ESTROGEN RECEPTOR TRANSCRIPTIONAL ACTIVATION (HUMAN CELL LINE – HeLa-9903)

Standard Evaluation Procedure (SEP)

ENDOCRINE DISRUPTOR SCREENING PROGRAM
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DEFINITIONS/ABBREVIATIONS

ER Estrogen Receptor

E2 Estradiol (positive control)

EC_{50} Effective dose of an estrogen receptor agonist that half maximally activates the estrogen receptor

PC_{10} Test chemical concentration eliciting transcriptional activity equivalent to 10% of the positive control value

PC_{50} Test chemical concentration eliciting transcriptional activity equivalent to 50% of the positive control value

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

PC_{max} Concentration associated with the RPC_{max}

PC Positive Control

VC Vehicle control

I. INTRODUCTION

A. Use of the Standard Evaluation Procedure

This Standard Evaluation Procedure (SEP) was developed by the U.S. Environmental Protection Agency (EPA) to provide guidance to EPA staff who will be reviewing the data submitted in response to Tier 1 Orders issued under the Endocrine Disruptor Screening Program (EDSP). The SEPs provide general guidance and are not binding on either EPA or any outside parties. The use of language such as "will," "is," "may," "can" or "should" in these documents does not connote any requirement for either EPA or any outside parties. As such, EPA may depart from the guidance where circumstances warrant and without prior notice. The SEPs are intended to be used in conjunction with the EDSP Test Guideline Series 890 and the Corrections and Clarifications document available on the EDSP web page.

This SEP provides guidance on how to review studies conducted using the OCSPP Guideline 890.1300 for the Estrogen Receptor Transcriptional Activation (Human Cell Line HeLa-9903) that are submitted to support requirements imposed under the EPA's EDSP. The product of the review will be a Data Evaluation Record (DER) that reflects how well the study was performed and conforms to the Guideline; and provides the appropriate conclusions supported by the data. The DER will include, for example, a list of any significant deviations from the protocol as well as their potential impacts, a list of significant information missing from the study report, and any other information about the performance of the study that affects interpretation of the data within the context of the EDSP.

The DER should contain adequate information to provide the EPA with the ability to determine whether the study was performed according to the Guideline. The objective of EDSP Tier 1 assays is to characterize the potential of a chemical to interact with the endocrine system.

The Guideline recommends the critical materials, methods, and analyses that lead to successful performance of the assay. If a particular material, method, or analysis is named in the Guideline, it is usually because other materials, methods, or analyses are either known to be inappropriate—or at least have not been validated—or that there is concern for their potential influence on results. The Agency has posted Corrections and Clarifications on Technical Aspects of the EDSP Tier 1 Assays (OCSPP Test Guideline Series 890) in the docket; the link to this document may be found by way of the EDSP web page (http://www.epa.gov/endo/). It is therefore important to note deviations from specific materials, methods, or analyses in the DER, and provide the Agency's opinion on whether the deviation/deficiency has an impact on the performance and results of the study or the acceptability of the study.

II. ESTROGEN RECEPTOR TRANSCRIPTIONAL ACTIVAITON ASSAY

A. Purpose of the Assay

The estrogen receptor transcriptional activation (ERTA) assay identifies chemicals that bind and activate the estrogen receptor (ER) *in vitro*. Estrogens regulate the expression of specific genes through interactions with the ER. Estrogen diffuses into the target cell and binds to the ER resulting in the dimerization of two estrogen-bound receptors. This ligand-bound estrogen receptor dimer complex can then interact with and activate specific DNA sequences called estrogen responsive elements (ERE) which regulate the transcription of estrogen responsive genes. *In vitro* transcriptional activation (TA) assays mimic this action by using cells that have been specially engineered to contain DNA constructs which contain an ERE promoter linked to a reporter gene, such as luciferase, that produces a gene product that can be easily measured. In some cases (as in the cell line used in this assay) a DNA construct is also introduced into the cell that expresses the ER protein within the cell.

In this assay, estrogen (or an estrogenic test compound) enters the cell binds the ER and activates this signalling pathway leading to the production of a luciferase enzyme. The luciferase product is measured by the addition of the luciferase substrate, luciferin. This causes a light emitting reaction to occur which is quantified as relative light units (RLU) using an instrument called a luminometer. The amount of light emitted is proportional to the potency and/or the concentration of the estrogen (or estrogenic test compound). Consequently this cell line can measure the ability of a test chemical to induce hERα-mediated transactivation of luciferase gene expression. Additionally, use of an immortalized cell line eliminates the use of live animals for this purpose. It should be noted that this particular assay evaluates estrogen agonist activity only.

B. Study Design

Test chemicals are evaluated utilizing a standardized protocol. The assay is performed under standard cell culture conditions in 96-well plates. After allowing the cells to attach for 3 hours, the cells are exposed to multiple concentrations of the test chemical (in triplicate wells) for 20-24 hours. Media is then removed, cells are lysed, luciferin substrate is added and luciferase activity measured using a luminometer.

C. Pre-test Preparation

Prior to conducting the assay, the Test Guideline recommends certain steps (discussed below) be taken to demonstrate that the cell line is properly maintained and that the assay responds appropriately. It is recommended that the laboratory demonstrate proficiency with running the assay.

1. Cell Line and Passages

The Estrogen Receptor Transcriptional Activation Assay uses the stably transfected, human cervical cancer hERα-HeLa-9903 cell line, obtained from the Japanese Collection of Research

Bioresources (JCRB) Cell Bank[1]. The human estrogen receptor-alpha- (hERα-)HeLa-9903 cell line was derived from a human cervical tumor and contains two stably transfected constructs: the hERα expression construct encoding the full-length human receptor; and 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.

Upon reaching 75-90% confluence, the cells can be subcultured. To maintain the integrity of the response, it is recommended that cells be grown for more than one passage from frozen stock before being used in an assay, and not cultured for more than 40 passages.

2. Laboratory Proficiency Test

Before testing unknown chemicals, the guideline recommends that a laboratory demonstrate that it is capable of achieving and maintaining appropriate cell culture and test conditions required for the successful conduct of the assay by successfully performing the laboratory proficiency test described in section (e) 5 of the Test Guideline. As the performance of an assay is directly linked to the laboratory personnel conducting the assay, it is recommended that these procedures be repeated if a change in laboratory personnel occurs.

III. EVALUATION OF STUDY CONDUCT

This section provides a summary description of the information that would generally be expected to be obtained from a study that had been conducted following the recommendations in the Test Guidelines. As described in this section, the DER reviewer is responsible for summarizing how the study was conducted, the extent to which that is consistent with the Guidelines, and how, if at all, that affected the validity of the study. This information will factor into the Agency's interpretations of the data contained in the study report. Specific points that are important for the DER to address are highlighted in the individual sections below, as appropriate.

The summary in this section is offered as a general outline to aid in preparation of the DER. The purpose of this section is not to serve as substitute for the Test Guideline, nor to provide any guidance on how the study should be conducted. Rather, the summary is intended to provide context and examples illustrating to the reviewer what the DER would be expected to contain.

A. Test Compound

EPA recommends that the Estrogen Receptor Transcriptional Activation Assay be performed with the technical form of the chemical intended for commercial use. It is recommended that the specifications of the test material be documented and the CAS number, percent purity, source, and lot number should be clearly indicated in the study report. Additionally, the percent purity should be ≥95% and the identity of any contaminants present at concentrations ≥1% should be reported. It is recommended that this information also be included in the study evaluation for comparison with material utilized in other studies. If a solvent other than DMSO, water, or ethanol is used, the study report will typically include a justification for the choice. Special considerations are generally applicable to highly volatile compounds. In such instances, control

[1] Catalog # JCRB1318.

wells adjacent to wells containing volatile compounds may generate false positive reactions. EPA recommends the use of plate sealers to isolate individual wells when testing of volatile compounds.

B. Cell Culture Conditions

The propagation medium specifically recommended by the protocol is Eagle's Minimum Essential Medium (EMEM) without phenol red, supplemented with 60 mg/L of Kanamycin and 10% dextran-coated-charcoal-treated fetal bovine serum (DCC FBS). The cells are cultured in a 5% CO_2 environment at 37±1ºC. The Guideline recommends that the cells be plated at a density of 1×10^4 cells/100 µL medium/well in a 96-well plate, and pre-incubated for 3 hours prior to exposure. The study report will typically document any deviations from the specified medium or cell culture conditions and provide a justification.

C. Vehicle

The recommended solvents are water, ethanol (95-100% pure) and dimethyl sulfoxide (DMSO). If DMSO is used, it is recommended that the concentration in the test plates not exceed 0.1% (v/v). For any vehicle, it is important to demonstrate that the maximum concentration used is not cytotoxic and does not negatively impact assay performance. The method for determining and reporting cytotoxicity is not specified, but it is generally recommended that the assay chosen provide information regarding a minimum of 80% cell viability. Test chemicals are dissolved in the selected solvent and serially diluted with the same solvent at a 1:10 ratio to prepare solutions for dilution with media.

D. Dose Preparation, Plating and Exposure

The Guideline recommends that each test chemical be serially diluted in the appropriate solvent as described above, and then further diluted by adding 1.5 µL of the test chemical dilution to 500 µL of medium. 50 µL of each new dilution (above) is then added in triplicate to the cells that contain 100 µL media for a total volume of 150 µL per well. This results in a 1:1000 dilution of the original serial dilutions, which would yield a final concentration of 0.1% (v/v) of the solvent. The plates are then incubated for 20-24 hours to allow induction and production of the reporter gene products. Example plate concentration assignments of the reference and test chemicals are presented in Tables 1 and 2.

Table 1. Plate concentration assignment of the reference chemicals and controls

	17α-Methyltestosterone			**Corticosterone**			**17α-Estradiol**			**Estradiol (E2)**		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	10 µM	→	→	100 µM	→	→	1 µM	→	→	10 nM	→	→
B	1 µM	→	→	10 µM	→	→	100 nM	→	→	1 nM	→	→
C	100 nM	→	→	1 µM	→	→	10 nM	→	→	100 pM	→	→
D	10 nM	→	→	100 nM	→	→	1 nM	→	→	10 pM	→	→
E	1 nM	→	→	10 nM	→	→	100 pM	→	→	1 pM	→	→
F	100 pM	→	→	1 nM	→	→	10 pM	→	→	0.1 pM	→	→
G	10 pM	→	→	100 pM	→	→	1 pM	→	→	0.01 pM	→	→
H	VC[a]	→	→	→	→	→	PC[b]	→	→	→	→	→

a VC = vehicle control
b PC = positive control

Table 2. Plate concentration assignment of the test chemicals and controls

	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	→	→	Conc 1	→	→	Conc 1	→	→	Conc 1	→	→
B	Conc 2	→	→	Conc 2	→	→	Conc 2	→	→	Conc 2	→	→
C	Conc 3	→	→	Conc 3	→	→	Conc 3	→	→	Conc 3	→	→
D	Conc 4	→	→	Conc 4	→	→	Conc 4	→	→	Conc 4	→	→
E	Conc 5	→	→	Conc 5	→	→	Conc 5	→	→	Conc 5	→	→
F	Conc 6	→	→	Conc 6	→	→	Conc 6	→	→	Conc 6	→	→
G	Conc 7	→	→	Conc 7	→	→	Conc 7	→	→	Conc 7	→	→
H	VC[a]	→	→	→	→	→	PC[b]	→	→	→	→	→

a VC = vehicle control
b PC = positive control

E. Range-Finding Test and Cell Viability

A preliminary range-finding test is necessary to determine the appropriate concentration range of the chemical to be tested, and to identify potential solubility and cytotoxicity issues. Initially, chemicals are tested up to the maximum concentration of 1 µl/ml, 1 mg/ml or 1 mM, whichever is the highest. Should the results of the cytotoxicity test show that a concentration of the test substance has reduced the cell number by 20% or more, this concentration is typically regarded as being cytotoxic, and the Agency recommends that cytotoxic concentrations be

excluded from evaluation. Based on cytotoxicity or lack of solubility, it is recommended that the first definite run use log serial dilutions starting at the minimum acceptable concentration. Multiple runs may be needed to better characterize the concentration-response curve or to avoid insolubility or excessive cytotoxicity.

F. Luciferase Assay

A commercial luciferase assay [e.g., Steady-Glo® Luciferase Assay System (Cat. # E2510, Promega Corporation, Madison, WI)] or a standard luciferase assay system [e.g., Cat. # E1500, Promega Corp.)] can be used interchangeably for the assay, as long as the results generally match the performance standards as defined in this document. A luminometer that is compatible with the luciferase assay system selected is used to obtain data in terms of relative light units

G. Data Analyses

To obtain the relative transcriptional activity as compared to the positive controls, it is recommended that the luminescence levels in relative light units from the each plate be analyzed by first calculating the mean value for the vehicle, and normalizing each well by the mean vehicle control. The normalized value of each well is then divided by the mean normalized positive control value to obtain the percent of control (PC) values. The mean value of the relative transcriptional activity for each concentration group of the test chemicals is then calculated.

For calculation of EC_{50} and the maximum induction level, it is recommended that an appropriate statistical program (*e.g.*, Graphpad Prism statistical software) be used. If the Hill's logistic equation is applicable to the concentration response data, the EC_{50} can be calculated using the following equation:

$$y = \frac{Bottom + (Top - Bottom)}{\left(1 + 10^{(\log EC_{50} - x) - Hillslope}\right)}$$

where:

- x is the logarithm of the concentration; and
- y is the response, and y starts at the bottom and goes to the top in a sigmoid curve.

Note: bottom is fixed at zero in the Hill's logistic equation.

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

$$\log(PC_x) = \log(c) + \frac{x - d}{d - b}$$

For each chemical the RPC_{max} (maximum level of response induced by a test chemical, expressed as a percentage of the response induced by the positive control on the same plate) and

PC_{max} (test compound concentration that provokes the RPC_{max}) are reported as well as the PC_{10} and PC_{50}.

H. Reporting data

The Guideline recommends that data be provided in tabular format. Data assessment is typically considered valid if two of two or two of three independent runs agree on a response. An experiment (or run) is typically considered independent if it was conducted on a different date and with cells from a different culture plate.

Raw and normalized data of luminescent signals are typically reported. The $EC_{50,}$ RPC_{max}, PC_{max}, PC_{50} and PC_{10} values (with appropriate standard error around each value) should be included in the DER, as appropriate. It is recommended that the data be presented in graphical form to show the full concentration response curve, where appropriate. The full concentration response curve is needed for the calculation of the EC_{50}, but this may not be achievable or practical due to limitations of the test concentration range. However, as the $EC_{50,}$ Hill Slope, and maximum induction level (corresponding to the top level of the Hill equation) are informative, it is recommended that these parameters be reported where possible.

IV. STUDY INTERPRETATION

This section of the DER is intended to address the interpretation of the study results, and any conclusions regarding the acceptability of the study. As part of that, the DER should describe how well the study conforms to the guideline and identify any deviations from the guideline-recommended test method, along with any rationale provided for deviations. This description is intended to clarify the reviewer's conclusions regarding whether, to what extent and how any deviation(s) affect the interpretation or acceptability of the study.

The following sections are based on a summary of the information generally expected to be obtained by a study that was conducted following the Test Guideline, and that would generally be relevant to interpreting the results of the Estrogen Receptor Transcriptional Activation assay. This summary is provided as a general outline to aid the reviewer in preparing a DER, and not as a substitute for the Test Guidelines, nor as guidance on how to conduct the assay.

A. Performance Criteria

1. Laboratory Proficiency Test

As indicated above, before testing unknown substances, a laboratory is typically expected to demonstrate that it is capable of achieving and maintaining appropriate cell culture and test conditions required for the successful conduct of the assay. The guideline recommends the following positive and negative reference chemicals be tested at least once for each newly prepared batch of cell stocks taken from frozen stocks (Table 3). The test is performed at least in

duplicate with each replicate preformed on different days. The guideline recommends that any deviations from the results in Table 3 be justified.

Table 3. Proficiency chemicals

Compound	Concentration range	Class
Diethylstilbestrol	$10^{-14} – 10^{-8}$ M	Positive[a]
17α-Ethynyl estradiol	$10^{-14} – 10^{-8}$ M	Positive
Hexestrol	$10^{-13} – 10^{-7}$ M	Positive
Genistein	$10^{-12} – 10^{-5}$ M	Positive
Estrone	$10^{-12} – 10^{-6}$ M	Positive
Butyl paraben	$10^{-11} – 10^{-4}$ M	Positive
1, 3, 5-Tris(4hydroxyphenyl)benzene	$10^{-12} –10^{-5}$ M	Positive
Dibutyl phthalate	$10^{-11} – 10^{-4}$ M	Negative[b]
Atrazine	$10^{-11} – 10^{-4}$ M	Negative
Corticosterone	$10^{-10} – 10^{-4}$ M	Negative

a Positive: if the RPC_{max} obtained is equal to or exceeds 10% of the response of the positive control in at least 2/2 or 2/3 runs. See page 6 for further explanation.
b Negative: if the RPC_{max} obtained fails to achieve at least 10% of the response of the positive control in at least 2/2 or 2/3 runs. See page 6 for further explanation.

2. Reference Chemicals and Quality Control (QC)

The guideline recommends prior to and concurrent with unknown chemicals, the responsiveness of the test system be tested using the appropriate concentrations of a strong agonist (17β-estradiol; E2), a weak agonist (17α-estradiol), a very weak agonist (17α-methyltestosterone), and a negative compound (corticosterone). It is recommended that these concurrent reference chemicals be included in each experiment to monitor the stability of the cell line, and the results would typically be expected to fall within the following limits (Table 4).

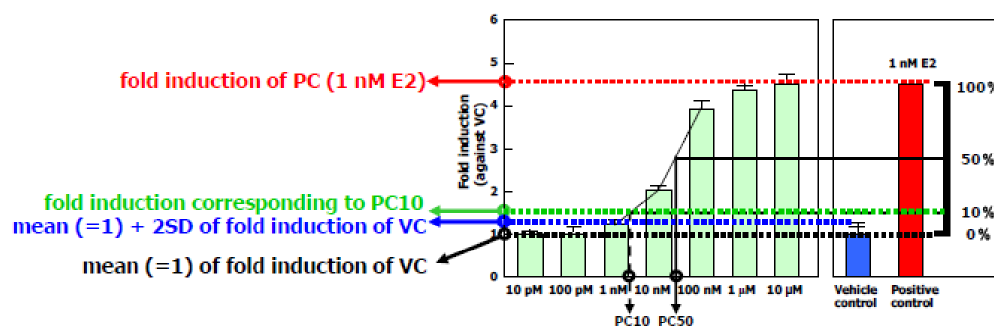
Table 4. Reference Chemicals

Compound	$logPC_{50}$	$logPC_{10}$	$logEC_{50}$	Hill slope	Concentration
17β-estradiol (E2)	-11.4 ~ -10.1	<-11	-11.3 ~ -10.1	0.7 ~ 1.5	$10^{-14} \sim 10^{-8}$ M
17α-estradiol	-9.6 ~ -8.1	-10.7 ~ -9.3	-9.6 ~ -8.4	0.9 ~ 2.0	$10^{-12} \sim 10^{-6}$ M
Corticosterone	–	–	–	–	$10^{-10} \sim 10^{-4}$ M
17α-Methyltestosterone	-6.0 ~ -5.1	-8.0 ~ -6.2	–	–	$10^{-11} \sim 10^{-5}$ M

The positive control (1 nM E2) is tested in at least triplicate on each test plate. A vehicle control is tested at least in triplicate on each plate. The vehicle is the solvent used to dissolve the compound, typically ethanol. In some instances, a test compound might require a different solvent as the vehicle if it does not dissolve in ethanol. If so, then both vehicles are tested on the same plate as described above. If the reference chemicals do not fall within the given limits, the reason for failure is determined and the assay repeated. This will ensure minimum variability of the EC_{50} (test compound concentration that provokes a response midway between the baseline and maximal test compound responses), PC_{10} (test compound concentration that provokes a response equivalent to 10% of the response induced by the positive control), and PC_{50} (test

compound concentration that provokes a response equivalent to 50% of the response induced by the positive control) values. A depiction of the PC values is provided in Figure 1.

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



B. Performance Standards

To be considered an acceptable run, the Agency recommends the following:

(1) The mean relative light units of the positive control be at least 4-fold greater than the mean vehicle control on each plate.

(2) The fold induction corresponding to the PC_{10} value of the concurrent positive control be greater than 1+2 SD of the fold induction value (=1) of the vehicle control.

(3) The results of the reference chemicals are within the acceptable ranges (Table 4).

(4) The results are consistent between the runs.

C. Data Interpretation

Based on the result of two (or three) independent runs, data are defined as being either positive or negative. Positive results will be characterized by both the magnitude of the effect and the concentration at which the effect occurs. Results are expressed as concentrations equivalent to the PC_{50} and PC_{10} values. A test chemical is generally considered to be positive if the RPC_{max} is equal to or exceeds the PC_{10} in at least two of two or two of three runs, while it would generally be considered to be negative if the RPC_{max} fails to achieve at least the PC_{10} value in two of two or two of three runs.

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

V. CHARACTERIZATION OF FINDINGS

The Estrogen Receptor Transcriptional Activation Assay is intended to identify xenobiotics that activate a recombinant estrogen receptor stably expressed within HeLa-9903 cells. This assay exclusively addresses transcriptional activation of an estrogen-regulated reporter gene by agonist binding to the hERα; therefore, it should not be directly extrapolated to the complex *in vivo* situation of estrogen regulation of cellular processes. This assay also does not address antagonist interaction with the hERα and subsequent effect on transcription. It is intended to be used in conjunction with other guidelines in the EDSP Tier 1 battery (890 Guideline Series) to determine on a weight-of-evidence basis if a chemical interacts with components of the endocrine system.