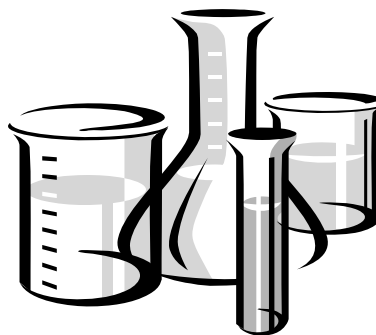


# Endocrine Disruptor Screening Program Test Guidelines

OPPTS 890.1300:  
Estrogen Receptor  
Transcriptional Activation  
(Human Cell Line (HeLa-  
9903))



## NOTICE

This guideline is one of a series of test guidelines established by the Office of Prevention, Pesticides and Toxic Substances (OPPTS), United States Environmental Protection Agency for use in testing pesticides and chemical substances to develop data for submission to the Agency under the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601, *et seq.*), the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*), and section 408 of the Federal Food, Drug and Cosmetic (FFDCA) (21 U.S.C. 346a).

The OPPTS test guidelines serve as a compendium of accepted scientific methodologies and protocols that are intended to provide data to inform regulatory decisions under TSCA, FIFRA, and/or FFDCA. This document provides guidance for conducting the test, and is also used by EPA, the public, and the companies that are subject to data submission requirements under TSCA, FIFRA and/or the FFDCA. As a guidance document, these guidelines are not binding on either EPA or any outside parties, and the EPA may depart from the guidelines where circumstances warrant and without prior notice. The procedures contained in this guideline are strongly recommended for generating the data that are the subject of the guideline, but EPA recognizes that departures may be appropriate in specific situations. You may propose alternatives to the recommendations described in these guidelines, and the Agency will assess them for appropriateness on a case-by-case basis.

For additional information about OPPTS harmonized test guidelines and to access the guidelines electronically, please go to <http://www.epa.gov/oppts> and select "Test Methods & Guidelines" on the left side navigation menu. You may also access the guidelines in <http://www.regulations.gov> grouped by Series under Docket ID #s: EPA-HQ-OPPT-2009-0150 through EPA-HQ-OPPT-2009-0159, and EPA-HQ-OPPT-2009-0576.



## OPPTS 890.1300: Estrogen Receptor Transcriptional Activation (Human Cell Line (HeLa-9903))

### (a) Scope.

- (1) **Applicability.** This guideline is intended to meet testing requirements of the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601, *et seq.*), the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*), and the Federal Food, Drug, and Cosmetic Act (FFDCA) (21 U.S.C. 346a).
- (2) **Background.** The Endocrine Disruptor Screening Program (EDSP) reflects a two-tiered approach to implement the statutory testing requirements of FFDCA section 408(p) (21 U.S.C. 346a). In general, EPA intends to use the data collected under the EDSP, along with other information, to determine if a pesticide chemical, or other substances, may pose a risk to human health or the environment due to disruption of the endocrine system.

This test guideline is intended to be used in conjunction with other guidelines in the OPPTS 890 series that make up the full screening battery under the EDSP to identify substances that have the potential to interact with the estrogen, androgen, or thyroid hormone (Tier 1 “screening”). The determination will be made on a weight-of-evidence basis taking into account data from the Tier 1 assays and other scientifically relevant information available. The fact that a substance may interact with a hormone system, however, does not mean that when the substance is used, it will cause adverse effects in humans or ecological systems.

Chemicals that go through Tier 1 screening and are found to have the potential to interact with the estrogen, androgen, or thyroid hormone systems will proceed to the next stage of the EDSP where EPA will determine which, if any, of the Tier 2 tests are necessary based on the available data. Tier 2 testing is designed to identify any adverse endocrine-related effects caused by the substance, and establish a quantitative relationship between the dose and that endocrine effect.

- (3) **Source.** OPPTS developed this guideline through a process of harmonization with the testing guidance and requirements published by the Organization for Economic Cooperation and Development (OECD) (Ref. 16).
- (b) **Purpose.** *In vitro* transcriptional activation (TA) assays are based upon the production of a reporter gene product induced by a chemical, following binding of the chemical to a specific receptor and subsequent downstream transcriptional activation. TA assays using activation of reporter genes are screening assays

that have long been used to evaluate the specific gene expression regulated by specific nuclear receptors, such as the estrogen receptors (ERs) (**Refs. 3, 4, 5 & 6**). They have been proposed for the detection of estrogenic transactivation regulated by the ER (**Refs. 7, 8, & 9**). The nuclear ERs exist as at least two subtypes, termed  $\alpha$  and  $\beta$ , encoded by distinct genes and with different tissue distribution, relative ligand binding affinities and biological functions. Nuclear ER $\alpha$  mediates the classic estrogenic response, therefore models currently being developed to measure ER activation mainly relate to ER $\alpha$ . The aim of this TA assay is to evaluate the ability of a chemical to function as an ER $\alpha$  ligand and activate an agonist response, for screening and prioritization purposes but can also provide mechanistic information that can be used in a weight of evidence approach. The definitions and abbreviations used in this Test Guidelines are described in Appendix 1.

- (c) **Initial Considerations and Limitations.** Estrogen agonists act as ligands for ERs, and may activate the transcription of estrogen responsive genes. This interaction may have the potential to trigger adverse health effects by disrupting estrogen-regulated systems. This Test Guideline describes an assay that evaluates TA mediated by the hER $\alpha$ . This process is considered to be one of the key mechanisms of possible endocrine disruption related health hazards, although there are also other important endocrine disruption mechanisms. These include:

- Actions mediated via other nuclear receptors linked to the endocrine system and interactions with steroidogenic enzymes
- Metabolic activation or deactivation of hormones
- Distribution of hormones to target tissues
- Clearance of hormones from the body

This Test Guideline exclusively addresses TA of an estrogen-regulated reporter gene by agonist binding to the hER $\alpha$ , and therefore it should not be directly extrapolated to the complex *in vivo* situation of estrogen regulation of cellular processes. Furthermore, this Test Guideline does not address antagonist interaction with the hER $\alpha$  and subsequent effect on transcription.

This test method is specifically designed to detect hER $\alpha$ -mediated TA by measuring chemiluminescence as the endpoint. However, non-receptor-mediated luminescence signals have been reported at phytoestrogen concentrations higher than 1  $\mu$ M due to the over-activation of the luciferase reporter gene (**Refs. 10 & 11**). While the dose response curve indicates that true activation of the ER system occurs at lower concentrations, luciferase expression obtained at high concentrations of phytoestrogens or similar compounds suspected of producing phytoestrogen-like over-activation of the luciferase reporter gene needs to be examined carefully in stably transfected ER TA assay systems (Appendix 2).

- (d) **Principle of the Test.** The TA assay using a reporter gene technique is an *in vitro* tool that provides mechanistic data. The assay is used to signal binding of the 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.

The test system provided in this guideline utilizes the hER $\alpha$ -HeLa-9903 cell line, which is derived from a human cervical tumor, with two stably inserted constructs:

- The hER $\alpha$  expression construct (encoding the full-length human receptor).
- 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 $\alpha$ -HeLa-9903 cell line can measure the ability of a test chemical to induce hER $\alpha$ -mediated transactivation of luciferase gene expression.

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 $\beta$  estradiol (E2) (*i.e.*, the PC10). Data analysis and interpretation are discussed in greater detail in section (f)(1) through (f)(3).

(e) **Procedure.**

- (1) **Cell Line.** Use the stably transfected hER $\alpha$ -HeLa-9903 cell line for the assay. The cell line can be obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank<sup>1</sup>.

Use only cells characterized as mycoplasma-free in testing. RT PCR (Real Time Polymerase Chain Reaction) is the method of choice for a sensitive detection of mycoplasma infection (**Refs. 12, 13 & 14**).

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

- (2) **Stability of the Cell Line.** To monitor the stability of the cell line, use E2, 17 $\alpha$ -estradiol, 17 $\alpha$ -methyltestosterone, and corticosterone as the reference chemicals, and include a complete concentration response curve in the test concentration range provided in Table 1 at least once each time the assay is performed. Comparative results to those provided in Table 1 are recommended.
- (3) **Cell Culture and Plating Conditions.** Maintain cells 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<sub>2</sub> incubator (5% CO<sub>2</sub>) at 37 $\pm$ 1 $^{\circ}$ C. Upon reaching 75-90% confluency, cells can be subcultured at 10 mL of 0.4 x 10<sup>5</sup> – 1 x 10<sup>5</sup> cells/mL for 100 mm cell culture dish. Suspend cells with 10% FBS-EMEM (which is the same as EMEM with DCC-FBS) and then plate into wells of a microplate at a density of 1 x 10<sup>4</sup> cells/100  $\mu$ L/well. Next, pre-incubate the cells in a 5% CO<sub>2</sub> incubator at 37 $^{\circ}$  $\pm$ 1 $^{\circ}$ C for 3 hours before the chemical exposure. Use plastic-ware free of estrogenic activity.

To maintain the integrity of the response, grow the cells for more than one passage from the frozen stock in the conditioned media and do not culture them for more than 40 passages. For the hER $\alpha$ -HeLa-9903 cell line, this will be less than three months.

The DCC-FBS can be prepared as described in Appendix 3, or obtained from commercial sources.

- (4) **Acceptability Criteria.**
- (i) **Positive and Negative Reference Chemicals.** Prior to and during the study, verify the responsiveness of the test system using the appropriate concentrations of a strong estrogen: E2, a weak estrogen (17 $\alpha$ -estradiol), a very weak agonist (17 $\alpha$ -methyltestosterone) and a negative compound (corticosterone). Acceptable range values derived from the validation study are given in Table 1 (**Ref. 2**). Include these 4 concurrent reference chemicals with each experiment. It is recommended that the results fall within the given limits. If this is not the case, it is suggested that the reason for the failure to meet the acceptability criteria be determined (*e.g.*, cell handling, and serum and antibiotics for quality and concentration) and the assay repeated. Obtaining values within the recommended range will help ensure minimum variability of EC<sub>50</sub>, PC<sub>50</sub> and PC<sub>10</sub> values. Consistent use of materials for cell culturing is also essential. The four concurrent reference chemicals, which are included in each experiment (conducted under the same conditions including the materials, passage level of cells and technicians), ensure the sensitivity of the

assay when the PC<sub>10</sub>s of the three positive reference chemicals, and the PC<sub>50</sub>s and EC<sub>50</sub>s (when they can be calculated (see Table 1)) fall within the recommended ranges.

**Table 1.** Acceptable Range Values of the 4 Reference Chemicals for the STTA Assay (means ± 2 standard deviations).

Name	logPC <sub>50</sub>	logPC <sub>10</sub>	logEC <sub>50</sub>	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 <sup>-14</sup> ~ 10 <sup>-8</sup> 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 <sup>-12</sup> ~ 10 <sup>-6</sup> M
Corticosterone CAS No: 50-22-6	–	–	–	–	10 <sup>-10</sup> ~ 10 <sup>-4</sup> M
17α-Methyltestosterone CAS No: 58-18-4	-6.0 ~ -5.1	-8.0 ~ -6.2	–	–	10 <sup>-11</sup> ~ 10 <sup>-5</sup> M

- (ii) **Positive and Vehicle Controls.** Test the positive control (PC) (1 nM of E2) at least in triplicate in each plate. Test the vehicle that is used to dissolve a test chemical as a vehicle control (VC) at least in triplicate in each plate. If the PC uses a different vehicle than the test chemical, include another vehicle control at least in triplicate on the same plate with the PC in addition to this original vehicle control.
- (iii) **Fold-induction.** The target mean luciferase activity of the PC (1 nM E2) is at least 4-fold that of the mean vehicle control 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).

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

- (5) **Chemicals to Demonstrate Laboratory Proficiency.** Prior to testing unknown chemicals in the STTA assay, confirm the responsiveness of the test system by each laboratory, at least once for each newly prepared batch of cell stocks taken from the frozen stock by independent testing of the 10 proficiency chemicals listed in Table 2. Perform this at least in



duplicate, on different days, and compare the results to Table 2. Please justify any deviations.

**Table 2.** List of Proficiency Chemicals.

Compound	CAS No.	Class <sup>2</sup>	Test concentration range	Note
Diethylstilbestrol (DES)	56-53-1	Positive	10 <sup>-14</sup> - 10 <sup>-8</sup> M	
17 $\alpha$ -Ethinyl estradiol (EE)	57-63-6	Positive	10 <sup>-14</sup> - 10 <sup>-8</sup> M	
Hexestrol	84-16-2	Positive	10 <sup>-13</sup> - 10 <sup>-7</sup> M	
Genistein	446-72-0	Positive	10 <sup>-12</sup> - 10 <sup>-5</sup> M	Cytotoxic at (0.01) <sup>4</sup> , 0.1 and 1 mM
Estrone	53-16-7	Positive	10 <sup>-12</sup> - 10 <sup>-6</sup> M	
Butyl paraben	94-26-8	Positive	10 <sup>-11</sup> - 10 <sup>-4</sup> M	Cytotoxic at (0.1) <sup>4</sup> and 1 mM
1,3,5-Tris(4hydroxyphenyl)benzene <sup>1</sup>	15797-52-1	Positive	10 <sup>-12</sup> - 10 <sup>-5</sup> M	Cytotoxic at 100 $\mu$ M. PCmax approx 15% of PC Binds to hER $\alpha$ and has ER antagonist activity
Dibutyl phthalate (DBP)	84-74-2	Negative <sup>3</sup>	10 <sup>-11</sup> - 10 <sup>-4</sup> M	Cytotoxic at 1 mM
Atrazine	1912-24-9	Negative	10 <sup>-11</sup> - 10 <sup>-4</sup> M	Cytotoxic <sup>4</sup> at 1 mM
Corticosterone	50-22-6	Negative	10 <sup>-10</sup> - 10 <sup>-4</sup> M	If not cytotoxic at 1 mM, then that is to be the highest tested concentration

<sup>1</sup>Compound selected to challenge solubility and cytotoxicity.

<sup>2</sup>See Table 5 for definitions of positive and negative.

<sup>3</sup>Negative for ER $\alpha$  mediated transcriptional activation but may not be negative for non-ER $\beta$  mediated transcriptional activation. Thus a positive result in this assay with DBP would indicate that the system is detecting other than pure ER $\alpha$  mediated activity and is therefore unacceptable.

<sup>4</sup>Cytotoxicity is close to 80%.

- (6) **Vehicle.** Use dimethyl sulfoxide (DMSO), or appropriate solvent, at the same concentration used for the different positive and negative controls and the test chemicals as the concurrent vehicle control. Dissolve each test substance 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, do not exceed 0.1% (v/v). For any vehicle, demonstrate that the maximum volume used is not cytotoxic and does not interfere with assay performance.
- (7) **Preparation of Test Chemicals.** Generally, dissolve the test chemicals in DMSO or other suitable solvent, and serially dilute with the same solvent at a common ratio of 1:10 in order to prepare solutions for dilution with media.
- (8) **Solubility and Cytotoxicity: Considerations for Range Finding.** Conduct a preliminary test 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, perform the first definite run for the test chemical at log serial dilutions starting at the maximum acceptable concentration (e.g., 1 mM, 100 µM, 10 µM, etc.). Note the presence of cloudiness, precipitate or cytotoxicity. Adjust concentrations in the second, and if necessary third run as appropriate to better characterize the concentration-response curve and to avoid concentrations which are found to be insoluble or to induce excessive cytotoxicity.

For ER agonists, the presence of increasing levels of cytotoxicity can significantly alter or eliminate the typical sigmoidal response and are a consideration when interpreting the data. Use cytotoxicity testing methods that can provide information regarding 80% cell viability, utilizing an appropriate assay based upon laboratory experience.

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

- (9) **Chemical Exposure and Assay Plate Organization.** The procedure for chemical dilutions (Steps-1 and 2) and exposure to cells (Step-3) can be conducted as follows:

Step 1: Dilute each test chemical by serial dilution in DMSO, or appropriate solvent, and add 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 $\alpha$ -Methyltestosterone			Corticosterone			17 $\alpha$ -Estradiol			E2		
	1	2	3	4	5	6	7	8	9	10	11	12
<b>A</b>	conc 1 (10 $\mu$ M)	→	→	100 $\mu$ M	→	→	1 $\mu$ M	→	→	10 nM	→	→
<b>B</b>	conc 2 (1 $\mu$ M)	→	→	10 $\mu$ M	→	→	100 nM	→	→	1 nM	→	→
<b>C</b>	conc 3 (100 nM)	→	→	1 $\mu$ M	→	→	10 nM	→	→	100 pM	→	→
<b>D</b>	conc 4 (10 nM)	→	→	100 nM	→	→	1 nM	→	→	10 pM	→	→
<b>E</b>	conc 5 (1 nM)	→	→	10 nM	→	→	100 pM	→	→	1 pM	→	→
<b>F</b>	conc 6 (100 pM)	→	→	1 nM	→	→	10 pM	→	→	0.1 pM	→	→
<b>G</b>	conc 7 (10 pM)	→	→	100 pM	→	→	1 pM	→	→	0.01 pM	→	→
<b>H</b>	VC	→	→	→	→	→	PC	→	→	→	→	→

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

Test the reference chemicals (E2, 17 $\alpha$ -Estradiol, 17 $\alpha$ -methyl testosterone and corticosterone) in every run (Table 3). Include 1) PC wells treated with 1 nM of E2 that can produce maximum induction of E2, and 2) VC wells treated with DMSO (or appropriate solvent) alone, 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 test the reference chemicals with 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
<b>A</b>	conc 1 (10 $\mu$ M)	→	→	1 mM	→	→	1 $\mu$ M	→	→	10 nM	→	→
<b>B</b>	conc 2 (1 $\mu$ M)	→	→	100 $\mu$ M	→	→	100 nM	→	→	1 nM	→	→
<b>C</b>	conc 3 (100 nM)	→	→	10 $\mu$ M	→	→	10 nM	→	→	100 pM	→	→
<b>D</b>	conc 4 (10 nM)	→	→	1 $\mu$ M	→	→	1 nM	→	→	10 pM	→	→
<b>E</b>	conc 5 (1 nM)	→	→	100 nM	→	→	100 pM	→	→	1 pM	→	→
<b>F</b>	conc 6 (100 pM)	→	→	10 nM	→	→	10 pM	→	→	0.1 pM	→	→
<b>G</b>	conc 7 (10 pM)	→	→	1 nM	→	→	1 pM	→	→	0.01 pM	→	→
<b>H</b>	VC	→	→	→	→	→	PC	→	→	→	→	→

Confirm the lack of edge effects, as appropriate, and if edge effects are suspected, alter the plate layout to avoid such effects. For example, a plate layout excluding the edge wells can be employed.

After adding the chemicals, incubate the assay plates in a 5% CO<sub>2</sub> incubator at 37 $\pm$ 1 $^{\circ}$ C for 20-24 hours to induce the reporter gene products.

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 it is important they 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.

Conduct repeat definitive tests for the same chemical on different days, to ensure independence.

- (10) **Luciferase assay.** A commercial luciferase assay reagent [*e.g.*, Steady-Glo® Luciferase Assay System (Promega, E2510, or equivalents)] or a standard luciferase assay system (*e.g.*, Promega, E1500, or equivalents) can be used for the assay, as long as the results match the acceptability criteria as defined in this assay. Select the assay reagents based on the sensitivity of the luminometer to be used. When using the standard luciferase assay system, use the Cell Culture Lysis Reagent (*e.g.*, Promega, E1531, or equivalents) before adding the substrate. Follow the manufacturer’s instructions when using the luciferase reagent.
- (f) **Analysis of Data.** To obtain the relative transcriptional activity to PC (1 nM of E2), the luminescence signals from the same plate can be analyzed according to the following steps (other equivalent mathematical processes are also acceptable):
- Step 1: Calculate mean value for the VC.
  - Step 2: Subtract the mean value of the VC from each well value to normalize the data.
  - Step 3: Calculate the mean for the normalized PC.
  - Step 4: Divide the normalized value of each well in the plate by the mean value of the normalized PC (PC=100%). The final value of each well is the relative transcriptional activity for that well compared to the PC response.
  - Step 5: Calculate the mean value of the relative transcriptional activity for each concentration group of the test chemical. There are two dimensions to the response: the averaged transcriptional activity (response) and the concentration at which the response occurs (see following section).
- (1) **Considerations for Induction of EC<sub>50</sub>, PC<sub>50</sub> and PC<sub>10</sub>.** The full concentration response curve is required for the calculation of the EC<sub>50</sub>, 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<sub>50</sub> and maximum induction level (corresponding to the top value of the Hill-equation) are informative parameters, report these parameters where possible. For the calculation

of EC<sub>50</sub> and maximum induction level, use appropriate statistical software (e.g., Graphpad Prism statistical software).

If the Hill's logistic equation is applicable to the concentration response data, calculate the EC<sub>50</sub> by the following equation (**Ref 15**):

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{\exp((\log \text{EC}_{50} - X) \times \text{Hillslope}))}$$

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.

For each test chemical, provide the following:

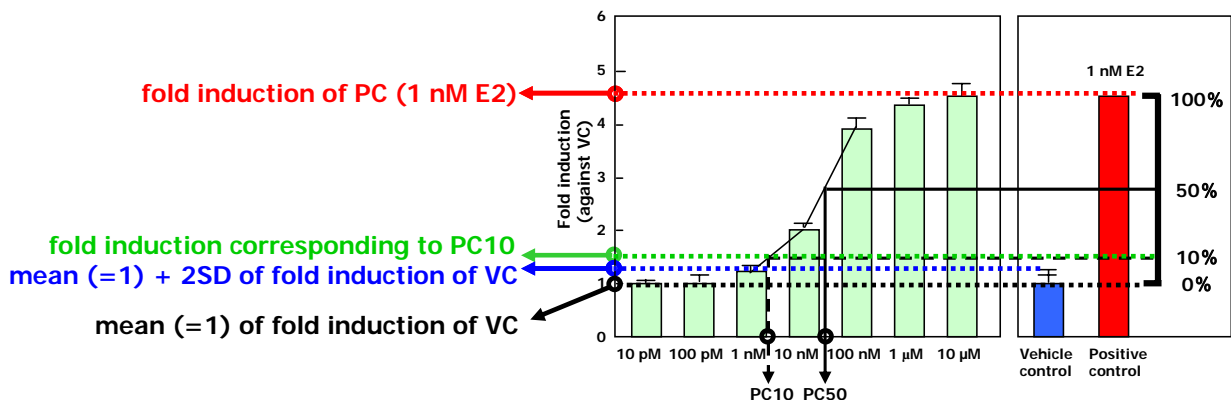
- The RPC<sub>Max</sub> which is the maximum level of response induced by a test chemical, expressed as a percentage of the response induced by 1 nM E2 on the same plate, as well as the PC<sub>Max</sub> (concentration associated with the RPC<sub>Max</sub>).
- For positive chemicals, the concentrations that induce the PC<sub>10</sub> and, if appropriate, the PC<sub>50</sub>.

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

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

Descriptions of PC values are provided in Figure 1 below.

**Figure 1.** Example of How to Derive PC-values. The PC (Positive control; 1 nM of E2) is included on each assay plate.



(2) **Performance Standards.**

To be acceptable, the following are considered:

- The mean luciferase activity of the positive controls (1 nM E2) is at least 4-fold that of the mean vehicle control on each plate.
- The fold induction corresponding to the PC<sub>10</sub> value of the concurrent PC (1 nM E2) is greater than 1+2SD of the fold induction value (=1) of the VC (vehicle control).
- The results of 4 reference chemicals are within the acceptable range (Table 1).
- Be reproducible.

(3) **Data Interpretation Criteria.** Base the results on two (or three) independent runs. If two runs give comparable and therefore reproducible results, it is not necessary to conduct a third run. Data interpretation criteria are shown in Table 5. Positive results will be characterized by both the magnitude of the effect and the concentration at which the effect occurs. Expressing results as a concentration at which a 50% (PC<sub>50</sub>) or 10% (PC<sub>10</sub>) of positive control 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 (RPC<sub>Max</sub>) is equal to or exceeds 10% of the response of the positive control in at least two of two or two of three runs, while a test chemical is considered negative if the RPC<sub>Max</sub> fails to achieve at least 10% of the response of the positive control in two of two or two of three runs.

**Table 5.** Positive and Negative Decision Criteria.

<b>Positive</b>	If the RPC <sub>Max</sub> 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.
<b>Negative</b>	If the RPC <sub>Max</sub> fails to achieve at least 10% of the response of the positive control in two of two or two of three runs.

The EPA intends to provide a calculation spreadsheet with the posting of this guideline on the Agency's Web site (**Ref. 17**) that may be utilized to determine PC<sub>10</sub>, PC<sub>50</sub> and PC<sub>Max</sub>.

Obtaining PC<sub>10</sub> or PC<sub>50</sub> values at least twice is sufficient, unless the resulting base-line for data in the same concentration range shows variability with an unacceptably high coefficient of variation (CV; %). In such a case, the data may not be considered reliable and it is recommended that the source of the high variability be identified. The target CV of the raw data triplicates (i.e. luminescence intensity data) of the data points that are used for the calculation of PC<sub>10</sub> is less than 20%.

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 above).

Where more information is required in addition to the screening and prioritization purposes of this TG for positive test compounds, particularly for PC<sub>10</sub>-PC<sub>49</sub> chemicals, as well as chemicals suspected to over stimulate luciferase, it can be confirmed that the observed luciferase-activity is solely an ER $\alpha$ -specific response, using an ER $\alpha$  antagonist (see Appendix 3).

(g) **Test Report.** Include the following information in the test report:

**Test substance:**

- Identification information (e.g., molecular weight, lot, supplier, expiration date) and CAS Number, if known
- Physical nature and purity
- Physicochemical properties relevant to the conduct of the study
- Stability of the test substance

**Solvent/Vehicle:**

- Characterization (nature, supplier and lot)
- Justification for choice of solvent/vehicle
- Solubility and stability of the test substance in solvent/vehicle, if known

**Cells:**

- Type and source of cells
- Number of cell passages
- Methods for maintenance of cell cultures

**Test conditions:**

- Report cytotoxicity data (and justifications for the method of choice) and solubility limitations, as well as:

- Composition of media, CO<sub>2</sub> concentration
- Concentration of test chemical
- Volume of vehicle and test substance added
- Incubation temperature and humidity
- Duration of treatment
- Cell density during treatment
- Positive and negative reference chemicals
- Duration of treatment period
- Luciferase assay reagents (Product name, supplier and lot)
- Acceptability and data interpretation criteria.

**Reliability check:**

- Fold inductions for each assay plate
- Actual logEC<sub>50</sub>, logPC<sub>50</sub>, logPC<sub>10</sub> and Hill slope values for concurrent reference chemicals

**Results:**

- Raw and normalized data of luminescent signals
- Concentration-response relationship, where possible
- RPC<sub>Max</sub>, P<sub>Max</sub>, PC<sub>50</sub> and/or PC<sub>10</sub> values, as appropriate
- EC<sub>50</sub> values, if appropriate
- 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.**

(h) **References.**

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## Appendix 1

### Definitions and Abbreviations

**Agonist:** A substance that binds to a specific [receptor](#) and triggers a response in the [cell](#). It mimics the action of an [endogenous ligand](#) that binds to the same 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 $\beta$ -estradiol mediated through estrogen receptors.

**CV:** Coefficient of variation

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

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

**DMSO:** Dimethyl sulfoxide

**E2:** 17 $\beta$ -estradiol

**EC<sub>50</sub> value,** the concentration of agonist that provokes a response halfway between the baseline (Bottom) and maximum response (Top).

**EE:** 17 $\alpha$ -ethynyl estradiol

**ER;** Estrogen receptor

**ERE:** Estrogen Response Element

**Estrogenic activity,** the capability of a chemical to mimic 17 $\beta$ -estradiol in its ability to bind to and activate estrogen receptors. hER $\alpha$  mediated specific estrogenic activity can be detected in this Test Guideline.

**FBS:** Fetal bovine serum

**hER $\alpha$ :** Human estrogen receptor alpha

**MT:** Metallothionein

**OHT:** 4-Hydroxytamoxifen

**PC:** Positive control

**PC<sub>10</sub>:** the concentration of a test chemical at which the response in an agonist assay is 10% of the response induced by positive control (E2 at 1nM) in each plate

**PC<sub>50</sub>:** the concentration of a test chemical at which the response in an agonist assay is 50% of the response induced by positive control (E2 at 1nM) in each plate

**PC<sub>Max</sub>:** the concentration of a test chemical inducing the RPCMax

**RPC<sub>Max</sub>**: maximum level of response induced by a test chemical, expressed as a percentage of the response induced by 1 nM E2 on the same plate

**RT PCR**: Real Time polymerase chain reaction

**SD**: Standard deviation

**STTA**: Stably Transfected Transcriptional Activation Assay.

**TA**: Transcriptional activation

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

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

## Appendix 2

### False Positives: Assessment of Non-receptor Mediated Luminescence Signals

#### 1. **False Positives**

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, examine the effect of an ER antagonist (e.g., 4-hydroxytamoxifen (OHT) at non-toxic concentration) on the response. The pure antagonist ICI 128780 may not be suitable for this purpose as a sufficient concentration of ICI 128780 may decrease the vehicle control value, and this will affect the data analysis.

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  $\mu$ M of OHT
- Vehicle Control (VC)(in triplicate)
- OHT (in triplicate)
- 1 nM of E2 (in triplicate) as agonist Positive Control (PC)
- 1 nM of E2 + OHT (in triplicate)

#### 3. **Data Interpretation Criteria**

**Note:** Treat all wells 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 considered as the true response to 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.
- Calculate the mean of the VC.

- Subtract the mean of VC from each well value **not** treated with OHT.
- Calculate the mean of OHT.
- Subtract the mean of the VC from each well value treated with OHT.
- Calculate the mean of the PC.
- Calculate the relative transcriptional activity of all other wells relative to the PC.

## Appendix 3

### Preparation of Serum treated with Dextran Coated Charcoal (DCC)

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

The following materials and equipment will be needed:

##### *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

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

**[Day-1]** Prepare dextran-coated charcoal suspension with 1 litre of ultrapure water containing 1.5 mM of  $\text{MgCl}_2$ , 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 sterilize the supernatant by filtration through 0.2  $\mu\text{m}$  sterile filter. This DCC treated FBS should be stored at -20°C and can be used for up a year.