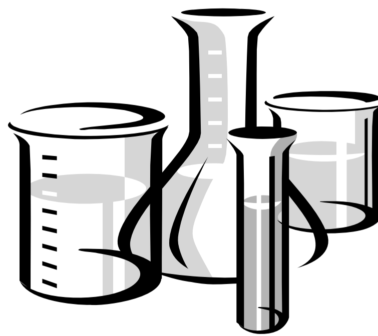
United States Environmental Protection Agency

Prevention, Pesticides and Toxic Substances (7101)

EPA 740-C-09-0010
October 2009

Endocrine Disruptor Screening Program Test Guidelines

OPPTS 890.1600: Uterotrophic Assay



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 890.1600: Uterotrophic Assay

(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.** The source material used in developing this guideline is Test Guideline No. 440 published by the Organization for Economic Cooperation and Development (OECD) (**Ref. 37**). OECD initiated a high-priority activity in 1998 to revise existing guidelines and to develop new guidelines for the screening and testing of potential endocrine disrupters (**Ref. 1**). One element of the activity was to develop a test guideline for the rodent Uterotrophic Assay. The rodent Uterotrophic Assay then underwent an extensive validation program including the compilation of a detailed background document (**Ref. 2 & 3**) and the conduct of extensive intra- and interlaboratory studies to show the relevance and reproducibility

of the assay with a potent reference estrogen, weak estrogen receptor agonists, a strong estrogen receptor antagonist, and a negative reference chemical (**Refs. 4, 5, 6, 7, 8, & 9**). The OECD Test Guideline 440 was the outcome of the validation test program and is the basis of this OPPTS Test Guideline. The only differences in this OPPTS Test Guideline and the OECD TG 440 is in the expressed preference for using the ovariectomized rat and subcutaneous injection dosing method which reflects the specific role of the assay in the EDSP Tier 1 Battery of Assays.

(b) **Purpose.** The Uterotrophic Assay is intended as an *in vivo* screening assay providing data about a single endocrine mechanism, *i.e.*, estrogenicity. It is intended to be included in a battery of *in vitro* and *in vivo* tests to identify substances with potential to interact with the endocrine system, ultimately leading to hazard and risk assessments for human health or the environment.

(c) **Overview.** The Uterotrophic Assay is a short-term screening test that originated in the 1930's (**Refs. 27 & 28**) and was first standardized for screening by an expert committee in 1962 (**Refs. 32 & 35**). It is based on the increase in uterine weight or uterotrophic response (for review, see **Ref. 29**). It evaluates the ability of a chemical to elicit biological activities consistent with agonists or antagonists of natural estrogens (*e.g.*, 17ß-estradiol), however, its use for antagonist detection is much less common than for agonists. The uterus responds to estrogens in two ways. An initial response is an increase in weight due to water imbibition. This response is followed by a weight gain due to tissue growth (**Ref. 30**). The uterus responses in rats and mice qualitatively are comparable.

The OECD validation program used both strong and weak estrogen agonists to evaluate the performance of the assay to identify estrogenic compounds (**Refs. 4, 5, 6, 7 & 8**). Thereby the sensitivity of the test procedure for estrogen agonists was well demonstrated besides a good intra- and interlaboratory reproducibility.

With regard to negative compounds, only one "negative" reference chemical already reported negative by uterotrophic assay as well as *in vitro* receptor binding and receptor assays was included in the validation program, but additional test data, not related to the OECD validation program, have been evaluated, giving further support to the specificity of the Uterotrophic Assay for the screening of estrogen agonists (**Ref. 16**).

(d) **Initial Considerations and Limitations.** Estrogen agonists and antagonists act as ligands for estrogen receptors α and β and may activate or inhibit, respectively, the transcriptional action of the receptors. This may have the potential to lead to adverse health hazards, including reproductive and developmental effects. Therefore, the need exists to rapidly assess and evaluate a chemical as a possible estrogen agonist or antagonist. While informative, the affinity of a ligand for an estrogen receptor or transcriptional activation of reporter genes *in vitro* is only one of several determinants of possible hazard. Other

determinants can include metabolic activation and deactivation upon entering the body, distribution to target tissues, and clearance from the body, depending at least in part on the route of administration and the chemical being tested. This leads to the need to screen the possible activity of a chemical *in vivo* under relevant conditions, unless the chemical's characteristics regarding Absorption – Distribution – Metabolism – Elimination (ADME) already provide appropriate information. Uterine tissues respond with rapid and vigorous growth to stimulation by estrogens, particularly in laboratory rodents, where the oestrous cycle lasts approximately 4 days. Rodent species, particularly the rat, are also widely used in toxicity studies for hazard characterization. Therefore, the rodent uterus is an appropriate target organ for the *in vivo* screening of estrogen agonists and antagonists.

This Guideline is based on those protocols employed in the OECD validation study which have been shown to be reliable and repeatable in intra- and interlaboratory studies (**Refs. 5 & 7**). Currently two methods, namely, the ovariectomized adult female method (ovx-adult method) and the immature non-ovariectomized method (immature method) are available. It was shown in the OECD validation test program that both methods have comparable sensitivity and reproducibility. However, in the EDSP battery the ovariectomized (OVX) animal is preferred due to its increased specificity over the immature model. The immature rat has an intact hypothalamic-pituitary-gonadal (HPG) axis may cover a larger scope of investigation than the ovariectomized animal at the expense of specificity because it can respond to substances that interact with the HPG axis rather than just the estrogen receptor. The HGP axis of the rat is functional at about 15 days of age. Prior to that, puberty cannot be accelerated with treatments like GnRH. As the females begin to reach puberty, prior to vaginal opening, the female will have several silent cycles that do not result in vaginal opening or ovulation, but there are some hormonal fluctuations. If a chemical stimulates the HGP axis directly or indirectly, precocious puberty, early ovulation and accelerated vaginal opening result. Not only chemicals that act on the HPG axis do this but some diets with higher metabolizable energy levels than others will stimulate growth and accelerate vaginal opening without being estrogenic. Such substances would not induce an uterotrophic response in OVX adult animals as their HPG axis doesn't work.

The Uterotrophic Assay in the EDSP Tier 1 battery is intended to specifically detect estrogenic substances and complement the rat pubertal assays in the battery which have a functionally intact HPG axis.

The uterotrophic response is not entirely of estrogenic origin, *i.e.,*compounds other than agonists or antagonists of estrogens may also provide a response. For example, relatively high doses of progesterone, testosterone, or various synthetic progestins may all lead to a stimulative response (**Ref. 30**). Any response may be analyzed histologically for keratinization and cornification of the vagina (**Ref. 30**). Irrespective of the possible origin of the response, a positive outcome of an Uterotrophic Assay should normally initiate actions for further

clarification. Additional evidence of estrogenicity could come from in vitro assays, such as the ER binding assays and transcriptional activation assays, or from other in vivo assays such as the female pubertal assay.

Taking into account that the Uterotrophic Assay serves as an in vivo screening assay, the validation approach taken, served both animal welfare considerations and a tiered testing strategy. To this end, effort was directed at rigorously validating reproducibility and sensitivity for estrogenicity - the main concern for many chemicals-, while little effort was directed at the antiestrogenicity component of the assay. Only one antiestrogen with strong activity was tested since the number of substances with a clear antiestrogenic profile (not obscured by some estrogenic activity) is very limited. Thus this Test Guideline is dedicated to the estrogenic protocol, while the protocol describing the antagonist mode of the assay is included in a guidance document. The reproducibility and sensitivity of the assay for substances with purely anti-estrogenic activity will be more clearly defined later on, after the test procedure has been in routine use for some time and more substances with this modality of action are identified.

It is acknowledged that all animal based procedures will conform to local standards of animal care; the descriptions of care and treatment set forth below are minimal performance standards, and will be superseded by local regulations. Further guidance of the humane treatment of animals is given by the OECD (**Ref. 25**).

The intended use of the Uterotrophic Assay is as a component of the EDSP Tier 1 Battery of Assays. Other uses should consider whether the data are truly necessary prior to the start of the assay and whether another experimental option is appropriate.

Definitions used in this Test Guideline are given in Appendix 1.

(e) **Principle of the Test.** The Uterotrophic Assay relies for its sensitivity on an animal test system in which the hypothalamic-pituitary-ovarian axis is not functional, leading to low endogenous levels of circulating estrogen. This will ensure a low baseline uterine weights and a maximum range of response to administered estrogens. A young adult female after ovariectomy with adequate time for uterine tissues to regress is preferred for use in the EDSP Tier 1. For other purposes, an immature female after weaning and prior to puberty is an option.

The test substance is administered daily preferably by subcutaneous injection. Alternatively, oral gavage may be considered. Subcutaneous injection is preferred for the assay's screening role in EDSP to provide ADME insight and contrast with the rat pubertal assays which employ oral gavage. Graduated test substance doses are administered to a minimum of two treatment groups (see subsection (h)(5) for guidance) of experimental animals using one dose level per group and an administration period of a minimum administration period of three

consecutive days for ovx-adult method and three consecutive days for the immature method. The animals are necropsied approximately 24 hours after the last dose. For estrogen agonists, the mean uterine weight of the treated animal groups relative to the vehicle group is assessed for a statistically significant increase. A statistically significant increase in the mean uterine weight of a test group indicates a positive response in this assay.

(f) **Description of the Method.**

(1) **Selection of Animal Species.** Commonly used laboratory rodent strains may be used. As an example, Sprague-Dawley and Wistar strains of rats were used during the validation. Strains with uteri known or suspected to be less responsive should not be used. The laboratory should demonstrate the sensitivity of the strain used as described in section (g).

The rat and mouse have been routinely used in the Uterotrophic Assay since the 1930s. The OECD validation studies were only performed with rats based on an understanding that both species are expected to be equivalent and therefore one species should be enough for the world-wide validation in order to save resources and animals. The rat is the species of choice in most reproductive and developmental toxicity studies. Taking into consideration that a vast historical data base exists for mice and thus to broaden the scope of the Uterotrophic Assay Test Guideline in rodents to the use of mice as test species, a limited follow-up validation study was carried out in mice (**Ref. 16**). A bridging approach with a limited number of test chemicals, participating laboratories and without coded sample testing has been selected in keeping with the original intent to save resources and animals. This bridging validation study shows for the Uterotrophic Assay in young adult ovariectomized mice that qualitatively and quantitatively, the data obtained in rats and mice correspond well with each other. Where the Uterotrophic Assay result may be preliminary to a long-term study, this allows animals from the same strain and source to be used in both studies. The bridging approach was limited to the OVX mice and the report doesn't provide a robust data set to validate the immature model, thus the immature model for mice is not considered under the scope of the current Test Guideline.

Thus, in some cases mice may be used instead of rats. A rationale should be given for this species, based on toxicological, pharmacokinetic, and/or other criteria. Modifications of the protocol may be necessary for mice. For example, the food consumption of mice on a body weight basis is higher than that of rats and therefore the phytoestrogen content in food should be lower for mice than for rats (**Refs. 9, 20, & 22**).

(2) **Housing and Feeding Conditions.** All procedures should conform with local standards of laboratory animal care. These descriptions of care and treatment are minimum standards and will be superseded by local

regulations, when present. The temperature in the experimental animal room should be 22°C (with an approximate range ± 3°C). The relative humidity should be a minimum of 30% and preferably should not exceed a maximum 70%, other than during room cleaning. The aim should be relative humidity of 50-60%. Lighting should be artificial. The daily lighting sequence should be 12 hours light, 12 hours dark.

Laboratory diet and drinking water should be provided *ad libitum*. Young adult animals may be housed individually or be caged in groups of up to three animals. Due to the young age of the immature animals, social group housing is recommended.

Very high levels of phytoestrogens in laboratory diets have been known to increase uterine weights in rodents to a degree enough as to interfere with the Uterotrophic Assay (**Refs. 13, 14, & 15**). High levels of phytoestrogens and of metabolized energy in laboratory diets may also result in early puberty, if immature animals are used. The presence of phytoestrogens results primarily from the inclusion of soy and alfalfa products in the laboratory diets. Body weight is an important variable, as the quantity of food consumed is related to body weight. Therefore, the actual phytoestrogen dose consumed from the same diet may vary among species and by age (**Ref. 9**). For immature female rats, food consumption on a body weight basis may be approximately double that of ovariectomized young adult females. For young adult mice, food consumption on a body weight basis may be approximately quadruple that of ovariectomized young adult female rats.

Uterotrophic Assay results (**Refs. 9, 17, 18, &19**), however, show that limited quantities of dietary phytoestrogens are acceptable and do not reduce the sensitivity of the assay. As a guide, dietary levels of phytoestrogens should not exceed 350 µg of genistein equivalents/gram of laboratory diet for immature female Sprague Dawley and Wistar rats (**Refs. 6 & 9**). Such diets should also be appropriate when testing in young adult ovariectomized rats because food consumption on a body weight basis is less in young adult as compared to immature animals. If adult ovariectomized mice or more phytoestrogen-sensitive rats are to be used, proportional reduction in dietary phytoestrogen levels must be considered (**Ref. 20**). In addition, the differences in available metabolic energy from different diets may lead to time shifts for the onset of puberty (**Refs. 21 & 22**).

Prior to the study, careful selection is required of a diet without an elevated levels of phytoestrogens (for guidance see **Refs. 6 & 9**) or metabolizable energy, that can confound the results (**Refs. 15, 17, 19, 22, & 36**). Ensuring the proper performance of the test system used by the laboratory as specified in section (g) is an important check on both of these factors. As a safeguard consistent with GLP, representative

sampling of each batch of diet administered during the study should be conducted for possible analysis of phytoestrogen content (*e.g.*, in the case of high uterine control weight relative to historic controls or an inadequate response to the reference estrogen, 17-alpha ethynyl estradiol). Aliquots should be analyzed as part of the study or frozen at -20°C or in such a way as to prevent the sample from decomposing prior to analysis.

Some bedding materials may contain naturally occurring estrogenic or antiestrogenic substances (*e.g.*, corn cob is known to affects the cyclicity of rats and appears to be antiestrogenic). The selected bedding material should contain a minimum level of phytoestrogens.

(3) **Preparation of Animals.** Experimental animals without evidence of any disease or physical abnormalities are randomly assigned to the control and treatment groups. Cages should be arranged in such a way that possible effects due to cage placement are minimized. The animals should be identified uniquely. Preferably, immature animals should be caged with dams or foster dams until weaning during acclimatization. The acclimatization period prior to the start of the study should be about 5 days for young adult animals and for the immature animals delivered with dams or foster dams. If immature animals are obtained as weanlings without dams a shorter duration of the acclimatization period may become necessary as dosing should start immediately after weaning (see subsection (h)(2)).

(g) **Verification of Laboratory Proficiency.**

(1) Two different options can be used to verify laboratory proficiency:

- ❑ Periodic verification, relying on an initial baseline positive control study (see subsection (g)(2)). 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 (animal model) should be verified using an appropriate dose (based on the baseline positive control study described below) of a reference estrogen: 17α-ethynyl estradiol (CAS No. 57-63-6) (EE).

- ❑ Use of concurrent controls, by including a group administered with an appropriate dose of reference estrogen in each assay.

If the system does not respond as expected the experimental conditions should be examined and modified accordingly. It is recommended that this dose of reference estrogen to be used in either approach be approximately the ED70 to 80.

(2) **Baseline Positive Control Study.** Prior to the study, laboratory proficiency should be demonstrated by testing the responsiveness of the animal model, by establishing the dose response of a reference estrogen: 17α-ethynyl estradiol (CAS No. 57-63-6) (EE) with a minimum of four doses. The uterine weight response will be compared to established historical data (see **Ref. 5**). If this baseline positive control study does not yield the anticipated results, the experimental conditions should be examined and modified.

(h) **Procedure.**

(1) **Number and Condition of Animals.** Each treated and control group should include at least 6 animals (for both immature and ovx-adult method protocols).

(2) **Age of Immature Animals.** For the Uterotrophic Assay with immature animals the day of birth must be specified. Dosing should begin early enough to ensure that, at the end of test substance administration, the physiological rise of endogenous estrogens associated with puberty has not yet taken place. On the other hand, there is evidence that very young animals may be less sensitive. For defining the optimal age each laboratory should take its own background data on maturation into consideration.

As a general guide, dosing in rats may begin immediately after early weaning on postnatal day 18 (with the day of birth being postnatal day 0). Dosing in rats preferably should be completed on postnatal day 21 but in any case prior to postnatal day 25, because, after this age, the hypothalamic-pituitary-ovarian axis becomes functional and endogenous estrogen levels may begin to rise with a concomitant increase in baseline uterine weight means and an increase in the group standard deviations (**Refs. 2, 4, 10, 11, & 12**).

(3) **Procedure for Ovariectomy.** For the ovariectomized female rat and mouse (treatment and control groups), ovariectomy should occur between 6 and 8 weeks of age. For rats, a minimum of 14 days should elapse between ovariectomy and the first day of administration in order to allow the uterus to regress to a minimum, stable baseline. For mice, at least 7 days should elapse between ovariectomy and the first day of administration. As small amounts of ovarian tissue are sufficient to produce significant circulating levels of estrogens (**Ref. 3**), the animals should be tested prior to use by observing epithelial cells swabbed from the vagina on at least five consecutive days (*e.g.*, days 10-14 after ovariectomy for rats). If the animals indicate any evidence entering oestrous, the animals should not be used. Further, at necropsy, the ovarian stubs should be examined for any evidence that ovarian tissue is present. If so, the animal should not be used in the calculations (**Ref. 3**).

The ovariectomy procedure begins with the animal in ventral recumbency after the animal has been properly anesthetized. The incision opening the dorso-lateral abdominal wall should be approximately 1 cm lengthways at the mid point between the costal inferior border and the iliac crest, and a few millimetres lateral to the lateral margin of the lumbar muscle. The ovary should be removed from the abdominal cavity onto an aseptic field. The ovary should be disconnected at the junction of the oviduct and the uterine body. After confirming that no massive bleeding is occurring, the abdominal wall should be closed by a suture and the skin closed by autoclips or appropriate suture. The ligation points are shown schematically in **Figure 1**. Appropriate post operative analgesia should be used as recommended by a veterinarian experienced in rodent care.

(4) **Body Weight.** In the ovx-adult method, body weight and uterine weight are not correlated because uterine weight is affected by hormones like estrogens but not by the growth factors that regulate body size. On the contrary, body weight is related to uterine weight in the immature model, while it is maturing (**Ref. 34**). Thus, at the commencement of the study the weight variation of animals used, in the immature model, should be minimal and not exceed ± 20% of the mean weight. This means that the litter size should be standardized by the breeder, to assure that offspring of different mother animals will be fed approximately the same. Animals should be assigned to groups (both control and treatment) by randomized weight distribution, so that mean body weight of each group is not statistically different from any other group. Consideration should be given to avoid assignment of littermates to the same treatment group as far as practicable without increasing the number of litters to be used for the investigation.

(5) **Dosage.** In order to establish whether a test substance can have estrogenic action *in vivo*, two dose groups and a control are normally sufficient and this design is therefore preferred for animal welfare reasons. If the purpose is either to obtain a dose-response curve or to extrapolate to lower doses, at least 3 dose groups are needed. If information beyond identification of estrogenic activity (such as an estimate of potency) is required, a different dosing regime should be considered. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the test group subjects. If a vehicle is used in administering the test substance, the control group should receive the same amount of vehicle used with the treated groups (or highest volume used with the test groups if different among groups).

The objective in the case of the Utertrophic Assay is to select doses that ensure animal survival and that are without significant toxicity or distress to the animals after three consecutive days of chemical administration up to a maximum dose of 1000 mg/kg/d. All dose levels should be proposed

and selected taking into account any existing toxicity and (toxico-) kinetic data available for the test compound or related materials. The highest dose level should first take into consideration the LD_{50} and/or acute toxicity information in order to avoid death, severe suffering or distress in the animals (**Refs. 24, 25, & 26**). The highest dose should represent the maximum tolerated dose (MTD); a study conducted at a dose level that induced a positive uterotrophic response would be accepted too. As a screen, large intervals (*e.g.*, one half log units corresponding to a dose progression of 3.2 or even up to one log units) between dosages are generally acceptable. If there are no suitable data available, a range finding study may be performed to aid the determination of the doses to be used.

Alternatively, if the estrogenic potency of an agonist can be estimated by in vitro (or in silico) data, these may be taken into consideration for dose selection. For example, the amount of the test chemical that would produce uterotrophic responses equivalent to the reference agonist (Ethynyl estradiol) is estimated by its relative in vitro potencies to ethynyl estradiol. The highest test dose would be given by multiplying this equivalent dose by an appropriate factor *e.g.*, 10 or 100.

(6) **Considerations for Range Finding.** If necessary, a preliminary range finding study can be carried out with few animals. In this respect, OECD Guidance Document No. 19 (**Ref. 25**) may be used defining clinical signs indicative of toxicity or distress to the animals. If feasible within this range finding study after three days of administration, the uteri may be excised and weighed approximately 24-hours after the last dose. These data could then be used to assist the main study design (select an acceptable maximum and lower doses and recommend the number of dose groups).

(7) **Administration of Doses.** As noted in section (e), the preferred method of test compound administration for the EDSP is by subcutaneous injection. However, animal welfare considerations as well as toxicological aspects such as the relevance to the human route of exposure to the chemical (*e.g.*, oral gavage to model ingestion, subcutaneous injection to model inhalation or dermal adsorption), the physical/chemical properties of the test material, existing toxicological information, data on metabolism and kinetics (*e.g.*, need to avoid first pass metabolism, better efficiency via a particular route), and especially the intended use of the assay (*e.g.*, EDSP Tier 1 battery) should also be taken into consideration when choosing the route of administration.

It is recommended that, wherever possible, the use of an aqueous solution/suspension be considered first. But as most estrogen ligands or their metabolic precursors tend to be hydrophobic, the most common approach is to use a solution/suspension in oil (*e.g.*, corn, peanut, sesame or olive oil). However, these oils have different caloric and fat content,

thus the vehicle might affect total metabolizable energy (ME) intake, thereby potentially altering measured endpoints such as the uterine weight especially in the immature method (**Ref. 33**). Thus, prior to the study, any vehicle to be used should be tested against controls without vehicles. Test substances can be dissolved in a minimal amount of 95% ethanol or other appropriate solvents and diluted to final working concentrations in the test vehicle. The toxic characteristics of the solvent must be known, and should be tested in a separate solvent-only control group. If the test substance is considered stable, gentle heating and vigorous mechanical action can be used to assist in dissolving the test substance. The stability of the test substance in the vehicle should be determined. If the test substance is stable for the duration of the study, then one starting aliquot of the test substance may be prepared, and the specified dosage dilutions prepared daily.

Dosage timing will depend of the model used (refer to subsection (h)(2) for the immature model and to subsection (h)(3) for ovx-adult model). Immature female rats are dosed with the test substance daily for three consecutive days. A three-day treatment is also recommended for ovariectomized female rats but longer exposures are acceptable and may improve the detection of weakly active substances. With ovariectomized female mice, an application duration of 3 days should be sufficient without a significant advantage by an extension of up to seven days for strong estrogen agonists, however, this relation was not demonstrated for weak estrogens in the validation study (**Ref. 16**) thus dosage should be extended up to 7 consecutive days in ovx-adult mice. The dose should be given at similar times each day. They should be adjusted as necessary to maintain a constant dose level in terms of animal body weight (*e.g.*, mg of test substance per kg of body weight per day). Regarding the test volume, its variability, on a body weight basis, should be minimized by adjusting the concentration of the dosing solution to ensure a constant volume on a body weight basis at all dose levels and for any route of administration.

When the test substance is administered by gavage, this should be done in a single daily dose to the animals using a stomach tube or a suitable intubation cannula. The maximum volume of liquid that can be administered at one time depends on the size of the test animal. Local animal care guidelines should be followed, but the volume should not exceed 5 ml/kg body weight, except in the case of aqueous solutions where 10 ml/kg body weight may be used.

When the test substance is administered by subcutaneous injection, this should be done in a single daily dose. Doses should be administered to the dorsoscapular or lumbar regions via sterile needle (*e.g.*, 23- or 25-gauge) and a tuberculin syringe. Shaving the injection site is optional. Any losses, leakage at the injection site or incomplete dosing should be recorded. The total volume injected per rat per day should not exceed 5

ml/kg body weight, divided into 2 injection sites, except in the case of aqueous solutions where 10 ml/kg body weight may be used.

(i) **Observations.**

(1) **General and Clinical Observations.** General clinical observations should be made at least once a day and more frequently when signs of toxicity are observed. Observations should be carried out preferably at the same time(s) each day and considering the period of anticipated peak effects after dosing. All animals are to be observed for mortality, morbidity and general clinical signs such as changes in behaviour, skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (*e.g.*, lacrimation, piloerection, pupil size, unusual respiratory pattern).

(2) **Body Weight and Food Consumption.** All animals should be weighed daily to the nearest 0.1 g, starting just prior to initiation of treatment *i.e.*, when the animals are allocated into groups. As an optional measurement, the amount of food consumed during the treatment period may be measured per cage by weighing the feeders. The food consumption results should be expressed in grams per rat per day.

(3) **Dissection and Measurement of Uterus Weight.** Twenty-four hours after the last treatment, the rats will be humanely killed. Ideally, the necropsy order will be randomized across groups to avoid progression directly up or down dose groups that could subtly affect the data. The assay objective is to measure both the wet and blotted uterus weights. The wet weight includes the uterus and the luminal fluid contents. The blotted weight is measured after the luminal contents of the uterus have been expressed and removed.

Before dissection the vagina will be examined for opening status in immature animals. The dissection procedure begins by opening the abdominal wall starting at the pubic symphysis. Then, uterine horn and ovaries, if present, are detached from the dorsal abdominal wall. The urinary bladder and ureters are removed from the ventral and lateral side of uterus and vagina. Fibrous adhesion between the rectum and the vagina is detached until the junction of vaginal orifice and perineal skin can be identified. The uterus and vagina are detached from the body by incising the vaginal wall just above the junction between perineal skin as shown in **Figure 2**. The uterus should be detached from the body wall by gently cutting the uterine mesentery at the point of its attachment along the full length of the dorsolateral aspect of each uterine horn. Once removed from the body, uterine handling should be sufficiently rapid to avoid desiccation of the tissues. Loss of weight due to desiccation becomes more important with small tissues such as the uterus (**Ref. 23**). If ovaries are present, the ovaries are removed at the oviduct avoiding

loss of luminal fluid from the uterine horn. If the animal has been ovariectomized, the stubs should be examined for the presence of any ovarian tissue. Excess fat and connective tissue should be trimmed away. The vagina is removed from the uterus just below the cervix so that the cervix remains with the uterine body as shown in **Figure 2**.

Each uterus should be transferred to a uniquely marked and weighed container (*e.g.*, a petri-dish or plastic weight boat) with continuing care to avoid desiccation before weighing (*e.g.*, filter paper slightly dampened with saline may be placed in the container). The uterus with luminal fluid will be weighed to the nearest 0.1 mg (wet uterine weight).

Each uterus will then be individually processed to remove the luminal fluid. Both uterine horns will be pierced or cut longitudinally. The uterus will be placed on lightly moistened filter paper (*e.g.,* Whatman No. 3) and gently pressed with a second piece of lightly moistened filter paper to completely remove the luminal fluid. The uterus without the luminal contents will be weighed to the nearest 0.1 mg (blotted uterine weight).

The uterus weight at termination can be used to assure that the appropriate age in the immature intact rat was not exceeded, however, the historical data of the rat strain used by the laboratory are decisive in this respect (see paragraph 56 for interpretation of the results).

(4) **Optional Investigations.** After weighing, the uterus may be fixed in 10% neutral buffered formalin to be examined histopathologically after Haematoxylin & Eosin (HE)-staining. The vagina may be investigated accordingly (see section (d)). In addition, morphometric measurement of endometrial epithelium may be done for quantitative comparison.

(j) **Data and Reporting.**

(1) **Data.** Study data should include:

- ❑ The number of animals at the start of the assay;
- ❑ The number and identity of animals found dead during the assay or killed for humane reasons and the date and time of any death or humane kill;
- ❑ The number and identity of animals showing signs of toxicity, and a description of the signs of toxicity observed, including time of onset, duration, and severity of any toxic effects; and
- ❑ The number and identity of animals showing any lesions and a description of the type of lesions.

Individual animal data should be recorded for the body weights, the wet uterine weight, and the blotted uterine weight. One-tailed statistical analyses for agonists should be used to determine whether the administration of a test substance resulted in a statistically significant (p <

0.05) increase in the uterine weight. Appropriate statistical analyses should be carried out to test for treatment related changes in blotted and wet uterine weight. For example, the data may be evaluated by an analysis of covariance (ANCOVA) approach with body weight at necropsy as the co-variable. A variance-stabilizing logarithmic transformation may be carried out on the uterine data prior to the data analysis. Dunnett and Hsu's test are appropriate for making pair wise comparisons of each dosed group to vehicle controls and to calculate the confidence intervals. Studentised residual plots can be used to detect possible outliers and to assess homogeneity of variances. These procedures were applied in the OECD validation program using the PROC GLM in the Statistical Analysis System (SAS Institute, Cary, NC), version 8 (6)(7).

(2) **Final Report.** A final report shall include:

- ❑ Testing Facility:
  - Responsible personnel and their study responsibilities.
  - Data from the Baseline Positive Control Test and periodic positive control data (see section (g)).
- ❑ Test Substance:
  - Characterization of test substances.
  - Physical nature and where relevant physicochemical properties.
  - Method and frequency of preparation of dilutions.
  - Any data generated on stability.
  - Any analyses of dosing solutions.
- ❑ Vehicle:
  - Characterization of test vehicle (nature, supplier and lot).
  - Justification of choice of vehicle (if other than water).
- ❑ Test Animals:
  - Species and strain and justification for their choice.
  - Supplier and specific supplier facility.
  - Age on supply with birth date.
  - If immature animals, whether or not supplied with dam or foster dam and date of weaning.
  - Details of animal acclimatization procedure.
  - Number of animals per cage.
  - Detail and method of individual animal and group identification.
- ❑ Assay Conditions:
  - Details of randomization process (*i.e.*, method used).
  - Rationale for dose selection.
  - Details of test substance formulation, its achieved concentrations, stability and homogeneity.
  - Details of test substance administration and rationale for the choice of exposure route.

- Diet (name, type, supplier, content, and, if known, phytoestrogen levels).
- Water source (*e.g.*, tap water or filtered water) and supply (by tubing from a large container, in bottles, *etc.*).
- Bedding (name, type, supplier, content).
- Record of caging conditions, lighting interval, room temperature and humidity, room cleaning.
- Detailed description of necropsy and uterine weighing procedures.
- Description of statistical procedures.

❑ Results:

- For individual animals:
    - All daily individual body weights (from allocation into groups through necropsy) (to the nearest 0.1 g).
    - Age of each animal (in days counting day of birth as day 0) when administration of test compound begins.
    - Date and time of each dose administration.
    - Calculated volume and dosage administered and observations of any dosage losses during or after administration.
    - Daily record of status of animal, including relevant symptoms and observations.
    - Suspected cause of death (if found during study in moribund state or dead).
    - Date and time of humane killing with time interval to last dosing.
    - Wet uterine weight (to the nearest 0.1 mg) and any observations of luminal fluid losses during dissection and preparation for weighing.
    - Blotted uterine weight (to the nearest 0.1 mg).

- For each group of animals:
    - Mean daily body weights (to the nearest 0.1 g) and standard deviations (from allocation into groups through necropsy).
    - Mean wet uterine weights and mean blotted uterine weights (to the nearest 0.1 mg) and standard deviations.
    - If measured, daily food consumption (calculated as grams of food consumed per animal).
    - The results of statistical analyses comparing both the wet and blotted uterine weights of treated groups relative to the same measures in the vehicle control groups.
    - The results of statistical analysis comparing the total body weight and the body weight gain of treated groups relative to the same measures in the vehicle control groups.

Table 1. Summary of the Important Guidance Facts of the Test Guideline.

	Rat	Mice
Animals		
Strain	Commonly used laboratory rodent strain	
Number of animals	*A minimum of 6 animals per dose group*	
Number of groups	A minimum of 2 test groups (see paragraph 33 for guidance) and a negative control group For guidance on positive control groups see paragraphs 26 and 27	
Housing and Feeding Conditions		
T° in animal room	22°C ± 3°C	
Relative humidity	50-60% and not below 30% or above 70%	
Daily lighting sequence	12 hours light, 12 hours dark	
Diet and drinking water	Ad libitum	
Housing	Individually or in groups of up to three animals (social group housing is recommended for immature animals)	
Diet and bedding	Low level of phytoestrogens recommended in diet and bedding	
Protocol		
Method	Ovariectomized adult female method (the US EDSP preferred one). Immature non-ovariectomized method.	Ovariectomized adult female method
Age of dosing for immature animals	PND 18 at the earliest. Dosing should be completed prior to PND 25	Not relevant under the scope of the current TG.
Age of ovariectomy	Between 6 and 8 weeks of age.	
Age of dosing for ovariectomized animals	A minimum of 14 days should elapse between ovariectomy and the 1st day of administration.	A minimum of 7 days should elapse between ovariectomy and the 1st day of administration.
Body weight	Body weight variation should be minimal and not exceed ± 20% of the mean weight.	
Dosing		
Route of administration	Subcutaneous injection(preferred) or oral gavage.	
Frequency of administration	Single daily dose	
Volume amount for gavage and injection	≤ 5ml/kg body weight (or up to 10 ml/kg body weight in case of aqueous solutions) (in 2 injection sites for subcutaneous route)	
Duration of administration	Minimum of 3 consecutive days for the OVX model 3 consecutive days for immature model	7 consecutive days for the OVX model
Time of necropsy	Approximately 24 hours after the last dose	
Results		
Positive response	Statistically significant increase of the mean uterus weight (wet and or blotted)	
Reference estrogen	17α-ethynyl estradiol	

(k) **Guidance for the Interpretation and Acceptance of the Results.** In general, a test for estrogenicity should be considered positive if there is a statistically significant increase in uterine weight ($p < 0.05$) at least at the high dose level as compared to the solvent control group. A positive result is further supported by the demonstration of a biologically plausible relationship between the dose and the magnitude of the response, bearing in mind that overlapping estrogenic and

antiestrogenic activities of the test chemical may affect the shape of the dose-response curve.

Care must be taken in order not to exceed the maximum tolerated dose to allow a meaningful interpretation of the data. Reduction of body weight, clinical signs, and other findings should be thoroughly assessed in this respect.

An important consideration for the acceptance of the data from the Uterotrophic Assay is the uterine weights of the vehicle control group. High control values may compromise the responsiveness of the assay and the ability to detect very weak estrogen agonists. Literature reviews and the data generated during the validation of the Uterotrophic Assay suggest that instances of high control means do occur spontaneously, particularly in immature animals (**Refs. 2, 3, 6, & 9**). As the uterine weight of immature rats depends on many variables like strain or body weight, no definitive upper limit for the uterine weight can be given. As a guide, if blotted uterine weights in immature control rats are comprised between 40 and 45 mg, results should be considered as suspicious and uterine weights above 45 mg may lead to rerun the test. However, this needs to be considered on a case by case basis (**Refs. 3, 6, & 8**). When testing in adult rats incomplete ovariectomy will leave ovarian tissue that can produce endogenous estrogen and retard the regression of the uterine weight.

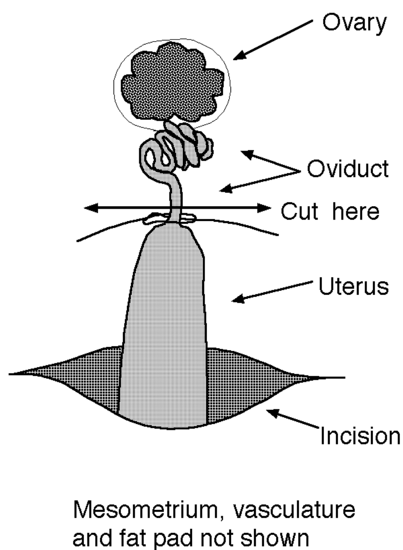
Blotted vehicle control uterine weights less than 0.09% of body weight for immature female rats and less than 0.04% for ovariectomized young adult females appear to yield acceptable results. If the control uterine weights are greater than these numbers, various factors should be scrutinized including the age of the animals, proper ovariectomy, dietary phytoestrogens, and so on, and a negative assay result (no indication for estrogenic activity) should be used with caution.

Historical data for vehicle control groups should be maintained in the laboratory. Historical data for responses to positive reference estrogens, such as 17α-ethynyl estradiol, should also be maintained in the laboratory. Laboratories may also test the response to known weak estrogen agonists. All these data can be compared to available data (**Refs. 2, 3, 4, 5, 6, 7, & 8**) to ensure that the laboratory's methods yield sufficient sensitivity.

The blotted uterine weights showed less variability in the course of the OECD validation study than the wet uterine weights (**Refs. 6 & 7**). However, a significant response in either measure would indicate that the test substance is positive for estrogenic activity.

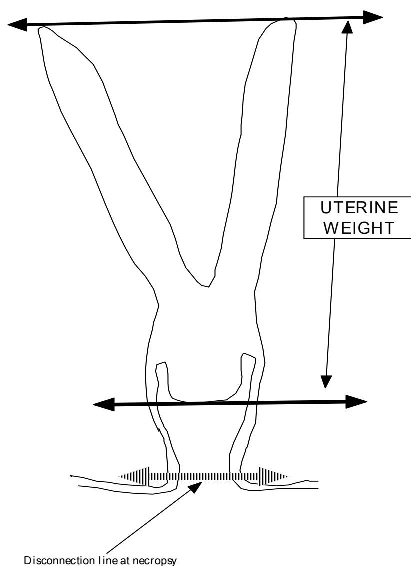
The uterotrophic response is not entirely of estrogenic origin, however, a positive result of the Uterotrophic Assay should generally be interpreted as evidence for estrogenic potential in vivo, and should normally initiate actions for further clarification (see paragraph 9 and the "OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals", Annex 2).

Figure 1. Schematic Diagram Showing the Surgical Removal of the Ovaries.



The procedure begins by opening dorso-lateral abdominal wall at the mid point between the costal inferior border and the iliac crest, and a few millimetres lateral to the lateral margin of the lumbar muscle. Within the abdominal cavity, the ovaries should be located. On an aseptic field, the ovaries are then physically removed from the abdominal cavity, a ligature placed between the ovary and uterus to control bleeding, and the ovary detached by incision above the ligature at the junction of the oviduct and each uterine horn. After confirming that no significant bleeding persists, the abdominal wall should be closed by suture, and the skin closed, *e.g.*, by autoclips or suture. The animals should be allowed to recover and the uterus weight to regress for a minimum of 14 days before use.

Figure 2. The Removal and Preparation of the Uterine Tissues for Weight Measurement.



The procedure begins by opening the abdominal wall at the pubic symphysis. Then, each ovary, if present and uterine horn is detached from the dorsal abdominal wall. Urinary bladder and ureters are removed from the ventral and lateral side of uterus and vagina. Fibrous adhesion between the rectum and the vagina are detached until the junction of vaginal orifice and perineal skin can be identified. The uterus and vagina are detached from the body by incising the vaginal wall just above the junction between perineal skin as shown in the figure. The uterus should be detached from the body wall by gently cutting the uterine mesentery at the point of its attachment along the full length of the dorsolateral aspect of each uterine horn. After removal from the body, the excess fat and connective tissue is trimmed away. If ovaries are present, the ovaries are removed at the oviduct avoiding loss of luminal fluid from the uterine horn. If the animal has been ovarectomized, the stubs should be examined for the presence of any ovarian tissue. The vagina is removed from the uterus just below the cervix so that the cervix remains with the uterine body as shown in the figure. The uterus can then be weighed.

(l) References.

1. OECD (1998). Report of the First Meeting of the OECD Endocrine Disrupter Testing and Assessment (EDTA) Task Force, 10th-11th March 1998, ENV/MC/CHEM/RA(98)5.
2. OECD (2003). Detailed Background Review of the Uterotrophic Assay: Summary of the Available Literature in Support of the Project of the OECD Task Force on Endocrine Disrupters Testing and Assessment (EDTA) to Standardise and Validate the Uterotrophic Assay. OECD Environmental Health and Safety Publication Series on Testing and Assessment No. 38. ENV/JM/MONO(2003)1.
3. Owens JW, Ashby J (2002). Critical Review and Evaluation of the Uterotrophic Assay for the Identification of Possible Estrogen Agonists and Antagonists: In Support of the Validation of the OECD Uterotrophic Protocols for the Laboratory Rodent. Crit. Rev. Toxicol. 32:445-520.
4. OECD (2001). Final Report of the Phase 1 of the Validation Study of the Uterotrophic Assay. [ENV/JM/TG/EDTA (2001)1/REV1].
5. Kanno, J, Onyon L, Haseman J, Fenner-Crisp P, Ashby J, Owens W (2001). The OECD program to validate the rat uterotrophic assay to screen compounds for in vivo estrogenic responses: Phase 1. Environ Health Perspect. 109:785-94.
6. OECD (2003). OECD Draft Report of the Validation of the Rat Uterotrophic Assay. Phase 2. Testing of Potent and Weak Oestrogen Agonists by Multiple Laboratories. [ENV/JM/TG/EDTA (2003)1].
7. Kanno J, Onyon L, Peddada S, Ashby J, Jacob E, Owens W (2003). The OECD program to validate the rat uterotrophic assay: Phase Two - Dose Response Studies. Environ. Health Persp. 111:1530-1549
8. Kanno J, Onyon L, Peddada S, Ashby J, Jacob E, Owens W (2003). The OECD program to validate the rat uterotrophic assay: Phase Two – Coded Single Dose Studies. Environ. Health Persp. 111:1550-1558.
9. Owens W, Ashby J, Odum J, Onyon L (2003). The OECD program to validate the rat uterotrophic assay: Phase Two – Dietary phytoestrogen analyses. Environ. Health Persp. 111:1559-1567.
10. Ogasawara Y, Okamoto S, Kitamura Y, Matsumoto K (1983). Proliferative pattern of uterine cells from birth to adulthood in intact, neonatally castrated, and/or adrenalectomized mice assayed by incorporation of [I^{125}]iododeoxyuridine. Endocrinology 113:582-587.
11. Branham WS, Sheehan DM, Zehr DR, Ridlon E, Nelson CJ (1985). The postnatal ontogeny of rat uterine glands and age-related effects of 17β-estradiol. Endocrinology 117:2229-2237.
12. Schlumpf M, Berger L, Cotton B, Conscience-Egli M, Durrer S, Fleischmann I, Haller V, Maerkel K, Lichtensteiger W (2001). Estrogen active UV screens. SÖFW-J. 127:10-15.

13. Zarrow MX, Lazo-Wasem EA, Shoger RL (1953). Estrogenic activity in a commercial animal ration. Science 118:650-651.
14. Drane HM, Patterson DSP, Roberts BA , Saba N (1975). The chance discovery of oestrogenic activity in laboratory rat cake. Fd. Cosmet. Toxicol. 13:425-427.
15. Boettger-Tong H, Murphy L, Chiappetta C, Kirkland JL, Goodwin B, Adlercreutz H, Stancel GM, Makela S (1998). A case of a laboratory animal feed with high estrogenic activity and its impact on *in vivo* responses to exogenously administered estrogens. Environ. Health Perspec. 106:369-373.
16. OECD (2006). Validation of the Uterotrophic Assay in mice by bridging data to rats
17. Degen GH, Janning P, Diel P, Bolt HM (2002). Estrogenic isoflavones in rodent diets. Toxicol. Lett. 128:145-157.
18. Wade MG, Lee A, McMahon A, Cooke G, Curran I (2003). The influence of dietary isoflavone on the uterotrophic response in juvenile rats. Food Chem. Toxicol. 41:1517-1525.
19. Yamasaki K, Sawaki M, Noda S, Wada T, Hara T, Takatsuki M (2002). Immature uterotrophic assay of estrogenic compounds in rats given different phytoestrogen content diets and the ovarian changes in the immature rat uterotrophic of estrogenic compounds with ICI 182,780 or antide. Arch. Toxicol. 76:613-620.
20. Thigpen JE, Haseman JK, Saunders HE, Setchell KDR, Grant MF, Forsythe D (2003). Dietary phytoestrogens accelerate the time of vaginal opening in immature CD-1 mice. Comp. Med. 53:477-485.
21. Ashby J, Tinwell H, Odum J, Kimber I, Brooks AN, Pate I, Boyle CC (2000). Diet and the aetiology of temporal advances in human and rodent sexual development. J. Appl. Toxicol. 20:343-347.
22. Thigpen JE, Lockear J, Haseman J, Saunders HE, Caviness G, Grant MF, Forsythe DB (2002). Dietary factors affecting uterine weights of immature CD-1 mice used in uterotrophic assays. Cancer Detect. Prev. 26:381-393.
23. Thigpen JE, Li L-A, Richter CB, Lebetkin EH, Jameson CW (1987). The mouse assay for the detection of estrogenic activity in rodent diets: I. A standardized method for conducting the mouse assay. Lab. Anim. Sci. 37:596-601.
24. OECD (2001). Acute oral toxicity – up-and-down procedure. OECD Guideline for the testing of chemicals, No. 425.
25. OECD (2000). Guidance document on the recognition, assessment and use of clinical signs as humane endpoints for experimental animals used in safety evaluation. Environmental Health and Safety Monograph Series on Testing and Assessment, No. 19. ENV/JM/MONO(2000)7.
26. OECD (2001). Guidance document on acute oral toxicity. Environmental Health and Safety Monograph Series on Testing and Assessment, No. 24. ENV/JM/MONO(2001)4.
27. Bulbring, E., and Burn, J.H. (1935). The estimation of oestrin and of male hormone in oily solution. J. Physiol. 85: 320 - 333.

28. Dorfman, R.I., Gallagher, T.F. and Koch, F.C (1936). The nature of the estrogenic substance in human male urine and bull testis. Endocrinology 19: 33 - 41.

29. Reel, J.R., Lamb IV, J.C. and Neal, B.H. (1996). Survey and assessment of mammalian estrogen biological assays for hazard characterization. Fundam. Appl. Toxicol. 34: 288 - 305.

30. Jones, R.C. and Edgren, R.A. (1973). The effects of various steroid on the vaginal histology in the rat. Fertil. Steril. 24: 284 – 291.

31. OECD (1982). Organization for Economic Co-operation and Development - Principles of Good Laboratory Practice, ISBN 92-64-12367-9, Paris.

32. R.I. Dorfman (1962). Methods in Hormone Research, Vol. II, Part IV: Standard Methods Adopted by Official Organization. New York, Academic Press.

33. J. E. Thigpen et al. (2004). Selecting the appropriate rodent diet for endocrine disruptor research and testing studies. ILAR J 45(4): 401-416.

34. L.E. Gray and J. Ostby (1998). Effects of pesticides and toxic substances on behavioral and morphological reproductive development: endocrine versus non-endocrine mechanism. Toxicol Ind Health. 14 (1-2): 159-184.

35. Booth AN, Bickoff EM and Kohler GO (1960). Estrogen-like activity in vegetable oils and mill by-products. Science 131:1807-1808.

36. Kato H, Iwata T, Katsu Y, Watanabe H, Ohta Y, Iguchi T (2004). Evaluation of estrogenic activity in diets for experimental animals using in vitro assay. J. Agric Food Chem. 52, 1410-1414.

37. OECD Test No. 440: Uterotrophic Bioassay in Rodents: A Short Term Test for (Anti)Androgenic Properties.

Appendix 1

Definitions

Antiestrogenicity is the capability of a chemical to suppress the action of estradiol 17ß in a mammalian organism.

Date of birth is postnatal day 0.

Dosage is a general term comprising of dose, its frequency and the duration of dosing.

Dose is the amount of test substance administered. For the Uterotrophic Assay, the dose is expressed as weight of test substance per unit body weight of test animal per day (*e.g.,*mg/kg body weight/day).

Maximum Tolerable Dose (MTD) is the highest amount of a substance that, when introduced into the body does not kill test animals (denoted by DL_0) (IUPAC, 1993).

Estrogenicity is the capability of a chemical to act like estradiol 17ß in a mammalian organism.

Postnatal day X is the Xth day of life after the day of birth.

Sensitivity is the proportion of all positive/active substances that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method.

Specificity is the proportion of all negative/inactive substances that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method.

Uterotrophic is a term used to describe a positive influence on the growth of uterine tissues.

Validation is a scientific process designed to characterize the operational requirements and limitations of a test method and to demonstrate its reliability and relevance for a particular purpose.

VMG mamm is the Validation Management Group on Mammalian Testing and Assessment.