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Part VII

Environmental Protection Agency

**Endocrine Disruptor Screening Program
(EDSP); Announcing the Availability of
the Tier 1 Screening Battery and Related
Test Guidelines; Notice**

ENVIRONMENTAL PROTECTION AGENCY

[EPA-HQ-OPPT-2008-0521; FRL-8432-6]

Endocrine Disruptor Screening Program (EDSP); Announcing the Availability of the Tier 1 Screening Battery and Related Test Guidelines

AGENCY: Environmental Protection Agency (EPA).

ACTION: Notice.

SUMMARY: EPA is announcing the availability of the Endocrine Disruptor Screening Program (EDSP) Tier 1 battery of assays and availability of test guidelines (protocols) for conducting the assays included in the battery. The EDSP was established under section 408(p) of the Federal Food, Drug, and Cosmetic Act (FFDCA), which directed EPA "to develop a screening program. . .to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen, or such other endocrine effect as the Administrator may designate." Coordinated by EPA, several *in vitro* and *in vivo* screening assays were developed, standardized, and validated to identify the potential of a chemical substance to interact with the estrogen, androgen or thyroid (E, A, or T) hormonal systems. Test chemicals that were thought to be potentially interactive as well as non-interactive with the E, A, or T hormonal systems were used to evaluate feasibility of the protocols, relevance of endpoints and reliability of results within and among independent contract laboratories. Subsequent independent peer review of individual assays helped to clarify the strengths and limitations of each assay and define their modes of action involving the E, A, or T hormonal systems within the context of the EDSP Tier 1 battery. EPA submitted a proposed battery of assays to the Federal Insecticide, Fungicide, and Rodenticide Act Scientific Advisory Panel (FIFRA SAP) for external peer review in March 2008. Based on the SAP recommendation, which found the proposed battery adequate to begin screening chemicals to detect the potential for interaction with the E, A, or T hormonal systems, EPA is finalizing the Tier 1 battery as proposed.

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SUPPLEMENTARY INFORMATION:

I. General Information

A. Does this Action Apply to Me?

This action is directed to the public in general. You may be potentially affected by this action if you produce, manufacture, use, consume, work with, or import pesticide chemicals. To determine whether you or your business may be affected by this action, you should carefully examine section 408(p) of FFDCA, 21 U.S.C. 346a(p).

Potentially affected entities may include, but are not limited to:

- Chemical manufacturers, importers and processors (NAICS code 325), e.g., persons who manufacture, import or process chemical substances.
 - Pesticide, fertilizer and other agricultural chemical manufacturers (NAICS code 3253), e.g., persons who manufacture, import or process pesticide, fertilizer and agricultural chemicals.
 - Scientific research and development services (NAICS code 5417), e.g., persons who conduct testing of chemical substances for endocrine effects.
- This listing is not intended to be exhaustive, but rather provides a guide for readers regarding entities likely to be affected by this action. Other types of entities not listed in this unit could also be affected. If you have any questions regarding the applicability of this action to a particular entity, consult the person listed under **FOR FURTHER INFORMATION CONTACT**.

B. How Can I Get Copies of this Document and Other Related Information?

1. *The Tier 1 battery announcement.* EPA has established a docket for this action under docket identification (ID) number EPA-HQ-OPPT-2008-0521. All documents in the docket are listed in the docket's index available at <http://www.regulations.gov>. Although listed in the index, some information is not publicly available, e.g., Confidential Business Information (CBI) or other information whose disclosure is restricted by statute. Certain other material, such as copyrighted material, will be publicly available only in hard copy. Publicly available docket materials are available electronically at <http://www.regulations.gov>, or, if only available in hard copy, at the OPPT Docket. The OPPT Docket is located in the EPA Docket Center (EPA/DC) at Rm. 3334, EPA West Bldg., 1301 Constitution Ave., NW., Washington, DC. The EPA/DC Public Reading Room hours of operation are 8:30 a.m. to 4:30

p.m., Monday through Friday, excluding Federal holidays. The telephone number of the EPA/DC Public Reading Room is (202) 566-1744, and the telephone number for the OPPT Docket is (202) 566-0280. Docket visitors are required to show photographic identification, pass through a metal detector, and sign the EPA visitor log. All visitor bags are processed through an X-ray machine and subject to search. Visitors will be provided an EPA/DC badge that must be visible at all times in the building and returned upon departure.

2. *The EDSP test guidelines.* For additional information about the test guidelines and to access the guidelines electronically, go to <http://www.epa.gov/oppts> and select "Test Methods & Guidelines" on the left side navigation menu. You may also access the EDSP guidelines in <http://www.regulations.gov> under docket ID number: EPA-HQ-OPPT-2009-0576.

II. Endocrine Disruptor Screening Program (EDSP)

The Food Quality Protection Act (FQPA) of 1996, which amended the Federal Food, Drug, and Cosmetic Act (FFDCA), directs EPA to:

develop a screening program, using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen, or such other endocrine effect as the Administrator may designate. (21 U.S.C. 346a(p)).

In 1998, after considering public comments, external consultations and peer review, EPA established the EDSP as a two-tiered approach to implement the statutory testing requirements of FFDCA section 408(p) (21 U.S.C. 346a). For additional information about the history of EDSP go to <http://www.epa.gov/endo>.

Under Tier 1 of the EDSP, the screening battery will be used to identify substances that have the potential to interact with the estrogen (E), androgen (A), or thyroid (T) hormonal systems (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 E, A, or T hormonal 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 the E, A, or T effect.

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.

III. Assay Validation Process

The use of validated assays is required by section 408(p) of the FFDCA. The process of assay validation used by the EDSP is based, in part, on principles developed by the Interagency Coordinating Committee for the Validation of Alternative Methods (ICCVAM). In addition to the ICCVAM approach to assay validation in the United States, EPA considered the European approach by the European Center for the Validation of Alternative Methods (ECVAM), as well as the international approach by the Organization for Economic Co-operation and Development (OECD) since some screening assays (Amphibian Metamorphosis, Estrogen Receptor Transcriptional Activation, Fish Short-term Reproduction, Hershberger, and Uterotrophic assays) involved a collaborative validation effort with OECD. Validation is still an ongoing process for EDSP Tier 2 tests, which are expected to be completed in 2011.

The purpose of assay validation is to establish relevance and reliability. In the context of the EDSP Tier 1 screening battery, relevance is the ability of an assay or endpoints within an assay to detect chemicals with the potential to interact with one or more of the E, A, or T hormonal systems, whereas reliability is the reproducibility of those results within and between or among laboratories. Throughout the validation process of individual assays between 2001 and 2007 and in accord with the FACA, the EDSP sought guidance on protocol development, selection of test chemicals, and interpretation of results from federal advisory committees such as the Endocrine Disruptor Methods Validation Sub-committee (EDMVS), Endocrine Disruptor Methods Validation Advisory Committee (EDMVAC) and the FIFRA SAP. Each committee meeting provided an opportunity for public comment. Materials from these meetings are available on the Agency's website.

In the **Federal Register** of July 13, 2007 (72 FR 13672) (FRL 8238-4), EPA announced the approach it intends to take for conducting peer reviews of the Tier 1 screening assays and Tier 2 testing assays that are being validated, as well as EPA's approach for conducting the peer review of the Tier 1 battery. For the Tier 1 screening assays, EPA followed a five-stage assay validation process as summarized:

1. *Test development.* A Detailed Review Paper (DRP) or an analogous document (e.g., Background Review Document) was first prepared as a comprehensive document to discuss the purpose of a proposed assay, the context in which it would be used, and the scientific basis on which an initial protocol design would be developed.

2. *Pre-validation.* With selected test chemicals, the initial protocols were refined, optimized, standardized and assessed for feasibility, transferability and performance in a number of independent laboratories based, in part, on the degree of intra-laboratory variability associated with relevant endpoints.

3. *Inter-laboratory validation.* With standardized protocols, each assay was assessed primarily for reliability (i.e., inter-laboratory variability) by running the same test chemicals in multiple, independent laboratories. Assay performance criteria and processes for data interpretation were also optimized during this stage.

4. *Peer review.* An independent scientific review of individual screening assays initially proposed for the EDSP Tier 1 battery was conducted by qualified experts using two processes. EPA conducted the peer review for six assays (i.e., the Androgen Receptor Binding, Aromatase, Estrogen Receptor Binding, Pubertal female, Pubertal male, and Steroidogenesis assays) in accord with EPA's Peer Review Handbook. The EDSP peer review process was published in a **Federal Register** notice of July 13, 2007 (72 FR 38577). In general, EPA prepared an Integrated Summary Report (ISR) for each of these six screening assays. Each ISR served as the main document during peer review, providing an overview of development, pre-validation and inter-laboratory testing of individual assays. Coordinated by an EPA contractor, each peer reviewer responded independently to a list of charges prepared by EPA. The peer reviewers' comments were compiled in a peer review record for each assay and submitted to the Agency. The five assays that were validated in collaboration with OECD (i.e., the Amphibian Metamorphosis, Estrogen Receptor Transcriptional Activation,

Fish Short-term Reproduction, Hershberger, and Uterotrophic assays) were peer reviewed by qualified experts using the OECD process, which includes the preparation of a peer review summary report for each of these five screening assays. EPA did not conduct a separate individual assay peer review of these assays. Assessment of the EDSP peer review records and OECD peer review summary reports for each screening assay provided an opportunity for EPA to clarify the strengths and limitations of each assay as well as the complementary nature among assays. This information was then used for selecting assays to include in the Tier 1 screening battery for SAP review.

5. *Regulatory acceptance.* Acknowledgment by EPA that the Agency accepts a test method for regulatory use. EPA adopted the EDSP Tier 1 screening battery (Table 1) in accord with recommendations made by the SAP who found the proposed suite of assays adequate to begin screening for E, A, or T effects as detailed in a final report to the Agency which can be found at the SAP website <http://www.epa.gov/scipoly/sap/meetings/2008/march/minutes2008-03-25.pdf>. The SAP report is summarized in Unit III.E.

IV. Peer Review of the Proposed EDSP Tier 1 Screening Battery

EPA announced the independent scientific peer review of the proposed EDSP Tier 1 screening battery by the FIFRA SAP in the **Federal Register** notice of January 24, 2008 (73 FR 4216) (FRL-8348-6), which was held March 25-26, 2008. The SAP serves as the primary peer review mechanism of EPA's Office of Prevention, Pesticides and Toxic Substances (OPPTS) and it provides comments, evaluations and recommendations to improve the effectiveness and quality of analyses made by Agency scientists. EPA provided the SAP with a technical review document that served as a basic guide and source of information about the proposed Tier 1 battery. Respective ISRs or summary reports and reviewer responses from individual review of each assay were also provided to the SAP as additional material for reviewing the proposed battery. The SAP was charged with commenting on whether the collection of assays comprising the proposed battery fulfills its intended purpose to identify the potential of a chemical to interact with the E, A, or T hormonal systems. For consideration during the peer review, the SAP received oral and written comments from EPA, the general public and

various stakeholders. The final SAP report to the Agency is summarized in this Unit and a copy can be found at the SAP website <http://www.epa.gov/scipoly/sap/meetings/2008/march/minutes2008-03-25.pdf>. EPA provided the SAP with two main charges:

1. Please comment on the ability of the proposed Tier 1 screening battery (Table 1 in Unit V.A.) to provide sufficient information to determine whether or not a substance potentially interacts with the E, A, or T hormonal systems based on the modes of action covered within the battery (Table 2 in Unit V.B.).

2. EPA proposed a Tier 1 screening battery that includes assays that are complementary in nature (i.e., the strengths of one assay offset the limitations of another) in their coverage of the E, A, or T hormonal systems, albeit by different taxa, life-stages, endpoints, exposure and use of *in vitro* and *in vivo* methods executed at different levels of biological organization (i.e., cellular and whole organism).

a. Please comment on how well the proposed battery minimizes the potential for “false negatives” and “false positives.”

b. Are there any unnecessary redundancies for Mode of Action (MOA) across the battery?

c. Please comment on whether a different combination of validated assays would be more effective in achieving the purpose of the battery than that proposed by EPA. In response to the charges, the panel discussed assays individually and as a complete set of assays regarding the ability to detect interactions with the E, A, or T hormonal systems with few false positives and false negatives as possible. The conclusions drawn upon completion of this review as quoted from the SAP report were:

- Chemicals testing positive in the battery of Tier 1 assays would be identified as potential estrogenic,

androgenic and thyroid hormone active substances.

- The ability to identify endocrine active substances is enhanced in the Tier 1 battery because the tests provide adequate replication and redundancy.

- It was clear that the inclusion of apical assays of amphibian metamorphosis and fish short-term reproduction were important to detect endocrine active substances that may operate by mechanisms of action yet to be discovered.

- The 15-day adult male rat assay proposed during some public comments would not be an appropriate substitute for the male and female pubertal assays because the pubertal assays provide for differences between the sexes and provide the only approach to testing for organizational effects during development.

Overall, the SAP agreed that the battery of Tier 1 assays in Table 1 in Unit V.A. is appropriate to begin screening for chemical substances that may interact with the E, A, or T hormonal systems. In addition, the SAP recommended that EPA continue to develop, refine and review the Tier 1 screening battery as the state of the science advances and to consider other hormonal systems that may be affected by exposure to environmental chemicals. After EPA considered the SAP final report and public comments, the Agency adopted the EDSP Tier 1 screening battery presented in Table 1 in Unit V.A.

V. The Final EDSP Tier 1 Screening Battery

A. Assays Included in the Tier 1 Screening Battery

The EDSP Tier 1 battery with its suite of *in vitro* and *in vivo* screening assays is indicated in Table 1 of this unit. The following discussion provides an overview of the nature and complementary aspects within and among assays that were selected to include in the battery.

TABLE 1.—SCREENING ASSAYS IN THE EDSP TIER 1 BATTERY

<i>In vitro</i>	<i>In vivo</i>
Estrogen receptor (ER) binding – rat uterine cytosol	Uterotrophic (rat)
Estrogen receptor α (hER α) transcriptional activation – Human cell line (HeLa-9903)	Hershberger (rat)
Androgen receptor (AR) binding – rat prostate cytosol	Pubertal female (rat)
Steroidogenesis – Human cell line (H295R)	Pubertal male (rat)
Aromatase – Human recombinant microsomes	Amphibian metamorphosis (frog)
	Fish short-term reproduction

B. Basis for Assay Selection for the Tier 1 Screening Battery

The EDSP Tier 1 battery was designed to work as a whole with all of the screening assays. The basis for selecting an assay to include in the battery involved two principal aspects: (1) The capacity of an assay to detect estrogen- and androgen-mediated effects by various modes of action including receptor binding (agonist and antagonist) and transcriptional activation, steroidogenesis, and hypothalamic-pituitary-gonadal (HPG) feedback, and (2) the degree that *in vitro* and *in vivo* assays complemented one another in the battery as summarized in Table 2 of this unit. In addition, rodent and amphibian *in vivo* assays were selected for the proposed battery based on their capacity to detect direct and indirect effects on thyroid function (hypothalamic-pituitary-thyroidal, HPT, feedback). Thus, the robustness of the proposed battery is based on the strengths of each individual assay and their complementary nature within the battery to detect effects on the E, A, or T hormonal systems.

TABLE 2.—COMPLEMENTARY MODES OF ACTION AMONG SCREENING ASSAYS IN THE EDSP TIER 1 BATTERY

Screening Assays	Modes of Action							
	Receptor Binding				Steroidogenesis		HPG ³ Axis	HPT ³ Axis
	E ²	Anti-E	A ²	Anti-A	E ²	A ²		
<i>In vitro</i>								
ER Binding ¹	•	• ⁴						
ER α Transcriptional Activation	•							
AR Binding ¹			•	•				

TABLE 2.—COMPLEMENTARY MODES OF ACTION AMONG SCREENING ASSAYS IN THE EDSP TIER 1 BATTERY—Continued

Screening Assays	Modes of Action							
	Receptor Binding				Steroidogenesis		HPG ³ Axis	HPT ³ Axis
	E ²	Anti-E	A ²	Anti-A	E ²	A ²		
Steroidogenesis H295R					•	•		
Aromatase Re-combinant					•			
<i>In vivo</i>								
Uterotrophic	•							
Hershberger			•	•				
Pubertal Male			•	•		•	•	•
Pubertal Female	•	• ⁴			•		•	•
Amphibian Metamorphosis								•
Fish Short-term Reproduction (male & female)	•	• ⁴	•	•	•	•	•	•

¹Estrogen and Androgen Receptor binding

²Estrogen and Androgen, respectively

³Hypothalamic-pituitary-gonadal or -thyroidal axis

⁴Estrogen receptor antagonists were not tested during the validation process, but the assay is expected to detect anti-estrogens.

1. *Assays for detection of compounds that affect the estrogen signaling pathway.* The earliest concern for endocrine disruptors was related to environmental chemicals that could bind to the estrogen receptor and thereby interfere with the estrogen signaling pathway. Estrogen is important for reproductive function in males and females, including sexual differentiation of the brain and development of secondary female sex characteristics. In addition, estrogen is involved in the structural and functional development of other bodily systems across genders and for maintaining homeostasis.

Five screening assays within the EDSP Tier 1 battery are capable of detecting whether or not a chemical interacts with the estrogen hormonal system and include: (1) Estrogen receptor (ER) binding; (2) ER transcriptional activation (ERTA); (3) uterotrophic; (4) pubertal female; and (5) fish short-term reproduction. Of the five assays, the two *in vitro* assays (ER binding and ERTA) identify the ability of the test chemical to interact with the estrogen receptor, thus providing mechanistic as well as some functional information. The three *in vivo* assays provide evidence for the effects of the chemical following exposure via subcutaneous injection (uterotrophic), oral gavage (pubertal female), and aquatic medium (fish short-term

reproduction). The different routes of exposure can provide information relevant to the effects of Absorption, Distribution, Metabolism and Excretion (ADME). Interpreting the results of the suite of estrogen-detecting assays within the battery is accomplished by examining the results of all the assays together using a weight-of-evidence approach. A brief description as well as value of each of the five assays for detection of compounds that can potentially interact with the estrogen signaling pathway is provided.

a. *ER binding assay.* The ER receptor binding assay utilizing rat uterine cytosol (RUC) is a rapid *in vitro* assay that measures the binding affinity of a chemical to the estrogen receptor. Although the ER RUC assay cannot distinguish between chemicals with agonistic, antagonistic and mixed activity or provide functional, transcriptional information, the technical simplicity of this assay is important for screening large numbers of chemicals. Thus, the assay is a valuable asset for identifying chemicals that can compete with endogenous estrogen for ER binding. The practical use of this assay and its relevance to *in vivo* effects is well documented in the scientific literature.

b. *ER transcriptional activation assay.* The ERTA assay is a method to detect the interaction and functional effect of a chemical on the estrogen receptor. The

ERTA assay is based on the expression of a reporter gene induced by a chemical following ligand-receptor binding and subsequent transcriptional activation. As part of the Endocrine Disruption Testing and Assessment Task Force activity under the OECD Test Guidelines Program, Chemical Evaluation and Research Institute (CERI) of Japan developed and validated a stably transfected transcriptional activation assay using the hER-HeLa-9903 (HeLa) cell line with the ER α . Although the ERTA assay is still being evaluated to detect ER antagonists, it complements the ER binding assay within the EDSP Tier 1 battery (Table 2 of this unit) and provides a functional component for identifying ER α agonists.

c. *Uterotrophic assay.* The uterotrophic assay is an *in vivo* assay that was designed to detect estrogenic activity of a chemical through uterine hypertrophy/hyperplasia. For the EDSP, it is preferred that the assay be conducted using ovariectomized female rats exposed via subcutaneous administration because of increased specificity and information gained on specific estrogen-related responses in the absence of first-pass liver metabolism. The sole endpoint is a change in uterine weight (i.e., increase) in response to estrogen-induced water imbibition and hypertrophy. Thus, data from the uterotrophic assay can complement the *in vitro* ER and ERTA

assays (Table 2 of this unit) where metabolic activity is either non-detectable (ER binding) or minimal (ERTA assay) and provide differential information in relation to first-pass effects through the liver since the uterotrophic assay uses subcutaneous exposure compared to the pubertal female assay that uses oral exposure.

d. *Pubertal female assay.* The pubertal female assay is an *in vivo* assay with an intact HPG axis that is sensitive to estrogens such that chemicals with estrogenic activity hasten the age of vaginal opening (VO). For example, when a selective estrogen receptor modulator (SERM) with mixed agonistic/antagonistic activity was examined in the pubertal female assay during the validation process, VO occurred earlier in the treated group than in controls. Although estrogens also accelerate the age at first estrus, the interval may or may not correspond to the time of VO. Nonetheless, the estrogen agonistic effect of the SERM test chemical was substantiated within the assay by increased uterine weight. Notably, the change in time to VO is not necessarily a specific ER binding effect; however, evaluation of results from the pubertal female and uterotrophic assays and *in vitro* ER and ERTA assays may allow distinction between an ER mechanism of action or other steroidogenic and HPG mechanisms. Since oral gavage is the route of exposure for the pubertal female assay and subcutaneous exposure is indicated for the uterotrophic assay, these screening assays can contribute differential information on specific estrogen-related responses taking into account ADME, which is crucial to the identification of compounds that need to be metabolized in order to interact with the estrogen pathway.

e. *Fish short-term reproduction assay.* The fish short-term reproduction assay with fathead minnows is designed to detect changes in spawning, reproductive morphology, and specific biochemical endpoints that reflect disturbances along the HPG axis in response to estrogen agonists and antagonists. Collectively, the endpoints allow for inferences with regard to possible endocrine disturbances involving the estrogen hormonal pathway. Vitellogenin is an egg yolk protein in which synthesis and secretion is primarily controlled through estrogen-receptor interaction. There are commercially available immunoassay kits specific to the fathead minnow that have made vitellogenin production readily measurable; hence, it is a well-established endpoint. Induction of vitellogenin in male fish is

an extremely sensitive and specific indication of ER agonists since males have very low circulating concentrations of endogenous estrogen. Reproductively active females have moderate circulating concentrations of vitellogenin, which can be decreased by ER antagonists. Estrogens and anti-estrogens can also affect egg production in the fish assay. Changes in fecundity combined with alterations in gonadal histopathology provide a good indication of reproductive health and have been demonstrated to be sensitive to estrogenic and anti-estrogenic exposures.

2. *Assays for detection of compounds that affect the androgen signaling pathway.* Androgens are critical for sexual differentiation and development of secondary sex characteristics in the male, as well as for a wide variety of reproductive and non-reproductive functions in both males and females. Four screening assays within the EDSP Tier 1 battery are capable of detecting whether or not a chemical interacts with the androgen hormonal pathway. Together these assays are expected to detect chemicals with androgenic and anti-androgenic activity and include: (1) AR binding; (2) Hershberger; (3) pubertal male; and (4) fish short-term reproduction assays.

Of the four assays, the one *in vitro* assay (AR binding) provides mechanistic information at the receptor level, while the three *in vivo* assays provide evidence for the effects of a chemical on the reproductive system at the whole organism level. Again, interpreting the results of the suite of androgen-detecting assays within the battery is accomplished by examining the results of all the assays together using a weight-of-evidence approach. A brief description as well as the value of each of the assays for detection of compounds that can potentially interact with the androgen signaling pathway is provided.

a. *AR binding assay.* The androgen receptor binding assay (AR binding), utilizing rat prostate cytosol, is a rapid *in vitro* assay that measures the affinity of a test chemical for the androgen receptor. As with the ER binding assay, the AR binding assay does not assess functional, transcriptional activity. Nevertheless, the assay's technical simplicity along with its rapid turnaround time is conducive for screening large numbers of chemicals. Thus, the assay is a valuable asset for identifying chemicals that have androgenic or anti-androgenic activity that can compete with endogenous androgens for receptor recognition. In addition to detecting androgen agonists and antagonists, the

AR binding assay is complementary in supporting an agonistic or antagonistic result in the Hershberger assay (Table 2 of this unit).

b. *Hershberger assay.* The Hershberger assay is a short-term *in vivo* screen that uses castrated peripubertal male rats exposed via oral gavage to assess biological activities consistent with either androgen agonists or antagonists (or 5 α -reductase inhibitors) by measuring changes in the weights of five androgen-dependent tissues: (i) Ventral prostate; (ii) seminal vesicle; (iii) levator ani-bulbocavernosus (LABC) muscle complex; (iv) Cowper's glands; and (v) glans penis. An increase in tissue weights is diagnostic of androgenic activity. In contrast, an anti-androgenic chemical will block any increase in tissue weights when co-administered with a potent androgen such as testosterone propionate. The Hershberger assay contributes to the battery by providing information on androgen-related responses that is complimentary with the intact pubertal male and fish short-term reproduction assays as well as AR binding and steroidogenesis assays (Table 2 of this unit).

c. *Pubertal male assay.* The male pubertal assay is an *in vivo* test system with an intact HPG axis that is sensitive for detecting chemicals that act as androgens or anti-androgens or interfere with androgen synthesis. Importantly, as an *in vivo* assay, it can detect chemicals which require metabolism in order to interact with the androgen hormonal system because of its oral route of exposure. The pubertal male assay is reproducible and sensitive for chemicals that alter androgenic hormone action which is necessary for preputial separation (PPS), associated with the onset of puberty, and growth and development of androgen dependent tissues (e.g., testes, prostate, seminal vesicles). The pubertal male assay is complementary with the Hershberger and AR binding assays (Table 2 of this unit).

d. *Fish short-term reproduction.* Secondary sex characteristics of fathead minnows are affected by androgenic and anti-androgenic substances. Specifically, females will develop external male secondary sex characteristics (nuptial tubercles) when exposed to an AR agonist. Not only is this endpoint specific for this mode of action, it is highly sensitive since female fathead minnows typically do not express these characteristics. In contrast, AR antagonists decrease the expression of male secondary sex characteristics in male fathead minnows. Changes in secondary sex

characteristics in fathead minnows are biologically relevant, unique, robust and reproducible. Androgens and anti-androgens also effectively inhibit egg production in the fish assay, with corresponding alterations in gonad histopathology. The fish short-term reproduction assay is complementary with *in vitro* assays and other *in vivo* assays (Table 2 of this unit).

3. *Assays for detection of compounds that affect steroid synthesis.* Numerous environmental compounds have been shown to interfere with the steroidogenic pathways for estrogens (e.g., estradiol) and androgens (e.g., testosterone) in various *in vitro* and *in vivo* test systems. In this regard, a number of *in vitro* assays for steroidogenesis were considered for the battery with the decision to include the H295R cell line as it offers the potential to identify chemicals that induce or inhibit estradiol and testosterone synthesis. In addition, since many environmental compounds are known to inhibit the conversion of androgen substrates to estrogen, a decision was made to include a human recombinant aromatase assay. A combination of *in vitro* and *in vivo* assays is expected to provide complementary information to be used in a weight of evidence approach for making decisions as to whether or not a compound interferes with the estrogen or androgen hormonal signaling pathways and includes: (1) Steroidogenesis; (2) aromatase; (3) pubertal female; (4) pubertal male; and (5) fish short-term reproduction assays. A brief description as well as the value of each of the five assays for detection of compounds that can potentially affect steroidogenesis is provided.

a. *H295R for steroidogenesis.* H295R is a human adrenocortical carcinoma cell line that possesses all of the key enzymes involved in the steroidogenic pathways. The assay provides a straightforward, inexpensive and specific way to detect chemicals that affect steroid hormone synthesis through enzyme induction or inhibition. The measurement of estradiol and testosterone in culture media are the essential hormonal endpoints in this assay. Chemical exposure may inhibit enzymes in the pathway, leading to decreased production of one or both of the hormonal endpoints or stimulate enzymes, leading to increased production of one or both of the endpoints.

b. *Human recombinant aromatase.* The recombinant aromatase assay using human recombinant microsomes is an inexpensive and rapid *in vitro* method to detect chemicals that inhibit aromatase activity, thus blocking the

conversion of androgens to estrogens. The aromatase and H295R steroidogenesis assays are complementary within the Tier 1 battery (Table 2 of this unit) and are the only *in vitro* assays that have been shown to detect the activity of chemicals that weakly inhibit aromatase and estrogen synthesis.

c. *The pubertal female and pubertal male assays.* A chemical that interferes with endogenous steroid hormone production by the ovaries or testes will produce changes in the numerous hormone-dependent endpoints measured by the female and male pubertal assays, respectively. Together, the pubertal female and male assays and H295R steroidogenesis and aromatase assays are complementary within the Tier 1 battery (Table 2 of this unit) and provide diagnostic information to discern impaired estrogen and androgen production.

d. *Fish short-term reproduction.* Interference in the steroid synthetic pathways is detected by several endpoints in the fish assay. Proliferation of testicular interstitial cells (males), decreased circulating concentrations of reproductive steroids (males and females) and vitellogenin (females), and impaired egg production (females) would all signal potential alteration in steroid synthesis.

4. *Assays for detection of chemicals that affect the HPG axis.* Environmental compounds have been found to interfere with endocrine function of the ovaries and testes by altering the hypothalamic regulation of pituitary hormone synthesis and secretion. By this mode of action, it has been shown that many of these same chemicals can interfere with reproductive development and fertility. The EDSTAC recommended and EPA agreed that the effect of environmental chemicals on the hypothalamic-pituitary-gonadal axis (HPG) be evaluated. To address this issue, the Tier 1 battery includes: (1) Pubertal male; (2) pubertal female; and (3) fish short-term reproduction assays.

The EDSP Tier 1 battery is designed to use the combined results of the *in vitro* and *in vivo* assays included in the battery to differentiate between hormone-receptor binding and non-receptor binding at the cellular and whole organism levels that may involve the HPG axis.

Hypothetically, if a test chemical is found to delay PPS and VO in the *in vivo* pubertal male and female assays, respectively, but none of the *in vitro* assays were altered, it would likely be concluded that the delay in male and female puberty is due to impaired hypothalamic-pituitary function. This

scenario has been demonstrated in the pubertal male and female assays with compounds that act on the central nervous system and alter gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH).

The fish short-term reproduction assay with fathead minnows is designed to detect changes in spawning, morphology and specific biochemical endpoints that reflect alterations in the HPG axis. Again, the combined results of the *in vitro* and *in vivo* assays included in the battery are to determine and differentiate if an alteration involves the HPG axis, which may be information for Tier 2 testing.

5. *Assays for detection of chemicals that affect the HPT axis.* In addition to identifying environmental compounds that have the potential to alter the hormonal regulation of reproductive function involving the estrogen and androgen hormonal pathways, certain assays included in the EDSP Tier 1 screening battery (Tables 1 and 2 of this unit) will also provide relevant information about the potential of a chemical to interfere with thyroid function. Thyroid hormones (thyroxine, T4 and triiodothyronine, T3) are essential for normal development and maintenance of physiological functions in vertebrates. Delivery of thyroid hormones to tissues and cells is highly regulated throughout life and is governed by complex physiological processes involving the HPT axis, including peripheral organs/tissues. Environmental factors, such as the presence of specific toxicants, can perturb this system at various points of regulation, inducing a variety of responses that can be detected with thyroid-related endpoints in the *in vivo* assays. Three screening assays have been designed for this purpose within the EDSP Tier 1 battery and include: (1) Pubertal female; (2) pubertal male; and (3) amphibian metamorphosis assays. A brief description as well as the value of each of the three assays for detection of compounds that can potentially interfere with thyroid development and function is provided.

a. *Pubertal male and female assays.* The pubertal male and female assays include multiple endpoints that can detect an interaction of a chemical with the thyroid hormonal system, including circulating concentrations of thyroid stimulating hormone (TSH) and T4, thyroid organ weight and histology, and liver weight. Both the male and the female assays have been shown to detect chemicals that act through various thyroid-related mechanisms. The male and female pubertal assays include the same thyroid endpoints; thus,

examining the thyroid axis in both sexes provides the opportunity to detect potential gender differences in response to treatment at a relatively early life stage.

b. *Amphibian metamorphosis assay.* The amphibian metamorphosis assay (AMA) is an *in vivo* screening assay intended to identify substances which interfere with the normal function of the HPT axis. The AMA represents a generalized vertebrate model based on the conserved structure and function of thyroid systems among species. The AMA is based on the principle that the dramatic morphological changes that occur during post-embryonic development are dependent upon the normal functioning of the HPT axis, and that interference with these processes leads to measurable effects. During tadpole metamorphosis, thyroid hormone (TH) influences virtually every tissue in the body initiating diverse morphological, physiological and biochemical changes that include cell proliferation, differentiation and death. The result is *de novo* organ formation, organ loss, and extensive tissue remodeling. Given the dependence of metamorphosis on TH and the strict biochemical control under which these processes occur, the transformations that occur can serve as endpoints representative of thyroid axis function. The primary endpoints in the AMA are the hindlimb length during the developmental stage and the thyroid histology. Each endpoint can be affected by chemicals that interact with the HPT axis. For example, antagonists of thyroid production, iodination and action have been shown to delay development and induce diagnostic lesions in the thyroid gland. Thyroid agonists (e.g., native thyroid hormone) will accelerate development. Additionally, unlike the mammalian assays that have been developed to detect interactions along the HPT axis, the AMA has the ability to detect chemicals that act on peripheral tissues. For example, inhibition of monodeiodinases that transform T4 to T3 can cause asynchronous development, detected by an inability to assign a developmental stage to a tadpole. Knowledge of this mechanism is important because development can be affected without concomitant effects on thyroid histology or circulating thyroid hormone concentrations. Although post-embryonic development is different between mammals and most amphibians (i.e., metamorphosis), there is a high level of evolutionary conservation of the thyroid system and underlying molecular and cellular

pathways among vertebrates. Hence, the AMA, particularly with the use of Anurans, is a general model for evaluating the interaction of chemicals with the HPT axis in the EDSP Tier 1 screening battery. In addition, the results can be used to complement or corroborate results in the pubertal male and female assays (Table 2 of this unit).

VI. Test Guidelines for EDSP Tier 1 Screening Battery

EPA is also announcing the availability of the test guidelines for conducting the assays included in the EDSP Tier 1 Screening Battery (Table 1 in Unit V.A.).

The Androgen Receptor Binding, Aromatase, Estrogen Receptor Binding (Rat Uterine Cytosol), Female Pubertal, Male Pubertal, and Steroidogenesis assays were developed and validated by the Agency.

The Amphibian Metamorphosis, Estrogen Receptor Transcriptional Activation, Fish Short-term Reproduction, Hershberger and Uterotrophic assays were developed and validated using a collaborative process involving EPA's Office of Science Coordination and Policy (OSCP), Office of Research and Development (ORD), and Office of Pesticide Programs (OPP) as well as OECD as previously outlined in a **Federal Register** notice of July 13, 2007 (72 FR 38577) (FRL-8138-4). The process took into account the harmonized testing strategy for the screening and testing of potential endocrine disrupting chemicals and consequences of such a strategy on the development and validation of test guidelines involving regulatory systems for new and existing substances according to OECD's Endocrine Disrupter Testing and Assessment (EDTA) Task Force in 1998.

In both cases, the draft protocols (and all related materials) were made available as part of the independent peer review. The draft protocols were revised to reflect comments received during the peer review process, and have been incorporated into the OPPTS compendium of harmonized test guidelines, under *Series 890-Endocrine Disruptor Screening Program Test Guidelines* as follows:

- 890.1100-Amphibian Metamorphosis (Frog)
- 890.1150-Androgen Receptor Binding (Rat Prostate Cytosol)
- 890.1200-Aromatase (Human Recombinant)
- 890.1250-Estrogen Receptor Binding (Rat Uterine Cytosol)
- 890.1300-Estrogen Receptor Transcriptional Activation (Human Cell Line — HeLa-9903)

- 890.1350-Fish Short-term Reproduction
 - 890.1400-Hershberger (Rat)
 - 890.1450-Female Pubertal (Rat)
 - 890.1500-Male Pubertal (Rat)
 - 890.1550-Steroidogenesis (Human Cell Line — H295R)
 - 890.1600-Uterotrophic (Rat)
- For information on accessing these guidelines see Unit I.B.2.

List of Subjects

Environmental protection, Chemicals, Chemical testing, Endocrine disruptors, Pesticides, Test guideline.

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ENVIRONMENTAL PROTECTION AGENCY

[EPA-HQ-OPP-2009-0634; FRL-8434-8]

Endocrine Disruptor Screening Program; Tier 1 Screening Order Issuing Announcement

AGENCY: Environmental Protection Agency (EPA).

ACTION: Notice.

SUMMARY: This action announces the Agency's initiation of the Endocrine Disruptor Screening Program (EDSP) Tier 1 screening for the first group of 67 chemicals by issuing orders between October 29, 2009, and February 26, 2010, pursuant to the authority provided to EPA under section 408(p)(5) of the Federal Food, Drug, and Cosmetic Act (FFDCA). The EDSP Tier 1 screening data required to satisfy an order are due within 2 years of the date of issuance of the order. This action also provides information for pesticide registrants, manufacturers and importers of inert chemicals used in pesticide products, and the public on how to obtain details about the orders (such as the date of issuance and the recipients), the "Pesticide Inert Ingredients Data Submitters and Suppliers List" (PIIDSSL), and how interested persons other than recipients of test orders may submit other scientifically relevant information on the chemicals subject to the orders.

DATES: Order recipients must respond according to the schedules contained in the order they receive. Persons other than order recipients who wish to submit other scientifically relevant information related to one of the chemical-specific orders should submit