October 10, 2007

Dr. Cliff Gabriel
Director, Office of Science Policy and Coordination
Office of Pollution Prevention and Toxic Substances
USEPA Headquarters, 7201 Ariel Rios Building
1200 Pennsylvania Avenue,
N. W. Washington, DC 20460

Re: Additional Information Relevant to EPA's Peer Review of the 15-Day Intact Adult Male Rat Assay

Dear Dr. Gabriel:

The American Chemistry Council (ACC or the "Council") has played an active role in the development and implementation of EPA's Endocrine Disruptor Screening Program (EDSP) for several years. The Council has consistently supported the Agency's efforts to develop, standardize and validate scientifically robust endocrine screening and testing methods[1]. In that regard, ACC and its members actively participated in Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), and the subsequent federal advisory committee's that the Agency established to provide technical advice and recommendations to EPA concerning the validation of endocrine disruptor screening and testing methods. We acknowledge the Agency's commitment to objectively evaluating each screening and testing method for compliance with the exacting standards of validation established by the scientific community and incorporated into guidance by the ICCVAM and the OECD. The Agency's approach to validation shows due consideration for the scientific standards of validation as well as pragmatism in design to achieve the validation objectives in a manner that reflects appropriate consideration of animal welfare concerns.

Within the Tier 1 endocrine screening battery, EPA's validation studies of the 15-Day Intact Adult Male Rat Assay as a Tier 1 screen are to be commended. This assay has several features that make it particularly suitable in the Tier 1 screening battery. It is designed to run in

[1] The American Chemistry Council (ACC) represents the leading companies engaged in the business of chemistry. ACC members apply the science of chemistry to make innovative products and services that make people's lives better, healthier and safer. ACC is committed to improved environmental, health and safety performance through Responsible Care®, common sense advocacy designed to address major public policy issues, and health and environmental research and product testing. The business of chemistry is a $635 billion enterprise and a key element of the nation's economy. It is one of the nation's largest exporters, accounting for ten cents out of every dollar in U.S. exports. Chemistry companies are among the largest investors in research and development. Safety and security have always been primary concerns of ACC members, and they have intensified their efforts, working closely with government agencies to improve security and to defend against any threat to the nation's critical infrastructure.

parallel with the uterotrophic assay and to complement the Hershberger assay. By comparing the "fingerprint" of the organ weight and hormonal endpoints for an unknown compound to a series of positive controls, the 15-Day Intact Adult Male Rat Assay not only identifies potential endocrine active substances, but aids in the characterization of their mode of action (thyroid, estrogen/anti-estrogen, androgen/anti-androgen, steroidogenesis biosynthesis inhibitors, aromatase inhibitors, dopaminergic effectors, etc.). The Agency's Integrated Summary Report of the Intact Adult Male Rat Assay provides a comprehensive summary of development, standardization and validation studies of this assay conducted to date. (http://www.epa.gov/scipoly/oscpendo/pubs/isr_adultmalerat.pdf)

The Agency's independent peer review of the 15-Day Intact Adult Male Rat Assay should provide meaningful feedback on the strengths and limitations of the validation studies dataset. With respect to this peer review, we feel it is important for the Agency to inform the peer reviewers of the additional validation studies of this assay that are planned to be initiated shortly under the auspices of ACC's Long Range Research Initiative. The goal of these investigations is to expand the dataset of validation studies of the Intact Adult Male Rat Screening Assay in order to more fully ascertain the specificity and sensitivity of this assay. Informing the peer reviewers of these planned studies would only be an informational matter, and should have no effect on the pace of the Agency's peer review of the 15-Day Intact Adult Male Rat Assay.

To expand the validation dataset of the 15-Day Intact Adult Male Rat Assay, ACC anticipates making two contract awards, each to separate testing facilities. Awardees will follow the identical study protocol and evaluate the same compounds at the same dose levels. This will enable comparison of reproducibility across laboratories. The chemicals selected were based upon consideration of: 1) the desire to collect data relevant to evaluating the specificity of the assay (negative control: allyl alcohol) in several labs; 2) the different modes of action which have been underrepresented in previous validation studies of this assay (steroidogenesis inhibition: fadrazole); 3) the importance of collecting data in the assay for chemicals run in related multi-modal endocrine assays (pubertal assays) to facilitate comparisons (thyroid toxicant: DE71); and 4) the need to expand the database to include additional thyroid agents (iopanoate). Further details of these planned studies are contained in the attached Request for Proposals (RfP). The deadline for submission of Proposals was Sept 28, 2007. ACC anticipates making awards within a few weeks and initiating the studies shortly thereafter. The study results should be available in spring of 2008.

The Council appreciates this opportunity to provide input on matters related to the EDSP. Please don't hesitate to call me at (703-741-5210) if you have questions.

Sincerely,

Original Signed By

Richard A. Becker, Ph.D., DABT
Public Health and Science Policy Team

Attachment: ACC's RfP for Validation Studies of the Intact Adult Male Rat Screening Assay

AMERICAN CHEMISTRY COUNCIL
LONG-RANGE RESEARCH INITIATIVE

REQUEST FOR PROPOSALS

RfP Title Validation Studies of the Intact Adult Male Rat Screening Assay

RfP Number MTH-07-09

Proposal Due Date: September 28, 2007

INTRODUCTION

The American Chemistry Council's (ACC) Long-Range Research Initiative (LRI) was created in 1999 to support research to better understand the potential impacts of chemicals on human health (see www.USLRI.org for more information). The LRI supports research in various ways, one of the most important of which is through a competitive research project program of which this RfP is a part.

PROJECT SCOPE

The goal for this project is to expand the dataset of validation studies of the Intact Adult Male Rat Screening Assay in order to more fully ascertain the specificity and sensitivity of this assay. Specifically, the studies of the Intact Adult Male Rat Screening Assay shall follow the standardized protocol, and study data shall be collected and results formatted and analyzed as specified in Attachment 1.

The study results will form part of the validation database for use in assessing the relevance and reliability of this screening method. ACC anticipates making two contract awards, each to separate testing facilities. Each awardee will follow the identical study protocol and evaluate the same compounds at the same dose levels. This will enable comparison of reproducibility across laboratories. In order to satisfy the scientific standards of method validation, it is necessary for ACC in this case to specify the detailed protocol as well as the test articles and dose levels.[1]

After the awardees are identified the Contractor shall prepare the testing facility's draft study protocol, and this shall be submitted to ACC for review.. This request is unusual for LRI RfPs; however, since the purpose of this RfP is to validate an assay protocol, it is essential to be assured that the protocols being tested are identical and match up to previous studies utilizing the exact same standardized protocol (see Attachment 1). Deviations from the standardized protocol could adversely effect the generation of the data needed to compare results across different labs and to judge performance of the method across time. Also, since these studies are to be conducted in accordance with Good Laboratory Practices (GLP) regulations (see FDA, 2006), the awardee's study protocol is the official record of each experimental investigation, and

[1] LRI research RfPs do not identify chemicals to be tested. However, because the purpose of this RfP is to validate a specific test method rather than to do research per se, the RfP must specify the substances to be tested and the test protocol to be validated in this case.

therefore it is critical that the draft study protocols be reviewed in advance of study initiation to assure full concordance with the standardized protocol, included herein as in Attachment 1.. ACC will provide comments on the draft protocol to the Contractor within 2 weeks of receipt. . Because two contractors are likely to be involved, a joint discussion may be needed to harmonize the individual draft protocols. Once agreements are reached, the contractors will finalize their respective study protocols, in accordance with GLP requirements (FDA, 2006). Upon completion of data collection and analysis, the Contractor shall submit a draft final report of the study results to ACC for review and ACC will provide comments to the Contractor within 2 weeks of receipt. These comments are intended to assist the Contractor and to assure that the data are presented in a manner that facilitates comparison with results from other validation studies. They are not requirements for change. The Contractor will then provide the final report to ACC (2 written copies, two electronic copies as pdf files, and the accompanying data as Excel files). The Contractor will also submit the results of the work for publication in a peer-reviewed journal. The ACC has no approval authority for such publications either.

In responding to this RfP, proposals shall include a section describing the activities and associated costs for conduct of the experiments described in this RfP. The substances, dose levels, vehicles and routes of administration will be specified by ACC. The chemicals selected were based upon consideration of: 1) the desire to collect data relevant to evaluating the specificity of the assay (negative control: allyl alcohol) in several labs; 2) the different modes of action which have been underrepresented in previous validation studies of this assay (steroidogenesis inhibition: fadrazole); 3) the importance of collecting data in the assay for chemicals run in related multi-modal endocrine assays (pubertal assays) to facilitate comparisons (thyroid toxicant: DE71); and 4) the need to expand the database to include additional thyroid agents (iopanoate). A summary of substances previously evaluated in validation studies of the Intact Adult Male Rat Screening Assay is included as Attachment 2. For comparison across assays, Attachment 3 lists the substances evaluated during validation studies of all of the EPA's Tier-1 Endocrine Disruptor Screening Program assays.

Table 1. Test Chemicals and Dose Levels

Chemical	**Dose Levels**	**Route of Exposure**	**Mode of Action**
0.25% Methylcellulose[1]	----------	Oral Gavage	Vehicle Control Group
Corn Oil[1]	----------	Oral Gavage	Vehicle Control Group
Allyl Alcohol	3 dose levels to be provided by ACC based on previous studies [4]	Oral Gavage	(Negative Control)
Fadrozole[2]	3 dose levels to be provided by ACC based on previous studies	Oral Gavage	Steroidogenesis (Aromatase) Inhibitor
DE-71	3 dose levels to be provided by ACC based on previous studies	Oral Gavage	Thyroid Toxicant
Iopanoate[3]	3 dose levels to be determined following range finding study	Oral Gavage	Thyroid Toxicant

[1] 0.25% Methylcellulose will be used as a vehicle for all substances, except DE-71 which will employ stripped corn oil.

[2] If fadrazole cannot be obtained, then testolactone shall be used. However, fadrazole is preferred because it is a more specific aromatase inhibitor.

[3] The Contractor will conduct a range finding study for this substance as part of the overall contract. Responses to the proposal shall also include plans for this study.
[4] Dose levels will be discussed with the contractors during the contract development process.

In responding to this RfP, proposals shall include a section on chemical procurement and handling. This section shall describe the activities and associated costs. It is envisioned that only one award will be made for chemical procurement and handling. The contractor receiving this chemical procurement award would be responsible for procuring test articles, developing standard operating procedures (SOPs) for dose preparation, preparing neat test articles for use in studies by both labs, blinding neat test articles and shipping the test articles to the second lab. The Contractor receiving this award shall also procure certified animal chow for use by both labs, so that the studies employ the same batch of animal feed to control for any possible variations. Chemicals, including vehicle control materials, are to be procured from commercial sources in quantities sufficient for use of the same batch of test articles/vehicles in studies in both labs. DE-71 shall be obtained from EPA's contractor at no cost to the validation contractor (this will assure use of the same exact batch of DE-71 that was run by EPA in the pubertal validation studies). This will permit direct comparison, which is important for substances such as DE-71 that can vary in composition from batch to batch. Test article identity and purity will be specified by the commercial supplier. This approach is consistent with the endocrine assay validation work of the OECD (OECD, 2007). Aliquots of the neat test articles and dose solutions will be preserved (-20^0C) to allow for analytical evaluation at a subsequent date if the ACC deems this necessary. Samples shall be kept for no more than three years after acceptance of the final report by the ACC.

BACKGROUND INFORMATION

ACC is engaged with the scientific community and government agencies in a broad effort to understand the potential of chemicals to interact with the endocrine system and the ability to cause adverse health and ecological effects at environmentally relevant exposure levels. The chemical industry contributes to basic scientific research on public health and safety through its LRI and holds to the fundamental tenet that public policy and product stewardship should be based on scientific evidence.

In response to public concern that certain substances may interfere with endocrine processes in humans and wildlife, Congress directed the EPA in 1996, through the Food Quality Protection Act (Public Law 104-170), to develop a screening program for evaluating the potential of substances to induce hormone-related health effects. To comply with the 1996 Congressional mandate to implement an endocrine screening program, the US EPA launched their Endocrine Disruptor Screening Program (EDSP) and initiated laboratory research to develop, standardize and validate a number of screening and testing methods needed for this program. As specified in the EPA's Federal Register Notices (EPA, 1998 b, c) the EDSP consists of both a Tier-1 screening battery of assays that are designed to identify substances capable of interacting with the endocrine system and Tier-2 tests designed for risk assessment. If, based on a weight-of-evidence evaluation of the results from the Tier-l screening battery, a test substance is identified as having the ability to interact with the endocrine system, Tier-2 *in vivo* tests would be required to provide detailed information on concentration response relationships and specific adverse effects that may result for use in risk assessment.

The ACC has been, and continues to be, involved in this effort by sponsoring technical studies, reviewing lab reports and providing comments and testimony on EPA's standardization and validation plans and reports. Working in collaboration with industry, academia, and government researchers, the Council has invested in research over the last 10 years to improve the scientific understanding of the relationship between chemicals and the endocrine system. At the core of the research is the question: Does the presence in the environment of low levels of certain chemicals interfere with naturally occurring hormones or with the normal function of the endocrine system to cause possible adverse health effects?

Phase 1 of the EPA's EDSP is progressing. In 2005, under Phase 1, EPA published a priority setting process that the Agency will use to identify candidate substances to undergo screening testing. On June 18, 2007 EPA published (EPA, 2007a) its list of candidate chemicals for Phase 1 of its EDSP. The list includes 73 pesticide chemicals, composed of 64 pesticide active ingredients plus 9 pesticide inert ingredients that are HPV chemicals. Although it can't be known with certainty, based on progress EPA has made with validating the necessary endocrine screens and tests, it is anticipated that EPA will complete validation studies by mid-2008 for Tier-1 assays, and it is reasonable to assume that Tier 1 EDSP screening will commence in the later part of 2008.

The Tier-1 battery of screening assays identified for use in the EPA's EDSP (EPA 1998 a,b,c) includes both *in vitro* and *in vivo* methods. The *in vitro* assays included in the Tier-1 battery are estrogen receptor (ER) and androgen receptor (AR) binding assays (and/or transactivational assays), a steroidogenesis assay and an aromatase assay. The *in vivo* screening assays in the Tier-1 battery include the uterotrophic, Hershberger, pubertal male and female, and Intact Adult Male Rat Screening Assay. The Intact Adult Male Rat Screening Assay is the subject of this RfP.

In their 1998 report (EPA, 1998a), the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) recommended to the EPA a Tier-1 screening battery that comprised 8 assays, but EDSTAC also proposed two alternative batteries with a different combination of assays that were deemed worthy of further evaluation. All three EDSTAC batteries incorporate an array of 6-8 *in vitro*, *in vivo* mammalian and environmental assays for examining effects of endocrine active compounds (EACs). In alternate Battery 1, the Hershberger, female pubertal, and *in vitro* steroidogenesis assays are replaced by the Intact Adult Male Rat Screening Assay. It is expected that the Intact Adult Male Rat Screening Assay will be run in parallel with the uterotrophic and the *in vitro* receptor binding and/or transcriptional activation assays; both are assays which are also in the EDSTAC-recommended Tier-1 screening battery. The primary purpose of the uterotrophic assay is identification of estrogenic/antiestrogenic compounds. While the Intact Adult Male Rat Screening Assay offers some redundancy with the uterotrophic assay, it is primarily designed to complement the uterotrophic assay by identifying several endocrine activities that alter the hypothalamic pituitary gonadal axis, through a comprehensive assessment of serum hormone concentrations, organ weights, and focused histopathology. The *in vitro* receptor/transactivation assays will aid in the identification of substances that directly interact with the steroid hormone receptors.

The Intact Adult Male Rat Screening Assay is designed as a mode-of-action (MOA) screening assay (O'Connor et al., 2002a). The MOA screening approach advances scientific

understanding over assays based on apical endpoints alone, as these essentially are reproductive effects screens that are not necessarily specific for endocrine activity. By identifying the potential MOA, critical endpoints can be included in Tier-2 studies that will be used to define dose-response curves and no observed adverse effect levels (NOAELs)/no observed effect levels (NOELs) for the compound. Another advantage of the Intact Adult Male Rat Screening Assay is that it can easily be adapted for the detection of other endocrine activities by the inclusion of other hormone measurements, organ weights, and/or histopathology evaluations (See O'Connor et al., 2002a). A complete list of references is included as Attachment 4.

The rationale for using a comprehensive hormonal assessment in the Intact Adult Male Rat Screening Assay is based on the observation that different classes of EACs produce distinct hormonal profiles; a comprehensive review of the Intact Adult Male Rat Screening Assay was published in 2002 in *Critical Reviews in Toxicology* (O'Connor et al., 2002a). This method has used this approach to identify modes of action for different compounds using components of the male *in vivo* battery. For example, ammonium perfluorooctanoate (C8), a peroxisome proliferator, was shown to induce aromatase and increase serum estradiol levels in two-week studies similar in design to the current Tier-1 battery (Cook et al., 1992). Using a similar design to the Tier-1 battery that includes the Intact Adult Male Rat Screening Assay, linuron (an herbicide) was identified as being a weak androgen receptor antagonist (Cook et al., 1993). In a mechanistic investigation that included the Intact Adult Male Rat Screening Assay, 1-methyl-3-propylimidazole-2-thione (PTI) was shown to alter thyroid function by directly inhibiting thyroid hormone synthesis and by enhancing thyroid hormone excretion via UDP-glucuronyltransferase induction (Biegel et al., 1995). In addition, the modes of action for several proprietary compounds were identified using the Intact Adult Male Rat Screening Assay study design, all of which produced endocrine tumors in two-year rat bioassays but did not produce adverse responses in developmental or multigeneration reproduction studies. To date, approximately 29 substances have been evaluated in the Intact Adult Male Rat Screening Assay (as summarized in Attachment 2; for a complete list of publications/references see Attachment 3).

In the Intact Adult Male Rat Screening Assay, adult male rats (15/group) are dosed daily by oral gavage for 15 days with the test substance and euthanized on the morning of test day 15, approximately 2 hours after the last administered dose. At the terminal necropsy, the liver, thyroid gland, and reproductive organs [testes, epididymides, prostate, seminal vesicles with fluid, ASG (prostate and seminal vesicles)] are weighed, and the testes, epididymides, and thyroid gland (formalin-fixed weight) are saved for histopathological evaluation. Blood is collected and serum is prepared for hormonal evaluation (testosterone, estradiol, dihydrotestosterone [DHT], luteinizing hormone [LH], follicle stimulating hormone [FSH], prolactin, triiodothyronine [T_3], thyroxine [T_4], and thyroid-stimulating hormone [TSH]). Since the in-life portion of the Intact Adult Male Rat Screening Assay is performed similarly to routine toxicology studies, there are no serious logistical issues with performing the assay. The one potentially challenging aspect of the Intact Adult Male Rat Screening Assay is inclusion of serum hormone analyses, as these may not be routinely performed in many toxicology testing laboratories. Nevertheless, commercial kits are available for each of these hormonal assays and most manufactures provide technical support.

By comparing the "fingerprint" of the organ weight and hormonal endpoints for an unknown compound to a series of positive controls, the Tier-1 battery that includes the Intact Adult Male Rat Screening Assay not only identifies potential EACs, but aids in the characterization of their MOA. The "fingerprints" that were obtained for flutamide and ketoconazole provide a useful example (O'Connor, 2005). Flutamide, an AR antagonist, competes with testosterone and DHT for binding to the AR. Essentially, flutamide blocks the recognition of testosterone and DHT. ASG weights, which are androgen-dependent, are decreased by flutamide treatment. The inability of the hypothalamus and pituitary to recognize androgens stimulates the secretion of gonadotropin-releasing hormone (GnRH) and LH by these two organs, which in turn, stimulates testosterone production by the Leydig cells. Therefore, AR antagonists such as flutamide decrease ASG weights and increase serum testosterone and LH concentrations. Ketoconazole, a steroid biosynthesis inhibitor, inhibits testosterone production by binding to the heme iron of the three cytochrome P450 isozymes of the testosterone biosynthetic pathway. Similar to flutamide, ketoconazole decreases ASG weights. However, since ketoconazole acts directly at the testis to inhibit testosterone production, serum testosterone concentrations are decreased, and secondary to the decreased serum testosterone concentrations, serum concentrations of LH are increased. Therefore, steroid biosynthesis inhibitors such as ketoconazole decrease ASG weights and serum testosterone concentrations, and increase serum LH concentrations. Hence, while organ weight measurements alone cannot distinguish between these two modes of action, the two different modes of action are easily distinguishable when the changes in organ weights are coupled with the serum hormone data.

O'Connor and co-workers completed a pre-validation exercise for an integrated Tier-1 screening battery which included the Intact Adult Male Rat Screening Assay (see O'Connor et al., 2002a). The two primary goals of the pre-validation exercise were to test the hypothesis that distinct "fingerprints" could be identified for each type of endocrine activity, and to determine which of the endpoints evaluated in the pre-validation exercise should be included in a final screen. To accomplish these goals, 15 positive controls with known endocrine activities were examined. Each endpoint was evaluated for variability, stability over time, predictability, and dose-dependency for each of the positive endocrine controls. The assay was effective at detecting a wide range of endocrine activities. In studies using the Intact Adult Male Rat Screening Assay, EACs that were identified include ER agonists and antagonists, AR agonists and antagonists, PR agonists and antagonists, thyroid modulators, steroid biosynthesis inhibitors (aromatase, 5α-reductase, and testosterone biosynthesis), and prolactin modulators. Since the initial pre-validation exercise, additional EACs have been examined in the Intact Adult Male Rat Screening Assay using the standardized protocol, bringing the total number of compounds examined to 29. Laboratories performing the Intact Adult Male Rat Screening Assay include both industrial laboratories (DuPont, BASF, Syngenta, Dow) and contract laboratories (Charles River, RTI and WIL). A significant number of studies have already been conducted as part of the protocol standardization and validation studies of the Intact Adult Male Rat Screening Assay. All of the results of the studies funded/conducted to date by industry, EPA, and contract laboratories are summarized in Attachment 2. In addition, Attachment 3 summarizes the substances, as of December 2005, which have been evaluated as part of EPA's program to validate all of the EDSP Tier 1 assays specified by EPA (EPA, 2005a).

In addition to the investigations discussed above, EPA has sponsored studies of the Intact Adult Male Rat Screening Assay as part of the Agency's efforts to validate screens for the EDSP. Prior to the EPA sponsored studies, a total number of six laboratories (including two contract research laboratories) had conducted the adult male rat assay. For validation purposes, EPA focused on developing a design to demonstrate that contract research laboratories can adopt this protocol with relative ease and success (i.e., obtain expected results). Considering available resources, EPA chose to test two of the same chemicals in three different contract research laboratories to take advantage of the results that had already been generated with these chemicals in previous studies conducted in industrial and contract research labs. The chemicals selected by EPA for this inter-laboratory phase of validation were chosen to represent different MOAs that the Intact Adult Male Rat Screening Assay is expected to detect.[2] The MOAs that were identified by EPA as critical for detection by the Intact Adult Male Rat Screening Assay included: an anti-androgen, a thyroid-active agent, and a steroid biosynthesis inhibitor. The substances evaluated in the EPA sponsored studies were methoxychlor and linuron in a pre-validation study (EPA, 2005b), and linuron and phenobarbital in an inter-laboratory validation study (EPA, 2006). In regard to the latter, (as cited in EPA, 2007b), "there was statistical agreement on the results for the multiple reproductive endpoints used for data interpretation in this study among the three laboratories and by inference with previous studies (EPA, 2005b; O'Connor et al., 1999a,b,c,d, 2002a,b,c,d) using this bioassay. The systemic toxicity endpoints, such as body weight effects, were not as consistent among the three laboratories. However, there was still an indication of a high level of reliability for this standardized protocol based on the results of this inter-laboratory validation exercise. The data combined across the three laboratories appeared to support the validation of this screening assay for potential androgen and thyroid disruptors when the full complement of endpoints, such as final body weight, organ weights (absolute and relative), and appropriate histopathology and serum hormones concentrations, are considered together."

SPECIAL REQUIREMENTS

A goal of the LRI is to share broadly the results of funded projects. Thus, it is expected that results be submitted for publication in peer-reviewed scientific journals and presented at scientific meetings, conferences, and/or symposia. The ACC's policy is to support the public release of research findings from the LRI.

All proposals should include costs for preparing manuscripts for submission to peer-reviewed scientific journals and supplying the ACC with three reprints of each journal article and an electronic version of the published article. Brief (250-word) annual progress reports are required. The final report will consist of information and data as specified under "Data Analysis and Statistical Evaluations" (Protocol Attachment 1), peer-reviewed publications, and published abstracts,. Specific reporting requirements will be negotiated as part of the development of the research contract. The Contractor will then provide the final report to ACC (2 written copies, two

[2] In EPA's EDSP Tier-1 battery, the Intact Adult Male Rat Screening Assay may be used as an alternative and possibly replace the steroidogenesis assay, the Hershberger assay and the pubertal female (male) assays. It is expected that the uterotrophic assay will be included in the Tier-1 screening battery that includes the Intact Adult Male Rat Screening Assay; therefore, EPA decided that it wasn't necessary to include estrogenic/anti-estrogenic chemicals as a mode of action as part of the EPA validation studies.

electronic copies as pdf files, and the accompanying data as Excel files)

All proposals should include reasonable and necessary travel and related expenses. Please include in the proposed travel plan at least one trip (for the cost proposal, assume it will be to Washington, DC) for the purpose of presenting research results to ACC, as well as to scientific meetings. Foreign travel costs (defined as any travel outside the country of the Contractor's principal place of business) will require additional approvals.

ELIGIBILITY

Proposals may be submitted by any domestic or foreign for-profit, not-for-profit, or non-profit organization, public or private entities, such as universities, colleges, laboratories, and contract research organizations; units of federal, state, and local governments with the necessary laboratory facilities; and research cooperatives.

FUNDS AVAILABLE/PROJECT DURATION

It is anticipated that the award from this solicitation will be two fixed price contracts. The total program cost (of all projects funded under this RfP for all years) has been budgeted in the range of $600,000-700,000. The project costs are expected to be commensurate with project scope. Proposals should include funds necessary to complete the full scope and deliverables described earlier, including direct and indirect costs (e.g., direct labor, fringe benefits, materials, subcontracts, purchased parts, shipping, indirect costs and rates, fees, status reports, publications, meeting presentations, travel expenses). Projects are expected to begin immediately upon execution of a contract. The duration of the project is expected to be commensurate with the goals of the project. One year is expected to be the maximum duration. Ideally, a project will be expected to provide a full preliminary report for presentation and discussion at a workshop, within 8 months.

PROPOSAL GUIDANCE

Proposals must be received electronically by the ACC no later than close of business on **XXXXX, 2007**. Receipt of the electronic version is deemed to be in confidence. The Project Plan section must be no longer than 15 pages in length, not including literature cited, attachments, and appendices. All proposals must be prepared using the Proposal Form (Attachment A). Budgets, biographies/curricula vitae for the Principal Investigator and all other key personnel, and other submissions specified in the Proposal Form are not part of the 15-page limit. The electronic copy of the proposal should be sent to the following address:

Tina Bahadori, D.Sc.
American Chemistry Council
Long-Range Research Initiative Team
1300 Wilson Blvd.
Arlington, VA 22209
(703) 741-5214
Tina_Bahadori@americanchemistry.com

The proposal must be signed by an individual who is authorized to sign on behalf of, and bind your organization to the proposed rates (including indirect costs).

Incomplete or nonresponsive proposals will be returned to applicants without further review. Proposals that are complete and within the framework of the RfP will be peer-reviewed for scientific merit by independent scientists with expertise appropriate to the subject RfP.

The following criteria will be used by peer reviewers to evaluate proposals:

- Scientific merit and feasibility relative to RfP;
- Expertise of investigator(s); and
- Quality Assurance (QA) and GLP processes and animal care considerations.

Peer reviewers will also assign each proposal an overall rating of "Excellent," "Very Good," "Good," "Satisfactory," or "Unsatisfactory." Only proposals that receive an overall rating of "Excellent" or "Very Good" by the peer reviewers will be considered for funding, according to the following programmatic criteria:

- Relevance to the chemical industry, as described in the RfP;
- Proposed milestones/timelines;
- Appropriateness of the budget/cost-effectiveness; and
- Use of collaborators/leveraging.

AWARD CRITERIA

The criteria that will be used in making awards include receipt of a sufficient number of proposals of scientific merit and programmatic merit as described in criteria above; availability of funds; and LRI program balance. The ACC reserves the right to make no awards under this RfP.

PROPOSAL REVIEW FEEDBACK PROCEDURES

Each applicant will receive an electronic notification of receipt of their proposal. Each applicant will receive a copy of the peer-reviewers' comments on the Peer-Review Forms (Attachment B) with the reviewer's identifying information deleted and the LRI's evaluation on the Proposal Selection Form (Attachment C). All applicants will receive a letter of notification regarding the award/non-award decision from the ACC on approximately 90 days after close of the RfP.

TYPE OF AWARD

The form of award under the LRI is a contract between the ACC and the awardee.

INQUIRIES

More details about the Long-Range Research Initiative can be found on www.USLRI.org. Questions regarding this RfP should be directed in writing, preferably by e-mail to the following address:

Tina Bahadori, D.Sc.
American Chemistry Council
Long-Range Research Initiative Team
1300 Wilson Blvd.
Arlington, VA 22209
(703) 741-5214
Tina_Bahadori@americanchemistry.com

ATTACHMENT 1.

15-DAY INTACT ADULT MALE RAT SCREENING ASSAY FOR IDENTIFYING POTENTIAL ENDOCRINE ACTIVE CHEMICALS IN SPRAGUE DAWLEY RATS

STANDARDIZED PROTOCOL
(from EPA, 2006; modified to reflect name of sponsor and identity of chemicals to be tested)

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INTRODUCTION

The Food Quality Protection Act of 1996 mandates EPA to determine whether "pesticide chemicals" have endocrine effects and the Safe Drinking Water Act Amendments of 1996 authorize the Agency to screen other substances that "may be found in sources of drinking water." EPA's federal advisory committee, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) issued their report (EPA, 1998a) which recommended to the EPA a tiered, hierarchical approach for endocrine screening and testing. EDSTAC recommended a primary Tier 1 battery of assays as well as two alternative batteries. All three EDSTAC recommended batteries are expected to incorporate a suite of *in vitro* and *in vivo* mammalian and non-mammalian screening assays for determining the potential of a substance to interact with one or more components of the endocrine system. In the first alternate screening battery, the EDSTAC recommended that the Hershberger, female pubertal and *in vitro* steroidogenesis assays in the primary battery be replaced by the Intact Adult Male Rat Screening Assay (O'Connor et al., 2002a) The Intact Adult Male Rat Screening Assay would be expected to be run in parallel with the uterotrophic assay, which may use intact immature or ovariectomized adult female rats for identification of estrogen agonists or antagonists, and the *in vitro* estrogen and androgen receptor binding or transcriptional activation assays, as well as the non-mammalian assays. In this regard, the Intact Adult Male Rat Screening Assay is designed to complement and expand on the results of other *in vivo* and *in vitro* assay results by identifying physiological endocrine-specific effects that occur in the presence of a functioning hypothalamic-pituitary-gonadal and –thyroidal model system.

The Intact Adult Male Rat Screening Assay has been used to detect estrogen receptor agonists/antagonists, androgen receptor agonists/antagonists, progesterone receptor agonists/antagonists, steroid biosynthesis inhibitors, gonadotrophin and thyroid modulators either directly or indirectly by altering the hypothalamic-pituitary-gonadal or -thyroidal axes, and prolactin modulators through neuroendocrine pathways. The extent of the diversity of this assay to detect a variety of endocrine active compounds (EACs), especially those related to the estrogen, androgen and thyroid hormonal pathways, has been hypothesized, tested and reported in numerous standardization and validation studies published in peer-reviewed scientific journals (O'Connor et al., 1998a,b; 1999a,b; 2000a,b; 2002a,b). Thus, within the EPA's EDSP alternative Tier-1 screening battery, the primary purpose of the Intact Adult Male Rat Screening Assay is to detect androgen and thyroid active substances, as well as substances that can inhibit steroid biosynthesis (aromatase and testosterone biosynthesis). The Intact Adult Male Rat Screening Assay can also be useful in detecting estrogen agonists and antagonists, and compounds that have the potential to bind to dopamine and progesterone receptors.

OBJECTIVES

The specific purpose of this study is to evaluate the relevance and reliability of the adult male rat assay in response to three positive test substances that have different known modes of action on the endocrine system and one negative test chemical that is known not to affect the endocrine system directly.

Additionally, this study is to evaluate the feasibility of this bioassay to detect EACs by measuring body and organ weight changes, histology and changes in circulating concentrations of hormones.

TEST FACILITY

The study will be conducted in compliance with Good Laboratory Practices (GLPs) with one exception. This exception is that the independent purity and identity of test articles and analytical quantitative evaluation of dose solutions will not be required. The test articles will be purchased from commercial sources and dose solutions prepared in accordance with the study protocol and the laboratory's standard operating procedures. It is expected the laboratory will comply with GLPs cited in Subpart J §160.185 of FIFRA or §792.185 of TSCA, the protocol and standard operating procedures (SOP) of the laboratory and that any deviations will be recorded and assessed to determine whether the integrity of the study has been jeopardized.

STUDY DESIGN

The dosage levels will be determined from the results of previous studies and provided by the ACC during contract negotiations. The following table (Table 1) presents the groups, including the vehicle control groups (0.25% methylcellulose in water for all substances except DE-71 for which the vehicle will be stripped corn oil) and each of the four test substances at three dose levels (low, medium and high). The actual substances and dose levels are described below (Section A).

The laboratory is to implement and follow procedures such that the test article dose solutions are coded and administered "blind." There will be 14 test groups (2 vehicle control groups, and 12 test article treatment groups (3 treatment groups x 4 test articles)).

Table 1.

Group	Test Substance and Dosage Level Code (A letter code shall be randomly assigned)	Test Substance and Dosage Level	Dose Volume (mL/kg)	Number of Males
1	A letter code shall be randomly assigned	Methylcellulose vehicle control	5	15
2	A letter code shall be randomly assigned	Allyl Alcohol Low Dose	5	15
3	A letter code shall be randomly assigned	Allyl Alcohol Medium Dose	5	15
4	A letter code shall be randomly assigned	Allyl Alcohol High Dose	5	15
5	A letter code shall be randomly assigned	Fadrazole Low Dose	5	15
6	A letter code shall be randomly assigned	Fadrazole Medium Dose	5	15
7	A letter code shall be randomly assigned	Fadrazole High Dose	5	15
8	A letter code shall be randomly assigned	Iopanoate Low Dose	5	15
9	A letter code shall be randomly assigned	Iopanoate Medium Dose	5	15
10	A letter code shall be randomly assigned	Iopanoate High Dose	5	15

11	A letter code shall be randomly assigned	DE-71 Low Dose	5	15
12	A letter code shall be randomly assigned	DE-71 Medium Dose	5	15
13	A letter code shall be randomly assigned	DE-71 High Dose	5	15
14	A letter code shall be randomly assigned	Corn oil vehicle control	5	15

Note: Each group will be assigned a letter designation for the purposes of collecting animal data blinded to the test and control substances and dose levels.

Dose administration will be performed blind to treatment group. The suspensions or solutions of formulated test and vehicle control substances will be administered once daily by oral gavage for 15 consecutive days (Test Day [TD] 1 through TD 15). Prior to dose administration, all formulation to be used for dosing that day will be removed from refrigeration and placed on a stir plate to vortex for at least 45 minutes. The formulations will continue to be stirred throughout dose administration.

Animals will be dosed beginning early in the morning so that at termination blood collection and necropsy can be completed within a 2-3 hour window after the last dose on TD 15. Typical necropsy times used in previous experiments were from 0700-1000 hours and, in some instances, the laboratories staggered the start of the study in a manner across dose groups to accommodate the number of animals scheduled for necropsy within a defined time (2-3 hours) after administration of last dose on TD 15. Compliance with this dosing and study termination schedule is absolutely mandatory.

MATERIALS AND METHODS

A. Test Substances

Chemical	Dose Levels	Route of Exposure	Mode of Action
Allyl Alcohol	3 dose levels to be provided by ACC based on previous studies	Oral Gavage	(Negative Control)
Fadrozole	3 dose levels to be provided by ACC based on previous studies	Oral Gavage	Aromatase Inhibitor
DE-71	3 dose levels to be provided by ACC based on previous studies	Oral Gavage	Thyroid Toxicant
Iopanoate[1]	3 dose levels to be provided by ACC based on previous studies	Oral Gavage	Thyroid Toxicant

[1]The Contractor will conduct a range finding study for this substance as part of the overall contract. Responses to the proposal shall also include plans for this study. This range finding study shall use the same specifics and procedures as the main test. The range finding study shall use 3 animals per dose group and 6 dose groups plus a vehicle control group (7 test groups total). Measurements shall include daily body weights, clinical observations, body weight change, absolute organ weights and organ weights relative to final body eight. **Blood shall not be collected and hormone analyses will not be conducted for the range finding study**. Once the range finding study is completed, dose levels for the main study will be set by consultation with the ACC.

B. Test Species

Adult male Crl:CD®(SD)IGS BR rats, approximately 10 weeks of age and weighing between 260 and 300 grams at the start of dosing, will be acquired from Charles River Laboratories, Raleigh, North Carolina.

C. Animal Husbandry

Animals will be housed individually in solid-bottom, polycarbonate cages fitted with stainless steel wire lids with Sani-Chip® cage bedding or wire-mesh cages. Water will be available *ad libitum* through plastic bottles with stainless steel sipper tubes or an automatic watering system. Animal rooms will be maintained on a 12:12 hrs light:dark cycle. Target conditions for temperature and relative humidity in the animal rooms will be between 64-79°F and 30-70%, respectively.

The laboratory must have an animal health monitoring program. The following procedures will be performed periodically to assure that contaminant levels are below those that would be expected to impact the scientific integrity of the study:

- Water samples will be analyzed for total bacterial counts, and the presence of coliforms, lead, and other contaminants.
- Feed samples will be analyzed for the presence of bacteria and fungi.
- Samples from freshly washed cages and cage racks will be analyzed to ensure adequate sanitation by the cagewashers.

Certified animal feed will be used, guaranteed by the manufacturer to meet specified nutritional requirements and to be free of impurities which might influence the results of the study. The chemical procurement lab shall identify the feed and procure sufficient feed from the commercial supplier to assure use by both labs. The manufacturer's specifications and analyses shall be included in the study records. In addition, a 200 gram sample of each batch of feed shall be preserved (at -20^0C) by each lab to allow for analytical evaluation at a subsequent date if the ACC deems this necessary. Samples shall be kept for no more than three years after acceptance of the final report by the ACC.

The animal health monitoring program will be administered by the laboratory animal veterinarian. Data will be maintained separately from study records and may be included in the final report at the discretion of the study director.

D. Pretest Period

Upon arrival at the laboratory, all rats will be removed from shipping cartons and housed one per cage in a quarantine room. The rats will be:

- Quarantined for approximately one week;
- Identified with cage card identification;
- Weighed three times; and
- Observed with respect to weight gain and any gross signs of disease or injury.

The rats will be released from quarantine by the laboratory veterinarian on the bases of body weights and freedom from clinical signs.

Any rats accidentally killed during the pretest period will be discarded without necropsy. All rats found dead or sacrificed *in extremis* during the pretest period will be necropsied but tissues will not be examined microscopically.

E. Assignment to Groups

During the pretest period, male rats will be divided by computerized, stratified randomization into treatment groups so that there are no statistically significant differences among group body weight means. Each rat will be housed individually and individually identified.

F. Dosage Preparation and Administration

All dosing solutions will be made within three days of study start and will be prepared weekly for the duration of the study. The dose solutions (or suspensions) will be stored in the refrigerator when not in use. The dose volume will be 5 mL/kg. The vehicle will be 0.25% methylcellulose for every test article except DE-71, which will use stripped corn oil as the vehicle, and the route of administration will be oral gavage. The same volume of vehicle will be given to the control group. Individual rat dose volumes will be based on the daily body weight except on TD15 which will use the previous day's weight.

G. Body Weights

Body weights will be recorded individually on a daily basis from TD1 to TD15, inclusively. On TD15, live body weights will be recorded after dosing due to time constraints. Therefore, the body weight recorded on Test Day 14 will be used to determine the dose volume used for TD 15 dose administration.

H. Food Consumption and Food Efficiency

Individual food consumption data will be recorded on a weekly basis and recorded on TD 1, 8, and 15. Food intake will be reported as g/kg body weight/day (g/kg/day) for each corresponding body weight interval.

I. Clinical Observations and Mortality

The rats will be observed twice daily for appearance, behavior, moribundity and mortality. A detailed physical examination will be conducted on the day of randomization and daily prior to dose administration (except on TD 15). Observations shall include, but are not limited to, evaluations for changes in appearance of the skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous system functions, somatomotor activity and behavior patterns. Observations will be recorded.

J. Pathological Evaluation

Animals not surviving until the scheduled euthanasia will be necropsied and cause of death recorded, if possible. Rats not expected to survive to the next observation period (moribund) will be euthanized by carbon dioxide inhalation and subjected to a gross necropsy. Tissues with unusual gross findings will be preserved in 10% neutral-buffered formalin. All carcasses will be discarded.

On the morning of TD15 following dosing, all surviving study animals will be moved to the necropsy holding room and held for at least 1 hour before euthanasia of study animals is scheduled to begin (to minimize stress-induced changes in hormone levels related to cage transport). Animals will not be fasted prior to euthanasia. Rats will be weighed and anesthetized by exposure to carbon dioxide for up to 60 seconds (no more), then each rat will be euthanized by decapitation and the time of euthanasia will be recorded. Blood will be collected via the site of decapitation as described below. Rapid euthanasia is necessary because of the likelihood that undue stress associated with anesthesia alone will interfere with the accurate measurement of the various hormones that are essential endpoints with this assay.

The order in which animals will be necropsied for blood and tissue collection will be stratified across all groups, corresponding to the order in which the animals were dosed. Time of euthanasia for all animals should occur between 0700 and 1000 hr (2 to 3 hours after final dose) in order to minimize variability associated with serum hormone measurements. Immediately following euthanasia, trunk blood will be collected (target volume of 8 mL) into serum separator tubes. Tubes containing blood will be kept on ice until serum is prepared. Blood samples will then be centrifuged for isolation of serum. Aliquots of serum should be made based on the number of different assays that will be run in a day to minimize the potential freeze and thaw effect on hormone concentrations. Serum will be stored in a freezer set to maintain ≤ -65°C for subsequent hormone analyses. Extra serum will be stored at ≤ -65°C. Remaining serum samples will be discarded after acceptance of the final report.

The necropsy examinations will include the external surface, all orifices, the external surface of the brain and the thoracic, abdominal and pelvic cavities including viscera. Organs/tissues to be weighed and preserved are described below. Tissues with gross findings will be preserved in 10% neutral-buffered formalin, for possible histopathologic examination (unless different fixative is described below).

The following tissues will be weighed (to the nearest 0.1 mg) from all animals:

Liver	Right Testis
Entire prostate[1]	Left Testis
Seminal vesicles with coagulating gland containing fluid[2]	Epididymides[2]
Thyroid[3]	

[1]- dorsolateral and ventral prostate
[2]- weighed as paired organs
[3]-weighed following fixation and dissection described in section 8.3 below

With the exception of the thyroid trimming described below, organ harvesting and weighing procedures will be divided as equally as possible among the prosecting and weighing technicians, such that all animals from a group are not processed by a single individual (operator number will be recorded) in order to minimize systematic bias in the weighing procedures.

The testes will be placed in Bouin's fixative for approximately 24 hours, after which they will be rinsed and stored in 70% alcohol until histological processing. The epididymides and liver from each rat will be placed in 10% neutral-buffered formalin. The thyroid, with attached trachea, is

fixed in 10% neutral-buffered formalin for at least 48 hours. Then the thyroid is dissected under a dissecting microscope from the trachea, blotted, weighed and placed in 10% neutral-buffered formalin until histological processing. The fixed thyroid dissection will be performed by one individual in order to reduce the variability of the dissection procedure and hence reduce the variability of the thyroid weights.

The thyroid from the control and all treatment groups for each test substance and the testes and epididymides from the control and high-dose animals for each test substance are then embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) for subsequent histological evaluations. Sections of 2-4 microns will be made for the testis (transverse) and for the epididymis (longitudinal). Histological processing of the fixed livers and the low and mid-dose of the testes and epididymides will be performed at the discretion of the Study Director and ACC. Testes and epididymides (left and right) and thyroid histology from the control and high dose groups should be evaluated for pathologic abnormalities and potential treatment-related effects. A minimum of two sections per thyroid should be evaluated. Microscopic evaluations of the thyroid from lower dose groups will be done for all treatment groups for each test substance. Liver only will be evaluated microscopically only at the discretion of the Study Director and ACC.

Interpretation of the histological changes will be done by a Board Certified Veterinary Pathologist knowledgeable of the control and high-dose groups for each of the test chemicals but not the nature of the chemicals.

K. Hormonal Evaluation

The following hormones will be analyzed from serum samples from all animals:

Testosterone	Luteinizing Hormone (LH)
Estradiol	Prolactin
Dihydrotestosterone (DHT)*	Thyroid-Stimulating Hormone (TSH)
Follicle-Stimulating Hormone (FSH)	Thyroxine (T_4)
	Triiodothyronine (T_3)

All hormones will be measured using commercially (Biotrak™, Amersham Biosciences and Diagnostics Systems Laboratory) available radioimmunoassay (RIA) kits; model numbers will be specified by the ACC. The sequence in which the hormones should be assayed is testosterone, LH, TSH, T_4, T_3, FSH, estradiol, prolactin and DHT. If serum is limiting, the Study Director should contact the ACC to establish a priority list of hormones to be measured.

Each assay should include all samples from the control group and each dose level for both chemicals, except for re-analysis of specific samples that may be out of range of the standard curve. Each serum sample should be run in duplicate. Each assay should include high and low quality control (QC) samples. The QC standards for FSH, LH, TSH and prolactin will be obtained from the National Hormone and Pituitary Program, and QC standards for testosterone, estradiol, dihydrotestosterone, T_4 and T_3 will be obtained from a commercial supplier. For the QC samples, the buffer/medium in which the reference standards are prepared will be spiked with respective QC hormones at concentrations that are expected to be within 70% B/B_0 (±10%)

and 30% B/B_0 (±10%). QC standards should be run in duplicate and in replicate at the beginning, middle and end of each assay.

DATA ANALYSES AND STATISTICAL EVALUATIONS

Once all of the data have been collected and the experimental portions (including histopathology evaluation) are concluded, then the code will be disclosed to the Study Directors of each lab. The final report shall include presentation of the data by both the experimental protocol code letter as well as the actual chemical and dose group. For data analyses and statistical evaluation (including graphs and tables), the final report shall provide this information by the actual chemical and dose group (not the coded letter).

Endpoints for the statistical analysis described below include the following:

- TD 15 body weight
- Body weight change, TD 1-8, 8-15, 1-15
- Food consumption (g/kg/day), TD 1-8, 8-15, 1-15
- Absolute organ weights (9 total)
- Organ weights relative to final body weight (9 total)
- Hormones (9 total)

In addition to the 6 organ weights collected per animal at necropsy and the formalin-fixed thyroid weights determined post necropsy, paired testes weights (left plus right testis) and the accessory sex gland unit weights (entire prostate plus the seminal vesicles with coagulating gland containing fluid) will be calculated per animal and analyzed. Based on 9 absolute organ weight values, 9 relative organ weight values, 9 possible hormones and the 7 body weight and food values, there is a total of 34 possible endpoints to be evaluated statistically. A test for extreme or outlying values will be carried out prior to analysis following Grubbs analysis and an evaluation of normal probability plots. Tests for heterogeneity of variance will be carried out on the data (excluding the values identified as potential outliers) using a one-way analysis of variance model fitted to the data including the fixed factor of treatment and the residual replicate per treatment. Following heterogeneity of variance evaluation, transformation of the data may be performed as appropriate to minimize heterogeneity of the data. Subsequent analyses will be carried out based on these transformed data.

A one-way analysis of variance (ANOVA) model will be fitted to the data to estimate treatment effects for each endpoint described above. Probability values will be indicated for each endpoint where the level of significance will be two-tailed at 0.05 and 0.01. The data used for the above-described analyses will exclude potential outliers and may be performed on transformed versions of the variables. The factors in the ANOVA models will include treatment and residual replicate (treatment). Linear trend statistics will also be evaluated for each endpoint using the means of two-sample t-tests. Summary statistics may be back transformed to the original scale for the purposes of data presentation.

Tables and Figures

To facilitate comparison with previous studies, the following tables and figures for each test substance along with the respective control shall be provided in the final report:

The first set of tables will display summary values for the final live body weight (TD 15), body weight change intervals (TD 1-8, 8-15, and 1-15), and food consumption (g/kg/day) intervals (TD 1-8, 8-15, and 1-15). For each endpoint and each dose and control group, the following will be reported:

- number of animals per group
- mean +/- SE
- coefficient of variation
- mean as a percent of control group mean +/- SE
- p-value

In addition, the linear trend slope contrast will be estimated for each test substance based on the control group and the three graded dose groups. The estimated treatment slope and its standard error will be reported.

The second set of tables will display summary statistics described above for the nine absolute organ weights.

The third set of tables will display summary statistics described above for the nine relative organ weights (ratio of organ weight to final body weight).

The fourth set of tables will display summary statistics described above for the nine hormones.

The first set of figures will display, in a line graph, the mean body weight for each Test Day from TD 1 through TD 15 for the control group and each of the three dose levels per test substance.

The second set of figures will display, in a scatter plot, the Test Day 15 mean absolute body weight, the 3 mean body weight change intervals, and the 3 mean food consumption intervals listed in Section 12.1, +/- 2 standard errors.

The third set of figures will display, in a scatter plot, the mean absolute organ weight for each organ, +/- 2 standard errors.

The fourth set of figures will display, in a scatter plot, the mean relative organ weight for each organ, +/- 2 standard errors.

The fifth set of figures will display, in a scatter plot, the mean hormone concentration +/- two standard errors for each hormone (as applicable), +/- 2 standard errors.

The Contractor should exercise professional judgment in deciding whether additional tables and figures would be useful.

SAFETY

The testing laboratory shall establish, maintain and implement appropriate health and safety, and housekeeping standard operating procedures. Some or all of the test articles may pose health or environmental hazards if not stored, handled, or disposed of properly. The lead lab shall provide

to the Study Director and the Health and Safety management of each test facility a sealed envelop containing the code of the blinded test articles. This will enable the lab to immediately break the code in case of an emergency situation. Samples shall be kept for no more than three years after acceptance of the final report by the ACC.

RECORDS AND SAMPLE RETENTION

Records and specimens from these studies shall be retained in accordance with Good Laboratory Practices procedures and requirements.

ATTACHMENT 2
TEST CHEMICALS RUN IN THE INTACT ADULT MALE RAT SCREENING ASSAY ADMINISTERED ORALLY OR INTRAPERITONEALLY (IP)

No.	Chemical	Endocrine Activity	Oral Route[1]	IP Route[1]	Laboratory
1	17β-estradiol	ER agonist (full or potent)	--	Pos	Dupont
2	Coumestrol	ER agonist (weak or partial)	--	Neg	Dupont
3	Methoxychlor	ER agonist (weak or partial)	Pos	--	RTI
4	Genistein	ER agonist (weak or partial)	Neg	--	Syngenta
5	Nonylphenol	ER agonist (weak or partial)	Pos	--	BASF
6	ICI-182,780	ER antagonist	--	Pos	Dupont
7	Testosterone	AR agonist	--	Pos	Dupont
8	Methyltestosterone	AR agonist	Pos	--	WIL
9	Flutamide	AR antagonist (full or potent)	Pos	Pos	Dupont, Dow
10	*p,p'*-DDE	AR antagonist (weak or partial)	Pos	Pos	Dupont
11	Vinclozolin	AR antagonist (weak or partial)	Pos	--	Dupont
12	Cyproterone Acetate	AR antagonist (weak or partial)	Pos	--	Dupont
13	Linuron	AR antagonist (weak or partial)	Pos	--	Dupont, RTI, WIL & Charles River
14	Di-n-butyl phthalate	Anti-androgen (non-receptor mechanism)	Pos	--	DuPont
15	Progesterone	PR agonist	--	Pos	Dupont
16	Mifepristone (RU486)	PR antagonist	--	Pos	Dupont
17	Apomorphine	D_2 receptor agonist	--	Neg	Dupont
18	Haloperidol	D_2 receptor antagonist	--	Pos	Dupont
19	Reserpine	Dopamine depletor (catecholamine depletion)	--	Pos	Dupont
20	Phenobarbital	Thyroid hormone excretion enhancer	Pos	Pos	Dupont, Dow, RTI, WIL & Charles River
21	Oxazepam	Thyroid hormone excretion enhancer	Pos	--	Dupont
22	Propylthiouracil	Thyroid hormone synthesis inhibitor	Pos	Pos	Dupont
23	Propylimidazole-2-thione (PTI)	Thyroid hormone synthesis inhibitor	Pos	--	Dupont
24	Finasteride	5α-Reductase inhibitor	--	Pos	Dupont
25	Ketoconazole	Testosterone biosynthesis inhibitor	Pos	Pos	Dupont
26	Anastrozole	Aromatase inhibitor	--	Pos	Dupont
27	Fadrozole	Aromatase inhibitor	Pos	--	Dupont
28	Ammonium perfluorooctanoate	Aromatase inhibitor	Pos	--	Dupont
29	Allyl Alcohol	Negative control chemical	Neg	--	Dupont

[1]Positive and negative effects were determined by comparing the pattern of effects observed in the intact male assay with the expected pattern of effects for the known mode of action of the positive test materials.

EPA's studies of the Intact Adult Male Rat Screening Assay

The main objective of EPA's inter-laboratory study was to continue the validation process of the test method by comparing responses across three independent laboratories. Two test chemicals known to affect the endocrine system (linuron and phenobarbital, affecting the androgen and thyroid hormonal pathways, respectively) were run concurrently in three different laboratories (WIL, RTI and Charles River) to evaluate reliability, relevance and transferability.

- *Reliability* – the ability of the assay to detect endocrine-mediated effects on prescribed target organs within laboratories with the expectation of consistency among laboratories

and that the observed results are comparable to expected results reported in previous studies and, secondarily

- *Relevance* - the ability of the assay, within and among laboratories, to detect known effects of linuron and phenobarbital on the endocrine system by primarily measuring changes in body and prescribed target organ weights, histology and circulating hormone concentrations
- *Transferability* – the feasibility of the assay protocol to be conducted in various CRO laboratories in a logistical and practical manner to be compliant with standard operating procedures (SOP) within laboratories, the study protocol and GLP conditions in such a manner as not to jeopardize study results.

EPA's studies add to the database of studies that are needed to complete the validation evaluation of the assay. The statistical and qualitative results from EPA's inter-laboratory validation study provide additional data supports the reliability, relevance and transferability of the Intact Adult Male Rat Screening Assay when the full complement of key targeted endpoints (final body weight, organ weights (absolute and relative), histology, and serum hormone concentrations) are considered together in a weight-of–evidence approach (EPA, 2007 b). To date, the totality of the datasets indicate that this assay could play a key role in the EPA EDSP and OECD endocrine frameworks for assessing androgenic and thyroidogenic activity of chemicals.

ATTACHMENT 3. EPA's Compilation of EDSP Reference Chemicals at a Glance as of December 2005 (EPA, 2005a)												
Modality	Chemical	ER binding	AR binding	Hersh-berger	Utero-trophic	Pubertal Female	Pubertal Male	Adult Male	Fish repro screen	Frog	Steroid	Aromatase
Androgen agonist	Methyl testosterone		X	X			X		X			
	Methyl trienolone (R1881)		X									
	5α dihydrotestosterone		X									
	Testosterone propionate		X	X				X				
	Trenbolone			X					X			
Androgen antagonist	Cyproterone acetate		X					X				
	DDE-p,p'		X	X			X	X	X			
	Flutamide			X			X	X	X			
	Linuron		X	X			X	X				
	Methoxychlor (HPTE)**		X**		X	X	X		X			
	Procymidone		X	X								
	Vinclozolin		X	X			X	X	X		X	
Estrogen agonist	17α Estradiol	X										
	17β Estradiol	X	X					X	X			
	2,4,5 Trichlorophenoxyacetic acid	X										
	2-sec-butylphenol	X										
	4-cumylphenol	X										
	4-tert-octylphenol	X	X									
	4-tert-pentylphenol								X			
	6a-methyl-17a-hydroxyprogesterone acetate		X									
	Bisphenol A	X			X	X			X			

	Bisphenol B	X										
	Clomiphene citrate	X										
	Coumestrol	X						X				
	Daidzein	X										
	DDT-o,p'				X							
	Estrone	X										
	Ethyl-4-hydroxybenzoate	X										
	Ethynyl estradiol	X			X	X						
	Genistein				X			X				X
	Kaempferol	X										
	Meso Hexestrol	X										
	Methoxychlor (HPTE)**		X**		X	X	X	X	X			
	Morin	X										
	Nonylphenol	X			X							X
	Norethynodrel	X										
	Phenolphthalin	X										
Estrogen antagonist	ICI 182,780							X				
	Tamoxifen citrate	X				X						
HPG	Atrazine		X			X	X		X	X	X	
Steroidogenesis	Aminoglutethimide										X	
	Dimethoate										X	
	Flutamide						X	X	X		X	
	Ketoconazole					X	X	X	X		X	
	Prochloraz								X		X	
	Spironolactone		X								X	
	Di-n-butylphthalate				X		X	X				
(5a reductase)	Finasteride			X				X			X	
Aromatase	4-OH Androstenedione											X
	4-androstene-3,17-dione		X									
	Aminoglutethimide											X
	Anastrazole							X				
	Chrysin											X
	Dicofol											X
	Econazole											X
	Fadrozole							X	X			
	Fenarimol					X						X

	Ketoconazole					X	X	X				X
	Prochloraz											X
Thyroid	Ammonium perchlorate					X	X					
	Iopanoic acid (monodeiodinases)									X		
	Methimazole									X		
	PBDE (DE-71)					X	X			X		
	Phenobarbital					X	X	X		X		
	Propylthiouracil					X	X	X		X		
	Sodium perchlorate								X	X		
	Thyroxine									X		
Other modalities	Bis(2-ethylhexyl)phthalate (DEHP)		X									
	Potassium permanganate								X			
	Progesterone	X	X					X				
Corticosteroids	Corticosterone	X	X									
	Dexamethasone		X							X		
	Pregnenolone-16-a-carbonitrile									X		
Ah receptor	dibenz(a,h)anthracene											X
Ca channel blocker	Verapamil										X	
Dopamine Receptor Antagonists	Apomorphine							X				
	Haloperidol							X				
	Mifepristone RU486							X				
	Oxazepam							X				
	Pimozide					X	X	X				
	Reserpine							X				
Methemoglobinemia inducer	2-chloronitrobenzene					X	X					

**Needs metabolism to the active form, which is not expected to occur in vitro.
X Chemical has been run using either pre-validation or validation protocols.

ATTACHMENT 4 REFERENCES
INCLUDING PUBLICATIONS OF RELEVANCE TO THE
DEVELOPMENT, STANDARDIZATION AND VALIDATION
OF THE
INTACT ADULT MALE RAT SCREENING ASSAY

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