COMMON ELEMENTS FOR ALL TEST METHODS

Estrogen TA Assay 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 *invariable* test method components.

Control substances

12. The basis for the proposed concurrent reference estrogen and 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 basis for experiment-to-experiment comparisons; they are usually part of the acceptability critera 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, 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 contaminates (e.g. fungi, yeast and viruses).

Demonstratation of Laboratory Proficiency

14. Prior to testing unknown chemicals with any of the test methods under this PBTG, the responsiveness of the test system should be confirmed by each laboratory with independent testing of the 14 proficiency chemicals listed in Table 2. This list is a subset of the Reference Chemicals provided in the Performance Standards for the ER TA (6). These chemicals are commercially available, represent the classes of chemicals commonly associated with ER agonist activity, exhibit a suitable range of potency expected for ER agonists (i.e., strong to weak) and negatives. Testing of these chemicals should be replicated at least twice, on different days. Proficiency is demonstrated by correct classification (positive/negative) of each proficiency chemical. Proficiency testing should be repeated by each technician when learning the test methods.

Table 2: List of (14) Proficiency Chemicals

Chemical Name	CASRN	Expected Response[1]	STTA ER TA			BG1Luc ER TA		MeSH Chemical Class[5]	Product Class[6]
			PC10 Value (M)[2]	PC_{50} Value (M)[2]	Test concentration range (M)	Bg1Luc EC_{50} Value (M)[3]	Highest Concentration for Range Finder (M)[4]		
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
17∝-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
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
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
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
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
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
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
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
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
Atrazine	1912-24-9	NEG	-	-	$10^{-10} - 10^{-4}$	-	4.64×10^{-4}	Heterocyclic Compound	Herbicide
Spironolactone	52-01-7	NEG	-	-	$10^{-11} - 10^{-5}$	-	2.40×10^{-3}	Lactone, Steroid	Pharmaceutical
Ketoconazole	65277-42-1	NEG	-	-	$10^{-11} - 10^{-5}$	-	9.41×10^{-5}	Heterocyclic	Pharmaceutical

								Compound	
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 chemical; NEG = negative; POS = positive; PC_{10} (and PC_{50}) = the concentration of a test chemical 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) (32) as well as empirical data and other information obtained from referenced studies published and reviewed after the completion of the ICCVAM BRDs (3) (18) (30) (31) (32) (33) (34) (35).

[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 BG1Luc ER TA validation study (XDS, ECVAM, and Hiyoshi) (3).

[4]Concentrations reported were the highest concentrations tested (range finder) during the validation of the BG1Luc ER TA. 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 (BG1Luc 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 recognized standardized 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)

Test Run Acceptability Criteria

15. Acceptance or rejection of a test run is based on the evaluation of results obtained for the reference estrogen and controls used for each experiment. Values for the PC_{50} or EC_{50} values for the reference estrogen should meet the acceptable criteria as provided for the selected test method (e.g, STTA (Annex 2) or BG1Luc ER TA (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 estrogen and controls. Standard deviations (SD) or coefficients of variation (CV) for the means of reference estrogen 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 (i.e., efficacy and potency).
- The mean reporter activity of the reference concentration of estrogne 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 BG1Luc 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 chemical and not demonstrate cytotoxicity.

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. There is currently no universally agreed method for interpreting ER-TA data. However, both qualitative (e.g., positive/negative) and/or quantitative (e.g., EC50, PC50) assessments of ER-mediated activity should be based on empirical data and sound scientific judgement. 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}, RPCMax , 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;
  - Species of origin of the receptorReporter 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;
- 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 $\log_{10}EC_{50}$, $\log_{10}PC_{50}$, 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, PC_{50} and/or EC_{50} 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

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ANNEX 1

Definitions and Abbreviations

Acceptability 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 (concordance): (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. 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.

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.

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

BG-1Luc4E2: The BG-1Luc4E2 cell line was derived from BG-1 immortalized 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.

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.

CF: The OECD Conceptual Framework for the Screening and Testing of Endocrine Disrupting Chemicals.

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: 17β-estradiol

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

ED: Endocrine Disruption

EE: 17α-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
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 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 hERα and a luciferase reporter gene have been stably transfected

hERα: Human estrogen receptor alpha

hERß: 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).

IC50: The half maximal effective concentration of an inhibitory test substance.

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

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 reproducibility: 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.

Me-too test: A colloquial expression for a test methods that is structurally and functionally similar to a validated and accepted reference test method. Such a test method would be a candidate for catch-up validation. Interchangeably used with similar test method.

MT: Metallothionein

MMTV: Mouse Mammary Tumor Virus

OHT: 4-Hydroxytamoxifen

PBTG: Performance-Based Test Guideline.

PC: Positive control (1 nM of E2)

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 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 (substances): A subset of the Reference Chemicals included in the Performance Standards that can be used by laboratories to demonstrate technical competence with a standardized 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 chemicals (substances): A set of twenty two chemicals to be used to demonstrate the ability of a new test method to meet the acceptability criteria demonstrated by the validated reference test methods. These chemicals representative the classes of chemicals for which ER agonism is commonly observed, and represents the full range of potencies (e.g., EC_{50}, PC_{50}) that may be expected for ER agonists (e.g, strong to weak) along with negatives.

Reference estrogen (Positive control, PC): The reference estrogen, 17ß-estradiol (E2, CAS 50-28-2). (single concentration for PC10/50 test chemical)

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

Reference test method: 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.

Reliability: Measures 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

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 ERα transcriptional activation assay using the HeLA 9903 Cell Line.

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: 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 solvent 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 chemicals list 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 hERα-HeLa-9903 cell line

INITIAL CONSIDERATIONS AND LIMITATIONS (See also General Introduction)

1. This transcriptional activation (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 Assay (STTA) by the Japanese Chemicals Evaluation and Research Institute (CERI) using the hERα-HeLa-9903 cell line to detect estrogenic agonist 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, 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 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. 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) 17β 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 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[1], 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 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 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/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.

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 hERα-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 (17α-estradiol), a very weak agonist (17α-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 included with each experiment and the results should fall within the given acceptable limits. If this is not the case, the cause for

[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

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 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 PC_{50}s and EC_{50}s where they can be calculated (see Table 1).

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

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	$10^{-14} \sim 10^{-8}$ M
17α-Estradiol CAS No: 57-91-0	-9.6 ~ -8.1	-10.7 ~ -9.3	-9.6 ~ -8.4	0.9 ~ 2.0	$10^{-12} \sim 10^{-6}$ M
Corticosterone CAS No: 50-22-6	–	–	–	–	$10^{-10} \sim 10^{-4}$M
17α-Methyltestosterone CAS No: 58-18-4	-6.0 ~ -5.1	-8.0 ~ -6.2	–	–	$10^{-11} \sim 10^{-5}$ 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	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	→	→	→	→	→

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

23. The reference chemicals (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 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 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	→	→	→	→	→

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% CO_2 incubator at 37±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).

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

30. 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).

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

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

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

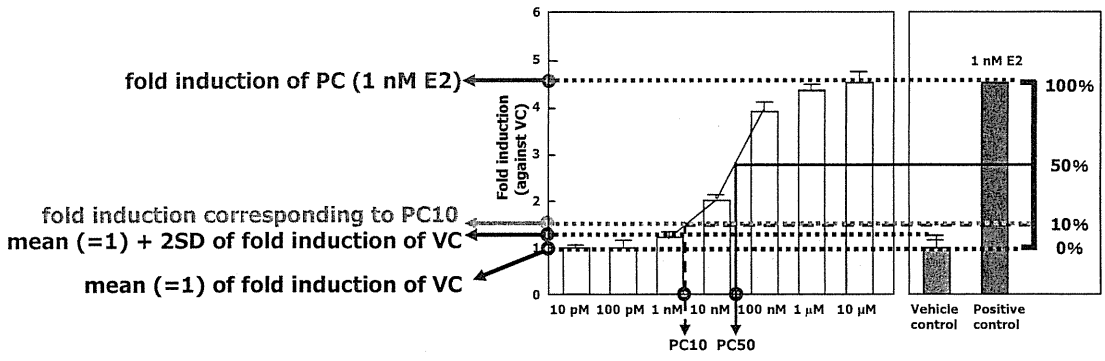
(ii) For positive chemicals, the concentrations that induce the PC10 and, if appropriate, the PC50.

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:

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

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.