Uterotrophic Assay
OCSPP Guideline 890.1600

Standard Evaluation Procedure (SEP)

ENDOCRINE DISRUPTOR SCREENING PROGRAM
U.S. Environmental Protection Agency
Washington, DC 20460

September 2011

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I. INTRODUCTION

A. Use of the Standard Evaluation Procedure (SEP)

This document was developed by the US 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). This document provides general guidance and is not binding on either EPA or any outside parties. The use of language such as "will," "is," "may," "can" or "should" in this document 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 Standard Evaluation Procedure (SEP) provides guidance on how to review studies conducted using the OCSPP Guideline 890.1600 for the Uterotrophic Assay that are submitted to support requirements imposed under the EDSP. The product of the review will be a Data Evaluation Record (DER) that reflects how well the study conforms to the Guideline, and evaluates how well the study was performed, and provides the appropriate conclusions supported by the data. The DER would typically be expected to 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. THE UTEROTROPHIC ASSAY

A. Purpose of the Assay

The Uterotrophic Assay is an *in vivo* screen that evaluates the ability of a test chemical to elicit biological activities consistent with agonists of estrogens (e.g., 17β-estradiol). This bioassay for estrogenicity is intended to be included in a battery of *in vitro* and *in vivo* tests to identify substances with the potential to interact with the endocrine system.

B. Background

Endocrine disrupting xenobiotics have the potential to interact with hormone systems. Possible mechanisms for these effects include hormone receptor binding, enzyme inhibition, interference in steroidogenesis, and *in vivo* effects on growth and development of reproductive and accessory sexual organs. Therefore, the EDSP has developed 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 substance may pose a potential hazard to human health or the environment due to disruption of the endocrine system.

Estradiol (17β-estradiol) is an endogenous hormone that stimulates cell division and growth of several tissues in female mammals (OECD, 2003). In particular, the reproductive tract tissues (e.g., uterus, cervix, and vagina) go through an estrous cycle of growth and regression. The duration of the entire cycle is relatively short in rats and mice, comprising only 4 to 5 days. The estrous cycle is controlled by the hypothalamic-pituitary-gonadal (HPG) axis. The hypothalamus secretes gonadotropin-releasing hormone (GnRH), which causes the anterior pituitary to secrete follicle-stimulating hormone (FSH) and luteinizing hormone (LH) into the peripheral circulation. FSH and LH regulate cyclic changes in the ovary, whereas cyclic changes in the uterus and vagina are dependent on ovarian steroids (Yuan and Foley, 2002). FSH stimulates the proliferation of granulosa cells and induces receptors for LH on these cells. LH stimulates thecal cells to secrete the androgen, androstenedione. During diestrus, FSH regulates ovarian follicle maturation and stimulates the follicular granulosa cells to produce aromatase, which converts androgens into 17β-estradiol. 17β-estradiol, released both into the follicle and into the peripheral circulation, then promotes cell proliferation and maturation in the uterus, cervix, and vagina. The primary site of 17β-estradiol synthesis is the ovary, although several tissues appear to produce low levels of 17β-estradiol that may act locally (e.g., bone and the male reproductive tract). The circulating 17β-estradiol also acts via a feedback mechanism with the HPG axis to maintain homeostasis.

The 17β-estradiol binds to the estrogen receptor (ER) as an agonist ligand (OECD, 2003). After binding, the active ligand-receptor complex binds to DNA sequences (i.e., response elements) specific for the ER, and up-regulates or down-regulates the transcription of specific genes. The end result of gene transcription modulation is a biological response of the target tissue, such as uterine cell division and growth, in response to 17β-estradiol. Receptor-mediated processes are also susceptible to inhibition by receptor antagonists. These

antagonists exhibit high affinity for the ER and competitively inhibit the binding of the native 17β-estradiol ligand and its subsequent biological activity.

The use of the increase in uterine weight as an endpoint encompasses earlier changes such as sequences of molecular, cellular, and tissue events in the uterus. Early changes include increased electrolytes and water imbibition; this increased fluid intake is one of the contributors to increased uterine weight. Additionally, the uterus responds to 17β-estradiol by increased cell division as part of uterine growth. The peak of mitotic division occurs approximately 24 hours after 17β-estradiol dose administration. The mitotic response is greatest in the uterine epithelium, followed by the stroma, and the myometrium. In addition to increased cell division, the morphology of uterine cells is transformed in response to 17β-estradiol. These morphological changes include increased epithelial cell height, epithelial gland cell height, and stromal thickness; and differentiation and extension of the luminal epithelium into a columnar shape.

During the normal estrous cycle, increased 17β-estradiol levels typically result in cell division and tissue growth of the uterus and vagina within 2 days. Administration of chemicals for 3 days would then exceed the natural response time for the growth phase of the uterus to endogenous estrogen. Therefore, the uterus appears to be an ideal tissue for detecting estrogenic effects because: its natural response time is relatively short (~2 days); its weight increase is easily measured; its sensitivity can be increased in the absence of endogenous estrogens (low baseline weight); and the magnitude of its potential response is at least several-fold. The Uterotrophic Assay uses either sexually mature ovariectomized female rats or sexually immature, intact female rats to determine the effect of exposure to potential estrogen agonists and antagonists on the weight of the uterus.

C. Study Design

The Uterotrophic Assay employs an animal test system in which the hypothalamic-pituitary-ovarian axis is not functional, which leads to low levels of endogenous circulating estrogen and concomitant low baseline uterine weights with minimal variation. This maximizes the response to administered 17β-estradiol. The non-functional HPG axis is achieved by using one of two versions of the assay. The first version uses young adult females after ovariectomy with adequate time for uterine tissues to regress. The second version uses immature females after weaning and prior to puberty. The EPA test guideline OCSPP 890.1600 is based directly on the OECD Test Guideline 440 for the Uterotrophic Assay (OECD 2007a). The only exception is that the EPA guideline states a preference for the adult ovariectomized version under EDSP.

The test substance is administered daily for a minimum administration period of three consecutive days. Although oral gavage may be considered, subcutaneous injection (s.c.) is preferred for this assay. Injection (s.c.) results in higher bioavailability than oral gavage due to bypass of absorption from the gut and first-pass metabolism. An additional advantage is that some information on relative bioavailability compared to the oral route can be gleaned when comparing to the rat pubertal assay which employs oral gavage. The animals are necropsied approximately 24 hours after the last dose, and the uterus is weighed. Six animals are included

in each test and control group. This guideline recommends screening for both estrogen agonist and antagonist activity.

For assays testing suspected estrogen agonists (U.S. EPA, 2009; OCSPP Guideline 890.1600), the assay is comprised of at least two treated dose groups (low and high), a vehicle control group, and a positive control group, EE. The uterine weights of the animals from the treated groups are compared to the vehicle control group, and a statistically significant increase indicates a positive response. The purpose of the reference estrogen agonist, EE, in this assay is to act as a positive control to verify that the model is working (i.e. appropriate uterine weight increase is observed).

The OECD provides an additional source of separate guidance for anti-estrogenic effects (OECD, 2007b). The anti-estrogen assay is comprised of at least three treated dose groups (low, mid, or high dose) concomitant with a constant dose of EE. Two other groups, a vehicle control group and an estrogenic control group are included. The vehicle control group establishes the baseline uterus weight. The estrogenic control group receives EE at 0.3 μg/kg/day by s.c. injection or 1.0 μg/kg/day if administration is by oral gavage. The purpose of the EE group is to verify an increase in uterus weight above vehicle control group. The uterine weights of the animals from the treated groups (test substance and EE) are compared to the estrogenic (EE) control group, and a statistically significant decrease indicates an anti-estrogenic response.

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 Guideline. 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 Guideline, 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 for the reviewer of what content would normally be expected in the DER.

A. Test Compound

The purity of the test compound used in the study, its source, Lot No. and/or Batch No. are reported in the DER along with data on the certificate of analyses. The stability of the test chemical in the vehicle is also reported in the DER for concentrations used in the study; the storage temperatures used for the stability analyses are specified. For test substances dosed as suspensions, the homogeneity of the test chemical suspensions is reported in the DER. The

DER should provide the range of values expressed as percent of nominal for the analysis of test chemical solutions/suspensions.

B. Controls and Vehicle

The recommended reference estrogen agonist is 17α-ethynyl estradiol (EE), CAS No 57-63-6. It is administered at a dose of 0.3 µg/kg/day by s.c. injection or 1.0 µg/kg/day if administration by oral gavage. When conducting the anti-estrogenicity component of the assay, it is not necessary to include a positive control antagonist. Similarly, a negative control for estrogenicity is not required for the Uterotrophic Assay. Information on the supplier, batch number, and purity of all reference materials should be reported. Additionally, information on any vehicle (identity, justification for selection, supplier, and lot number) should be included in the methods section of the DER.

C. Dose Formulations

An aqueous solution/suspension should be considered as the preferred solvent for preparation of the dose formulations. However, many estrogen ligands are hydrophobic, necessitating solution/suspension in oil (e.g. corn, peanut, sesame or olive oil). The vehicle choice and justification is provided in the DER. Test substances can be dissolved in a minimal amount of 95% ethanol or other appropriate solvent and diluted to final working concentrations in the test vehicle. The toxic characteristics of the solvent are typically tested by including a solvent-only control group. Information regarding the dose formulation preparation, storage, and analyses (for stability, homogeneity, and concentration) is included in the appropriate section of the DER.

D. Test Animals

The rat has been routinely used in versions of the Uterotrophic Assay since the 1930s, and this species was validated by the OECD. Sprague-Dawley and Wistar strains were used during the validation studies conducted by the OECD and are therefore recommended. A "bridging" study was conducted in mice, and results of the Uterotrophic Assay indicated similar responses between the species, both qualitatively and quantitatively (OECD, 2006d). Therefore, mice may be used instead of rats in some cases; however, a rationale for this choice should be provided, based on toxicology, pharmacokinetic, or other considerations. For all studies, the DER includes: the species and strain selected and the rationale for this choice; the source and supplier of the animals; and the number and age of the animals.

E. Animal Husbandry

The guideline recommends all procedures for animal care and use be documented and in compliance with regulatory standards. The recommended environmental conditions for the study are: temperature 22 ± 3ºC; relative humidity 30-70% (preferably 50-60%); and a 12-hour light/dark cycle. Housing may be individual for young adult females or in groups of up to three per cage. Group housing is preferred for immature animals. The guideline recommends bedding material contain a minimal amount of phytoestrogens; and corn cob bedding be

avoided, as it affects the estrous cycle in rats via an apparent anti-estrogenic mechanism (U.S. EPA, 2009).

Laboratory diet and water are provided *ad libitum*. The guideline recommends phytoestrogen levels in the diet be reported because phytoestrogens in laboratory diets, primarily from soy and alfalfa products, can increase uterine weights in rodents to an extent high enough to interfere with the Uterotrophic Assay. The guideline recommends diets low in phytoestrogens (<350 μg of genistein equivalents/gram diet for immature female Sprague Dawley and Wistar rats or <175 μg /gram diet for ovariectomized adult mice). Additionally, several semi-synthetic diets are available which are phytoestrogen-free, replacing soy and alfalfa with casein and vegetable oil. The laboratory diet name, source, batch number, and phytoestrogen content are reported in the DER.

The Corrections and Clarifications on Technical Aspects of the EDSP Tier 1 Assays (OCSPP Test Guideline Series 890) states that tap water is too variable and may contain potentially endocrine active substances. As such, tap water is generally not a recommended source of drinking water. Acceptable sources of drinking water typically include deionized, double-distilled water and charcoal-filtered water. Other sources may also be acceptable; however, the presence of soluble organic chemical contaminants such as natural and artificial hormones in drinking water has the potential to introduce variability into (and potentially compromise) the results (e.g. result in false negatives or false positives). Consequently, if an alternative source of water has been used, EPA recommends that the laboratory document that such contaminants have been removed from the drinking water.

F. Dose Selection

Previously conducted toxicity studies can be utilized to determine the appropriate doses of the test substance. However, a dose range-finding study may be necessary to aid in the dose-selection for the agonist component of the assay.

For the agonist component where two dose levels are recommended, the guideline recommends that the highest dose level be at or just below the Maximum Tolerated Dose (MTD) but need not exceed the Limit Dose (1000 mg/kg/day). However, the Agency typically also considers the toxicity profile of the chemical (i.e., cholinesterase inhibition, target organ toxicity, etc) when considering dose selection. The second dose level is spaced to produce a lesser degree of toxicity relative to the high dose unless justification is provided for testing at a different level. The DER contains the rationale for the selection of doses.

A similar logic applies to dose selection for the antagonist component where three dose levels are recommended. The highest dose level and the intermediate dose levels are spaced to produce a gradation of toxic effects and the lowest dose level typically produces no evidence of toxicity.

G. Duration and Timing of Dose Administration

Dosage duration will depend upon the animal model used. The guideline recommends three consecutive days of treatment for immature females or three to seven days for

ovariectomized adult female rats. For mice, three days is an acceptable duration for strong estrogen agonists. Animals are treated at the same time each day since necropsy occurs within 24 hours of the last dose.

H. Assay Procedures

1. Intact, Immature Female Version

For the Uterotrophic Assay with immature rats, the day of birth is stated in the DER. The day of birth is critical to provide maximum sensitivity and responsiveness to exogenous estrogens between post-natal day (PND) 18 and 25. Initial high circulating levels of α-fetoprotein (AFP) bind 17β-estradiol specifically and with high affinity, thereby reducing free 17β-estradiol and preventing its effect on uterine growth. However, AFP declines rapidly after birth, and rats become responsive to natural estrogens after PND 16-17. Therefore, dosing of immature, intact rats would be expected to begin on PND 18 and be completed by PND 21 or at the very latest by PND 25. After this age, the HPG axis becomes functional and endogenous estrogen levels may cause increases in baseline uterine weight and increases in variability (both of which reduce assay sensitivity). In the mouse, this window of sensitivity is from PND 16-22 (OECD, 2003). The DER typically states if animals were born in-house or shipped with the dam prior to weaning. If the latter, information on the acclimation period prior to dose initiation is included. The Reviewer should include information regarding co-housing of litters with dams, age at receipt, duration of acclimation, and age at initiation and termination of dosing in the appropriate sections of the DER.

2. Ovariectomized, Adult Female Version

An acclimation period of approximately five days after receipt is typically considered appropriate for young adult animals. The duration of acclimation and the age at ovariectomy and initiation of dosing should typically be included in the DER in the appropriate methods section. For the Uterotrophic Assay with ovariectomized adult rats or mice, ovariectomy is performed when the animals are between 6 and 8 weeks of age. For rats, 14 days is the minimum time allowed between ovariectomy and the first day of administration of the test material in order to allow the uterus to regress to a minimum, stable baseline. For mice, this time period is at least 7 days. The investigators examine epithelial cells swabbed from the vagina on at least five consecutive days (e.g., days 10-14 after ovariectomy for rats) to verify removal of all ovarian tissue that may otherwise produce significant circulating levels of estrogens. The Agency recommends a brief description of the ovariectomy procedure be included in the study report. The Reviewers should confirm that the procedure was conducted according to standard protocols, as depicted in Figure 1 of the U.S. EPA Guideline OCSPP 890.1600. It is not generally necessary to include details of the ovariectomy procedure, unless there are deviations from the standard procedure or specific situations such as concerns about quality assurance or inadequate removal of all ovarian tissue.

3. Animal Assignment

Animals at the extremes of the body weight range are excluded and the remaining animals randomly assigned to the treatment groups, stratified by body weight, so that there are

no statistically significant differences in initial mean body weight for each group. At the commencement of the study, the coefficient of variation for initial body weights typically is within 20% of the mean group weight. The Reviewer should typically include information regarding animal assignment in the methods section of the DER. Differences in body weights, whether treatment-related or not, can impact the assessment of the assay to a greater extent in the immature model. In the maturing rat, uterine weight is related to body weight because it is under the influence of growth factors prior to the effects of estrogen at the onset of puberty. However, in the adult ovariectomized females, body weight differences are less confounding because uterine weight is affected almost exclusively by hormone influence.

4. Dose Administration

The Reviewer should include a description of the dose administration for the test formulations, vehicle control, and positive/estrogenic control (EE) in the DER. This information includes the route of administration and its rationale, dose schedule, and dose volume. The route of administration for the test substance may be via s.c. injection or oral gavage, with the Agency preferring s.c. injection for this assay. Considerations during selection of the route of dose administration include: animal welfare; physical/chemical properties; relevance to expected route of exposure for humans; and existing toxicology data on metabolism and kinetics. Immature or ovariectomized adult rats are dosed for three consecutive days at approximately 24-hour intervals, with the dose level adjusted daily based on the concurrent body weight measurement. For adult ovariectomized mice, a seven-day administration period is required to detect weak estrogen agonists, and this extended dose administration period is acceptable (but not mandatory) for ovariectomized adult rats. For both s.c. injection and oral gavage, administration is via a single daily dose; the dose volume does not exceed 5 mL/kg body weight, except when using an aqueous solution for which 10 mL/kg body weight is acceptable. Specifically, for s.c. injection, doses are administered to the dorsoscapular or lumbar regions, divided into two injection sites.

In assays for anti-estrogenicity, the test substance and the reference estrogen are normally given within 15 minutes. For oral route, to avoid direct mixing of the test substance with the reference estrogen in the body, the reference estrogen is administered via s.c. injection. For the subcutaneous route, the test substance is typically injected on one side of the dorsum and the reference estrogen on the other side of the dorsum of the animal. It is recommended that the application volumes for both not exceed the guidelines for testing estrogen agonists.

5. Treatment Groups

A description of the study design, including treatment groups, dose levels, and number of animals should generally be included in the methods section of the DER. For the estrogen agonist assay, the treatment groups include positive control (EE), vehicle control, and the test substance groups. The recommended groups for the anti-estrogenic assay include the vehicle control, estrogenic control (EE), and test substance + EE. In addition to the control groups (vehicle and/or solvent), a minimum of two treatment groups are typically needed in order to detect an estrogenic effect or three treatment groups in order to detect an anti-estrogenic response.

6. Observations

Clinical observations are normally conducted at least once daily for mortality and signs of toxicity. If any clinical signs of toxicity were reported, the nature, incidence, severity and duration are included in the DER along with an assessment of whether the findings were considered adverse and related to treatment. If significant clinical signs of toxicity were noted in a dose-dependent manner, then the severity of the toxicity are taken into consideration with the results of the assay. Additionally, it is possible to observe clinical signs and/or mortality that are deemed unrelated to treatment due to a lack of dose-relationship and/or an undetermined cause of death. However, incidences of mortality and moribundity reduce sample size which results in a loss of power to detect statistical differences. Therefore, a lower sample size could result in false negatives. Body weights are obtained daily to determine dose volume and effects of treatment on the animals. Food consumption measurements (on a cage basis) are optional and are expressed as g/animal/day.

7. Necropsy and Uterus Weight

If immature animals are used, the animals are examined for vaginal opening prior to dissection. In either version of the assay (immature or young adult), the rats are euthanized and necropsied according to standard laboratory procedures approximately 24 hours after the final administration of the test substance. The recommended procedure for removal and handling of the uterus for weighing is detailed in Figure 2 in EPA Guideline OCSPP 890.1600 (U.S. EPA, 2009) and OECD Test Guideline 440 (OECD, 2007a). (For additional guidance refer to Korea FDA, 1999).

If immature animals are used, the ovaries are removed at the oviduct, avoiding loss of luminal fluid from the uterine horns. If animals are ovariectomized, the stubs are examined for the presence of any ovarian tissue. The rat has a duplex uterus with two separate uterine horns joined externally at their cervical ends, but with two independent cervical openings (Yuan and Foley, 2002). The vagina is removed just below the cervix, so that the luminal fluid is retained in the uterus. The "wet" uterus (i.e., containing the luminal fluid) is weighed. Subsequently, the uterine horns are pierced or cut longitudinally and gently blotted with moist filter paper to remove the luminal fluid but prevent desiccation. Wet and blotted uterine weights are measured and reported to the nearest 0.1 mg. It is imperative that the performing laboratory personnel exercise caution to prevent loss of fluids from the wet uterus or desiccation of the blotted uterus during this process. Previous work by Thigpen *et al* (1987), cited in the detailed background review document for the Uterotrophic Assay (OECD, 2003), showed that mouse uterine weight decreased by nearly 12% in three minutes of exposure to the open atmosphere.

8. Optional Measurements

Although wet and blotted uterine weights are the only terminal endpoints which are considered critical, the investigators sometimes choose to perform optional measurements. Examples of optional measurements include morphometric measurements of the endometrium and histopathological examination of the uterus, cervix and/or vagina. The Reviewer should evaluate the methodology in the study report and include a brief description in the methods section of the DER.

I. Statistical Analysis

Statistical analysis of uterine weights typically includes determination of homogeneity of group variance with appropriate data transformations as needed to achieve homogeneous variances (e.g., log transformation). Treatment groups with homogeneous variances should generally be analyzed using analysis of covariance (ANCOVA), using terminal body weight as the co-variable, to determine differences among groups followed by pair-wise comparisons with the controls using a post-hoc test that appropriately adjusts for multiple comparisons (e.g., Dunnett's test). If variances are not homogeneous, non-parametric procedures, such as Kruskal-Wallis and Dunn's tests, are typically employed. The criterion for statistical significance should be identified (e.g., $p \leq 0.05$), and groups attaining statistical significance are denoted. In the DER tables, the asterisk (*) is the conventional means of indicating statistical significance. Summary data tables reporting group mean, standard deviation and CV for wet and blotted uterine weights are included in the DER for each assay.

IV. STUDY INTERPRETATION

A. Results

1. Mortality

The DER should include any mortality and assess whether any animal deaths were due to treatment with the test substance. If treatment-related mortality occurred, the guideline recommends that the study be repeated at lower doses because the maximum tolerated dose was exceeded.

2. Clinical Signs of Toxicity

The DER should include any clinical signs of toxicity, including information on the nature, incidence, severity, onset, and duration. As with the mortality data, the reviewer should assess whether the findings are considered adverse and related to treatment and if they had an impact on dose selection.

Table 1. Incidence of Clinical Observations in the Estrogen Agonist Assay[a]

Observation	Dose (mg/kg/day)							
	Vehicle Control		Low (#)		High (#)		Reference Estrogen EE (#)	
	# Observed	# Examined	# Observed	# Examined	# Observed	# Examined	# Observed	# Examined

a Data were obtained from page [#] of the study report.

Table 2. Incidence of Clinical Observations in the Anti-Estrogenic Assay[a]

Observation	Dose (mg/kg/day)									
	Vehicle Control		EE		Low (#) (+EE)		Mid (#) (+ EE)		High (#) (+EE)	
	# Observed	# Examined	# Observed	# Examined	# Observed	# Examined	# Observed	# Examined	# Observed	# Examined

a Data were obtained from page [#] of the study report.

3. Body Weight and Weight Gain

The DERs will typically include at a minimum, body weights reported for the first and final days of dosing, along with the body weight gain for the overall study (3 days for the immature model, 3-7 days for the ovariectomized model). Any effects of treatment on body weights including information on statistical significance, magnitude difference from controls, and the onset and duration of the decreases will be reported.

Table 3. Selected Group Body Weights and Cumulative Body Weight Gains (g) In the Estrogen Agonist Assay[a]

Study Day #	Dose (mg/kg/day)										
	Vehicle Control			Low (#)			High (#)			Reference Estrogen EE (#)	
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean
#											
#											
#											
Body Weight Gain (# - #)											

a Data were obtained from page [#] of the study report.
N= No. of animals in the group
SD = Standard Deviation
* Significantly different from controls at $p<0.05$
**Significantly different from controls at $p<0.01$

Table 4. Selected Group Body Weights and Cumulative Body Weight Gains In the Anti-Estrogen Assay (g)[a]

Study Day	Dose (mg/kg/day)														
	Vehicle Control			EE (#)			Low (#) (+EE)			Mid (#) (+ EE)			High (#) (+EE)		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
#															
#															
#															
Body Weight Gain (# - #															

a Data were obtained from page [#] of the study report.
N= No. of animals in the group
SD = Standard Deviation
* Significantly different from controls at p<0.05
**Significantly different from controls at p<0.01

4. Food Consumption

Data should be reported as shown in the following tables. Food consumption information should also be documented as necessary to explain any effects on body weight or body weight gain.

Table 5. Food Consumption (g/kg/day) in the Estrogen Agonist Assay[a]

Study Day #	Dose (mg/kg/day)										
	Vehicle Control			Low (#)			High (#)			Reference Estrogen EE (#)	
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean
#											
#											
#											
Overall											

a Data were obtained from page [#] of the study report.
N= No. of animals in the group
SD = Standard Deviation
* Significantly different from controls at p<0.05
**Significantly different from controls at p<0.01

Table 6. Food Consumption (g/kg/day) in the Anti-Estrogen Assay[a]

Study Day	Dose (mg/kg/day)														
	Vehicle Control			EE (#)			Low (#) (+EE)			Mid (#) (+ EE)			High (#) (+EE)		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
#															
#															
#															
Body Weight Gain (#-#)															

a Data were obtained from page [#] of the study report.
N= No. of animals in the group
SD = Standard Deviation
* Significantly different from controls at $p<0.05$
**Significantly different from controls at $p<0.01$

5. Organ Weight

The DER will contain tables with mean wet uterine weights and mean blotted uterine weights (to the nearest 0.1 mg) and standard deviations for each group. Results of statistical analyses comparing both the wet and blotted uterine weights of treated groups relative to the same measures in the reference estrogen agonist control group would be provided as shown below.

Table 7. Uterine Weights from Estrogen Agonist Assay in [SD or Wistar] Rats[a]

Parameter	Dose (mg/kg/day)									
	Vehicle Control			Low (#)		High (#)		Reference Estrogen EE (#)		
	N	Mean	SD	Mean	SD	Mean	SD	N	Mean	SD
Terminal BW										
Wet, absolute (mg)										
Wet, relative (%)										
Blotted, absolute (mg)										
Blotted, relative (%)										

a Data were obtained from page [#] of the study report.
BW= body weight
N= No. of animals in the group
SD = Standard Deviation
* Significantly different from controls at $p<0.05$
**Significantly different from controls at $p<0.01$

Table 8. Uterine Weights from Anti-Estrogenic Assay in [SD or Wistar] Rats[a]

Parameters	Dose (mg/kg/day)														
	Vehicle Control			EE (#)			Low (#) (+EE)			Mid (#) (+ EE)			High (#) (+EE)		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
Terminal BW															
Wet, absolute (mg)															
Wet, relative (%)															
Blotted, absolute (mg)															
Blotted, relative (%)															

a Data were obtained from page [#] of the study report.
BW= body weight
N= No. of animals in the group
SD = Standard Deviation
* Significantly different from controls at $p<0.05$
**Significantly different from controls at $p<0.01$

6. Microscopic Examination (Optional)

Following fixation in10% buffered formalin and HE-staining, the vagina may be examined histologically for keratinization and cornification (Jones and Edgren, 1973). In addition, morphometric measurement of endometrial epithelium may be done for quantitative comparison.

Table 9. Microscopic Examination of the Vagina in the Estrogen Agonist Assay[a]

Parameter	Dose (mg/kg/day)							
	Vehicle Control		Low (#)		High (#)		Reference Estrogen EE (#)	
	# Observed	# Examined	# Observed	# Examined	# Observed	# Examined	# Observed	# Examined
Keratinization								
Cornification								

a Data were obtained from page [#] of the study report.
* Significantly different from controls at $p<0.05$
**Significantly different from controls at $p<0.01$

Table 10. Microscopic Examination of the Vagina in the Anti -Estrogen Assay[a]

Observation	Dose (mg/kg/day)									
	Vehicle Control		EE		Low (#) (+EE)		Mid (#) (+ EE)		High (#) (+EE)	
	# Observed	# Examined	# Observed	# Examined	# Observed	# Examined	# Observed	# Examined	# Observed	# Examined
Keratinization										
Cornification										

a Data were obtained from page [#] of the study report.
* Significantly different from controls at p<0.05
**Significantly different from controls at p<0.01

Table 11. Microscopic Examination of the Ovaries in the Estrogen Agonist Assay[a]

Parameter	Dose (mg/kg/day)							
	Vehicle Control		Low (#)		High (#)		Reference Estrogen EE (#)	
	# Observed	# Examined	# Observed	# Examined	# Observed	# Examined	# Observed	# Examined
Epithelial cell height								

a Data were obtained from page [#] of the study report.
* Significantly different from controls at p<0.05
**Significantly different from controls at p<0.01

Table 12. Microscopic Examination of the Ovaries in the Anti -Estrogen Assay[a]

Observation	Dose (mg/kg/day)									
	Vehicle Control		EE		Low (#) (+EE)		Mid (#) (+ EE)		High (#) (+EE)	
	# Observed	# Examined	# Observed	# Examined	# Observed	# Examined	# Observed	# Examined	# Observed	# Examined
Epithelial cell height										

a Data were obtained from page [#] of the study report.
* Significantly different from controls at p<0.05
**Significantly different from controls at p<0.01

B. Data Interpretation Procedure

The guideline recommends that the highest dose level be at or just below the Maximum Tolerated Dose (MTD) but need not exceed the Limit Dose (1000 mg/kg/day); however, typically, the Agency also considers the toxicity profile of the chemical (i.e., cholinesterase inhibition, target organ toxicity, etc) in dose selection. Typically the second dose level is spaced to produce a lesser degree of toxicity relative to the high dose unless justification is provided for testing at a different level.

The Reviewer should report any mortality and/or clinical signs of toxicity in the DER. It is important to determine whether any observed mortalities or clinical observations were treatment-related and adverse. Necropsy data and dose-response increases in incidence, duration, and/or severity of clinical signs are important factors to report and consider when determining if findings are related to treatment. Body weights and body weight change from the beginning of dosing until necropsy is also normally included in the DER. If measured, food consumption data is included.

The Reviewer should include summary data for weights of the wet and blotted uterus, in addition to any optional measurements in the results section in the DER. The organ weight data also may be presented after covariance adjustment for body weight but this would not replace presentation of the unadjusted (absolute) data.

For assays testing for potential estrogenicity, the reviewers should compare the data from the groups treated with the test material to the vehicle control; increased uterine weights generally can indicate a positive estrogenic response. The EE group serves as a reference estrogen and provides a demonstration of increased organ weights relative to vehicle controls.

For assays testing for potential anti-estrogenicity, the reviewers should compare the data from the groups co-administered the test material and estrogen (EE) to the EE-only control; decreased organ weights generally can indicate a positive anti-estrogenic response. The vehicle control group is included for comparison with the EE group in order to demonstrate a positive estrogenic response.

A statistically significant increase ($p < 0.05$) in uterine weight, at least at the high dose level, compared to the vehicle control group generally can indicate a positive result for estrogenicity. The guideline includes optional measures; supporting histopathology data from the uterus and/or cervix is helpful in corroborating the increased uterine weight as an estrogenic response. Mortality, clinical signs of toxicity and treatment-related effects on body weights, food consumption or other parameters are analyzed in the context of how these changes may affect the interpretation of uterine weight.

Overall, ovariectomized animals have a consistent, non-functioning HPG axis. An alteration of the HPG axis in sexually immature rats can be less specific for ER-mediated effect on uterine growth because chemicals may impact other areas of HPG axis. In addition, age and the onset of puberty can be slightly different among sexually immature rats and this can result in more variation/fluctuations in estrogen production during the peripubertal period. Thus where sexually immature animals are used, a strict adherence to the age range used in the study

would be necessary in order to prevent observations that are mediated by endogenous estrogen from normal peripuberty.

An important consideration for the acceptance of the data from the Uterotrophic Assay is the uterine weights of the vehicle control group. High control uterine weight values may compromise the responsiveness of the assay and the ability to detect very weak estrogen agonists. Any remaining ovarian tissue could have produced endogenous estrogen and retard the regression of the uterine weight; thereby making it less sensitive to influence of potential exogenous estrogens. Therefore, it is important that when the investigators note if any residual ovarian tissue (remaining from ovariectomy) is discovered at the time of removal of the uterus at the end of the assay that the information is included in the DER.

Although not included as part of the OCSPP Guideline 890.1600 test guideline, a guidance document to test for anti-estrogenicity is available (OECD, 2007b). In this protocol, the uterine weights of the animals from the treated groups (test substance and EE) are compared to the estrogenic (EE) control group, and a statistically significant decrease indicates an anti-estrogenic response. This report stated that the study design for estrogen antagonism need not include a positive control, as one had not been identified. The antagonistic effect will be monitored as a decrease in uterine weight from the reference estrogen group. Information from the detailed background review document (OECD, 2003) and Phase 1 of the OECD validation studies (OECD, 2006a) may be useful to the Reviewer to verify quality assurance and adequate performance of the assay.

The results of any histopathology or morphometric data are evaluated in the context of changes in wet and blotted uterine weights, and any correlation or corroboration of endpoints are detailed in the discussion section of the DER.

Additional guidance on data interpretation are provided in USEPA OCSPP Guideline 890.1600 for the Uterotrophic Assay and in the Agency's document titled "Corrections and Clarifications on Technical Aspects of the EDSP Tier 1 Assays (OCSPP Test Guideline Series 890)" which can be found on the EDSP web page (http://www.epa.gov/endo/).

C. Laboratory Performance

Prior to the study, a baseline positive control study is normally conducted to demonstrate laboratory proficiency. For this purpose, the Uterotrophic Assay is conducted by using at least four dose levels of the positive control reference estrogen, EE, to establish a dose response curve and to compare the uterine weight response with historical data. If this baseline positive control study did not yield the anticipated results, the experimental conditions would typically be examined and modified. It is recommended that the performing laboratory subsequently demonstrate competence in conducting the Uterotrophic Assay by one of two available options: periodic verification or use of concurrent controls.

The Test Guideline recommends periodic verification that relies on an initial baseline positive control study. At least every 6 months and each time there is a change that may influence the performance of the assay (e.g., a new formulation of diet, change in personnel performing dissections, change in animal strain or supplier, etc.), the responsiveness of the test

system is verified using an appropriate dose of EE, based on the baseline positive control study. The second option in the guideline for verification of laboratory proficiency recommends including a concurrent control group administered an appropriate dose of reference estrogen in each assay. EPA guideline recommends that this dose of reference estrogen used in either approach be approximately 70% to 80% of the effective dose determined from the dose response curve established in the baseline positive control study.

D. Assay Validation

From 1999-2001, the rat Uterotrophic Assay was subjected to an extensive validation program by the OECD. Validation of the rat Uterotrophic Assay was comprised of two phases. Phase 1 (OECD, 2006a) evaluated the sensitivity and reliability of four different protocols using EE; and Phase 2 (OECD, 2006b) tested the adult ovariectomized and immature rat models using EE, five weak estrogen agonists and a negative chemical. Understanding of the history of the validation process of the assay is helpful in evaluating and interpreting studies conducted under the current finalized guideline.

V. CHARACTERIZATION OF FINDINGS

On completion of the review of this assay, the Agency will conduct a weight of evidence analysis to consider the potential of the chemical to disrupt the estrogen, androgen, or thyroid hormone systems. Chemicals with demonstrated evidence of a potential to interact with the estrogen, androgen, and/or thyroid hormone systems will be considered as candidates for Tier 2 testing.

VI. DATA EVALUATION REPORT

Once the study has been reviewed using the principles described in the previous section of this SEP, a DER will be prepared. A DER template that provides additional guidance for the preparation of the DER is available.

VII. REFERENCES

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/).

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OECD (2006a) Series on Testing and Assessment No. 65. OECD Report of the Validation of the Rodent Uterotrophic Assay – Phase 1. ENV/JM/MONO(2006)33, OECD, Paris.

OECD (2006b) Series on Testing and Assessment No. 66. OECD Report of the Initial Work Towards the Validation of the Rodent Uterotrophic Assay: Phase 2. Testing of Potent and Weak estrogen Agonists by Multiple Laboratories. ENV/JM/MONO(2006)34, OECD, Paris.

OECD (2006d) Validation of the Uterotrophic Assay in Mice by Bridging Data to Rats.

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OECD (2007b) Series on Testing and Assessment No. 71. Guidance Document on the Uterotrophic Bioassay – Procedure to Test for Antiestrogenicity. ENV/JM/MONO(2007)15, OECD, Paris.

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