (2) (3) 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.

8. 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 37°C \pm 1°C, 90% \pm 5% humidity, and 5.0% \pm 1% CO₂/air.

9. Upon reaching ~80% confluence, BG1Luc4E2 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 substances and analysis of estrogen dependent induction of luciferase activity. The estrogen-free medium (EFM) contains 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. All plasticware should be free of estrogenic activity [see detailed protocol (10)].

Acceptability Criteria

10. 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. Results are compared to quality controls (QC) for these parameters that were derived from the 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.

Range Finder Test

11. Acceptability criteria for the range finder test are as follows:

- 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, and must be greater than three-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

12. It should include acceptability criteria from the 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 must be greater than the DMSO mean plus three times the standard deviation from the DMSO mean.
 - An experiment that fails any single acceptance criterion will be discarded and repeated.

Reference Standards, Positive, and Vehicle Controls

Vehicle Control

13. The vehicle control should be a 1% (v/v) DMSO diluted in EFM.

Reference Standard (Range Finder)

14. The reference standard is E2 and for range finder testing, is comprised of four concentrations of E2 (Table 1), with each concentration tested in duplicate wells.

Table 1 Range Finder Reference Standard Concentrations

	Concentration (µg/mL)	Concentration (M)
1	5.00×10^{-5}	1.84×10^{-10}
2	1.25×10^{-5}	4.59×10^{-11}
3	3.13×10^{-6}	1.15×10^{-11}
4	7.83×10^{-7}	2.87×10^{-12}

Abbreviations: µg/mL = micrograms per milliliter; M = Molar

Reference Standard (Comprehensive)

15. E2 for comprehensive testing is comprised of a serial dilution consisting of 11 concentrations (Table 2) of E2 in duplicate wells.

Table 2 Comprehensive Reference Standard Concentrations

	Concentration (µg/mL)	Concentration (M)
1	1.00×10^{-4}	3.67×10^{-10}
2	5.00×10^{-5}	1.84×10^{-10}
3	2.50×10^{-5}	9.18×10^{-11}
4	1.25×10^{-5}	4.59×10^{-11}
5	6.25×10^{-6}	2.29×10^{-11}
6	3.13×10^{-6}	1.15×10^{-11}
7	1.56×10^{-6}	5.73×10^{-12}

8	7.83×10^{-7}	2.87×10^{-12}
9	3.92×10^{-7}	1.44×10^{-12}
10	1.95×10^{-7}	7.16×10^{-13}
11	9.78×10^{-8}	3.59×10^{-13}

Abbreviations: $\mu\text{g/mL}$ = micrograms per milliliter; M = Molar

Weak Positive Control

16. The weak positive control is 3.13 $\mu\text{g/mL}$ (9.06×10^{-6} M) *p,p'*-methoxychlor (methoxychlor; CASRN 72-43-5) in EFM.

Fold-Induction

17. 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 must be greater than three-fold.

Demonstration of Laboratory Proficiency

18. To demonstrate proficiency with the BG1Luc ER TA test method, a laboratory should compile historical databases with reference standard and control data generated from at least 10 separate experiments, conducted on different days.

19. Once the historical databases are compiled, the agonist and antagonist proficiency substances listed in Table 2 of the Common Elements for all test methods, should be tested. For each test substance, starting concentrations should first be selected based on range finder test results, and then at least two comprehensive tests conducted. Proficiency is demonstrated by correct classification of each proficiency substance. Proficiency testing should be repeated by each technician learning the test methods, but does not have to be repeated by laboratory personnel more than once.

Vehicle

20. The vehicle is DMSO. Test substances are dissolved in 100% DMSO and then diluted to 1% v/v in EFM. 1% v/v DMSO is used as the solvent for the positive and negative controls, and is also used as the concurrent vehicle control.

Preparation of Test Substances

21. The solvent used for dissolution of test substances is 100% DMSO. All test substances should be allowed to equilibrate to room temperature before being dissolved and diluted. Test substance solutions (except for reference standards and controls) should not be prepared in bulk for use in subsequent tests. Test substances are to be used within 24 hours of preparation. Solutions should not have noticeable precipitate or cloudiness.

Solubility and Cytotoxicity: Considerations for Range Finding

22. Range finder testing consists of seven point, 1:10 serial dilutions run in duplicate. Initially, test substances are tested up to the maximum concentration of 1 mg/ml (~1 mM). Range finder experiments are used to determine the following:

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

23. An assessment of cell viability/cytotoxicity is included in the test method protocol (4) and is incorporated into range finder and comprehensive testing. The cytotoxicity method that was used to assess cell viability during the validation of the BG1Luc ER TA (1) was a qualitative visual observation method that assesses viability on a scale of 1 (normal) to 4 (significant loss of viability; [Table 3](#)).

Table 3 Visual Observation Scoring Table for Cell Viability

Viability Score	Brief Description
1	Normal Cell Morphology and Cell Density
2	Altered Cell Morphology and/or Small Gaps between Cells
3	Altered Cell Morphology and/or Large Gaps between Cells
4	Few (or no) Visible Cells
P	Wells containing precipitation are to be noted with “P”

24. Test substance concentrations with a viability score of 2 or greater should be excluded from data evaluation.

25. If desired, a more quantitative method for the determination of cytotoxicity can be used.

Test Substance Exposure and Assay Plate Organization

26. Cells are counted and plated into 96-well tissue culture plates (2 x 10⁵ 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 and incubated for 19-24 hours. Special considerations will need to be applied to those compounds 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 is therefore recommended in such cases.

Range Finder Tests

27. Range finder testing uses all wells of the 96-well plate to test up to six substances as seven point 1:10 serial dilutions in duplicate (see [Figure 1](#)). It uses four concentrations of E2 ([Table 1](#)) in duplicate as the reference standard and four replicate wells for the DMSO control.

Figure 1 Range Finder Test 96-well Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	TS1-1	TS1-1	TS2-1	TS2-1	TS3-1	TS3-1	TS4-1	TS4-1	TS5-1	TS5-1	TS6-1	TS6-1
B	TS1-2	TS1-2	TS2-2	TS2-2	TS3-2	TS3-2	TS4-2	TS4-2	TS5-2	TS5-2	TS6-2	TS6-2
C	TS1-3	TS1-3	TS2-3	TS2-3	TS3-3	TS3-3	TS4-3	TS4-3	TS5-3	TS5-3	TS6-3	TS6-3

D	TS1-4	TS1-4	TS2-4	TS2-4	TS3-4	TS3-4	TS4-4	TS4-4	TS5-4	TS5-4	TS6-4	TS6-4
E	TS1-5	TS1-5	TS2-5	TS2-5	TS3-5	TS3-5	TS4-5	TS4-5	TS5-5	TS5-5	TS6-5	TS6-5
F	TS1-6	TS1-6	TS2-6	TS2-6	TS3-6	TS3-6	TS4-6	TS4-6	TS5-6	TS5-6	TS6-6	TS6-6
G	TS1-7	TS1-7	TS2-7	TS2-7	TS3-7	TS3-7	TS4-7	TS4-7	TS5-7	TS5-7	TS6-7	TS6-7
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); TS1-1 to TS1-7 = concentrations (from high to low) of test substance 1 (TS1); TS2-1 to TS2-7 = concentrations (from high to low) of test substance 2 (TS2); TS3-1 to TS3-7 = concentrations (from high to low) of test substance 3 (TS3); TS4-1 to TS4-7 = concentrations (from high to low) of test substance 4 (TS4); TS5-1 to TS5-7 = concentrations (from high to low) of test substance 5 (TS5); TS6-1 to TS6-7 = concentrations (from high to low) of test substance 2 (TS2); VC = vehicle control (DMSO [1% v/v EFM.]).

28. The recommended final volume of media required for each well is 200 μ L. Only use test plates in which the cells in all wells give a viability score of 1 according to [Table 3](#).

29. To determine starting concentrations for comprehensive testing use the following criteria:

- If results in the range finder test suggest that the test substance is negative (i.e., if there are no points on the test substance concentration curve that are greater than the mean plus three times the standard deviation of the DMSO control, see Figure 12-2 in (4), comprehensive testing will be conducted using an 11-point 1:2 serial dilution starting at the maximum soluble concentration.
- If results in the range finder test suggest that the test substance is negative (i.e., if there are no points on the test substance concentration curve that are greater than the mean plus three times the standard deviation of the DMSO control), and the higher concentrations in the range finder are cytotoxic, comprehensive testing will be conducted using an 11-point 1:2 serial dilution with the lowest cytotoxic concentration as the starting concentration, see Figure 12-3 in (4).
- If results in the range finder test suggest that the test substance is positive (i.e., if there are points on the test substance 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:
 - (i) An 11-point 1:2 serial dilution should be used if the resulting concentration range (note: an 11-point 1:2 serial dilution will cover a range of concentrations over approximately three orders of magnitude [three logs]) will encompass the full range of responses based on the concentration response curve generated in the range finder test (see Figure 12-4 in (4)).
 - (ii) If the concentration range that would be generated with the 1:2 serial dilution will not encompass the full range of responses based on the concentration response curve in the range finder test (see Figures 12-5 and 12-6 in (4)), an 11-point 1:5 serial dilution should be used instead.
- If a substance exhibits a biphasic concentration response curve in the range finder test, both phases should also be resolved in comprehensive testing. In order to resolve both curves, the

starting concentration should be based on the peak associated with the higher concentration and should be one log higher than the concentration giving the highest adjusted RLU value in the range finder. As an example, an 11-point 1:5 serial dilution should be used based on the range finder results presented in Figure 12-7 of (4).

Comprehensive Tests

30. 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 [Figure 2](#)). Comprehensive testing uses 11 concentrations of E2 ([Table 2](#)) in duplicate as the reference standard. Four replicate wells for the DMSO control and three replicate wells for the methoxychlor control (3.13 µg/mL) are included on each plate.

Figure 2 Comprehensive Test 96-well Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	TS1-1	TS1-2	TS1-3	TS1-4	TS1-5	TS1-6	TS1-7	TS1-8	TS1-9	TS1-10	TS1-11	VC
B	TS1-1	TS1-2	TS1-3	TS1-4	TS1-5	TS1-6	TS1-7	TS1-8	TS1-9	TS1-10	TS1-11	VC
C	TS1-1	TS1-2	TS1-3	TS1-4	TS1-5	TS1-6	TS1-7	TS1-8	TS1-9	TS1-10	TS1-11	VC
D	TS2-1	TS2-2	TS2-3	TS2-4	TS2-5	TS2-6	TS2-7	TS2-8	TS2-9	TS2-10	TS2-11	VC
E	TS2-1	TS2-2	TS2-3	TS2-4	TS2-5	TS2-6	TS2-7	TS2-8	TS2-9	TS2-10	TS2-11	Meth
F	TS2-1	TS2-2	TS2-3	TS2-4	TS2-5	TS2-6	TS2-7	TS2-8	TS2-9	TS2-10	TS2-11	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: TS11-1 to TS1-11 = concentrations (from high to low) of test substance 1; TS2-1 to TS2-11 = concentrations (from high to low) of test substance 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

31. 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

32. 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. Light emission from each well is expressed as RLU per well.

ANALYSIS OF DATA

EC₅₀/IC₅₀ Determination

33. The EC₅₀ value (half maximal effective concentration of a test substance is determined from the concentration-response data. For substances that are positive at one or more concentrations, the concentration of test substance that causes a half-maximal response (EC₅₀) is calculated using a Hill

function analysis. The Hill function is a four-parameter logistic mathematical model relating the substance concentration to the response (typically following a sigmoidal curve) using the equation below:

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log \text{IC}_{50} - X) \text{Hillslope}}}$$

where Y = response (i.e., RLUs); X = the logarithm of concentration; Bottom = the minimum response; Top = the maximum response; log IC₅₀ (or log EC₅₀) = 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 EC₅₀ parameter.

Determination of Outliers

34. The Study Director will use good statistical judgment for determining “unusable” wells that will be excluded from the data analysis.

35. 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

36. 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:

- Step 1 Calculate mean value for the DMSO vehicle control (VC).
- Step 2 Subtract the mean value of the DMSO VC from each well value to normalize the data.
- Step 3 Calculate the mean fold induction for the reference standard (E2).
- Step 4 Calculate the mean EC₅₀ value for the test substances.

Collection and Adjustment of Luminometer Data for Comprehensive 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 are performed:

- Step 1 Calculate mean value for the DMSO VC.
- Step 2 Subtract the mean value of the DMSO VC from each well value to normalize the data.
- Step 3 Calculate the mean fold induction for the reference standard (E2).
- Step 4 Calculate the mean EC₅₀ value for E2 and the test substances.
- Step 5 Calculate the mean adjusted RLU value for methoxychlor.

Data Interpretation Criteria

38. The BG1Luc ER TA is intended as part of a weight of evidence approach to help prioritize substances for ED testing *in vivo*. Part of this prioritization procedure will be the classification of the test

substance as positive or negative for ER agonist activity. The classification system used in the BG1Luc ER TA validation study is described in [Table 4](#).

Table 4 Positive and Negative Decision Criteria

Positive	<ul style="list-style-type: none"> • All test substances 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 must contain at least three points with nonoverlapping 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 estrogen (i.e., 2000 RLU when the maximal response value of the reference estrogen is adjusted to 10,000 RLU). • If possible, an EC50 value should be calculated for each positive substance.
Negative	The average adjusted RLU for a given concentration is at or below the mean DMSO control RLU value plus three times its standard deviation.
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 substance is positive or negative.

39. Data interpretation criteria are shown in [Table 4](#). Positive results will be characterized by both the magnitude of the effect and the concentration at which the effect occurs, where possible. The calculations of EC_{50} can be made using a-parameter Hill Function (23). Duplicating the results of the first run is the best assurance that accurate data were produced.

Test Report

40. See paragraph 19 of the Common Elements to all methods.

LITERATURE

- (1) ICCVAM, ICCVAM Test Method Evaluation Report on the LUMI-CELL[®] ER (BG1Luc ER TA) Test Method An *In Vitro* Method for Identifying ER Agonists and Antagonists. 2011, National Institute of Environmental Health Sciences: Research Triangle Park, NC.
- (2) Balls, M., et al., The importance of good cell culture practice (GCCP). ALTEX, 2006. 23(Suppl): p. 270-273.
- (3) Coecke, S., et al., Guidance on good cell culture practice: a report of the Second ECVAM Task Force on good cell culture practice. Alternatives to Laboratory Animals, 2005. 33: p. 261-287.
- (4) NICEATM, 2011, Detailed protocols for the BG1Luc Estrogen Receptor Transcription Activation Assay (agonist and Antagonist)