UNITED STATES
CONSUMER PRODUCT SAFETY COMMISSION
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Memorandum

Date: July 11, 2012

TO : Kenneth R. Hinson, Executive Director

THROUGH: Cheryl A. Falvey, General Counsel
Robert J. Howell, Deputy Executive Director for Safety Operations

FROM : DeWane Ray, Assistant Executive Director
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SUBJECT : CPSC Staff Response to the ICCVAM Test Method Recommendations on the Usefulness and Limitations of the LUMI-CELL® Estrogen Receptor (BG1LucERTA) Screening Assay: A Test Method for the Identification of Chemical Substances with *In Vitro* Agonist (Activation) or Antagonist (Inactivation) Activity to the Human Estrogen Receptor

This memorandum discusses the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommendations regarding the LUMI-Cell assay's® ability to identify a chemical substance's potential for activating or inactivating human estrogen receptors (ERs).

It also discusses staff's consideration of whether these recommendations are acceptable in the regulatory context for the purpose of classification for labeling under the Federal Hazardous Substances Act (FHSA) (15 U.S.C. § 1261–1278).

I. Introduction

A. Background

The National Institutes of Health Revitalization Act of 1993 directed the National Institute of Environmental Health Sciences (NIEHS) to establish a method and criteria for the validation and regulatory acceptance of alternative testing methods (Public Law No. 103-43, Section 1301). To accomplish these goals, NIEHS created ICCVAM, which was made permanent by the ICCVAM Authorization Act of 2000 (Public Law 106-545). The Committee is composed of representatives from 15 federal regulatory and research agencies, including the U.S. Consumer Product Safety Commission (CPSC). These agencies generate, use, or provide information from toxicity test methods for risk-assessment purposes. The duties of ICCVAM are to review, optimize, and validate new, revised, or alternative test methods that encourage the reduction, refinement, or replacement of the use of animals in testing. In addition, ICCVAM is required to provide test recommendations to federal agencies and other stakeholders to facilitate appropriate interagency and international harmonization of toxicological test protocols. In 1998, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) was established to assist ICCVAM in performing the activities necessary for the validation and regulatory acceptance of alternative test methods. ICCVAM submits test recommendations for a test method to federal agencies that require or recommend acute or chronic toxicological testing. According to Public Law 106-545, these agencies should promote and encourage the development and use of alternatives to animal test methods for regulatory purposes and ensure that any new or revised acute or chronic toxicity test method is valid for its proposed use under the mandate of the ICCVAM Authorization Act of 2000.

On February 1, 2012, ICCVAM forwarded to the Commission for action, recommendations regarding the usefulness of the LUMI-CELL® estrogen receptor (BG1LucERTA) screening assay for identifying chemicals *in vitro* with the potential to activate (**agonize**) or inactivate (**antagonize**) the human estrogen receptor. The CPSC needs to determine whether the proposed recommendations would be acceptable for use under the Federal Hazardous Substances Act (FHSA). Under the mandate of the ICCVAM Authorization Act of 2000, federal agencies have 180 days to identify any relevant test methods for which the ICCVAM test recommendations may be added or substituted, review such test recommendations, and notify ICCVAM in writing if they will adopt the ICCVAM test recommendations.

The Commission recently delegated its authority on the evaluation and adoption or rejection of ICCVAM test recommendations to the Executive Director of CPSC. Regarding LUMI-CELL,® therefore, the Executive Director of CPSC needs to respond to ICCVAM by July 29, 2012.

B. Validation of Alternative Methods

Validation of alternative methods is required before regulatory acceptance and use by federal agencies. In general, for an alternative method to be considered valid, it must be reliable (*i.e.*, the toxicity predictions of test substances are repeatable within the same laboratory and reproducible across/among different laboratories) and relevant (*i.e.*, the alternative test method is useful for measuring the biological effect of interest, such as estrogen receptor activation or inactivation).

The reliability and relevance of an alternative test method can be assessed from the statistical analysis of data. The reliability of the alternative test method can be determined by calculating the reproducibility of test method results within and among laboratories. The relevance of an alternative test method can be determined by comparing the performance of the alternative test to the test that it is designed to replace. Both the reliability and relevance of the LUMI-CELL® assay (in terms of validation) can be found in Appendix A.

C. FHSA Requirements

Cautionary labeling of hazardous household substances is mandated by the FHSA (15 U.S.C. § 1261–1275). Under the FHSA, to be a hazardous substance, a product must present one or more of the hazards enumerated in the statute, and it must have the potential to cause substantial personal injury or substantial illness during, or as a result of, any customary or reasonably foreseeable handling or use, including reasonably foreseeable ingestion, by children.

1. FHSA and the LUMI-CELL® assay

Unlike some hazards discussed under the FHSA (*i.e.*, corrosives, irritants, strong sensitizers), the potential hazards associated with estrogen receptor (ER) activation or inactivation (as identified by the LUMI-CELL® assay) are not specifically addressed under the FHSA.

The hazards identified by the LUMI-CELL® assay can, however, still be of value in identifying a "chronic toxicant," which is defined in the FHSA (16CFR 1500.3(c)(2)(ii))[1].

As defined, a substance is termed a "chronic toxicant" if it meets the following definition:

> *(ii) Chronic toxicity. A substance is toxic because it presents a chronic hazard if it falls into one of the following categories. . .*
>
> *(A) For* ***Carcinogens****. A substance is toxic if it is or contains a known or probable human carcinogen.*

[1] The LUMI-CELL® assay has been validated by NICEATM and the associated laboratories as a method to screen chemicals for ER agonist and antagonist activity. Results are then expected to be used to prioritize chemicals for further review in programs such as EPA's Endocrine Disruptor Screening Program. Data and conclusions from the assay, however, may also be useful to CPSC in an additional manner. They may function as supplemental data to that generated from developmental, reproductive, cancer, or other *in vivo* studies, and then be part of a weight-of-evidence approach to support a determination of whether a chemical is a chronic toxicant. For CPSC, the results from the LUMI-CELL® assay, therefore, will not function solely as an endpoint for determining whether a chemical is a chronic toxicant, but will support data from other *in vivo* toxicity tests.

*(B) For **Neurotoxicological Toxicants**. A substance is toxic if it is or contains a known or probable neurotoxin.*
*(C) For **Developmental or Reproductive Toxicants**. A substance is toxic if it is or contains a known or probable human developmental or reproductive toxicant.*

Designation of a substance as a chronic toxicant (*i.e.*, carcinogen, neurotoxicant, developmental toxicant, or reproductive toxicant) is a complex process that requires interpretation of a variety of information, including human epidemiological testing and/or animal *in vivo* bioassays. These assays or studies are designed to determine toxic effects resulting from short- to long-term exposures.

2. Use of In-Vitro Tests

In vitro tests (such as the LUMI-CELL® assay) may be used to support or modify decisions regarding chronic toxicants made via interpretation of epidemiology and animal data. The use of *in vitro* tests is specifically referred to in 16 CFR Chronic Hazard Guidelines[2]:

- **Carcinogenicity** (Federal Register, 1992; 16 CFR part 1500 (VI)(B)(3)(b) – "Factors in the consideration of animal data". . .
  - (4) "results of short-term *in vivo*[3] and ***in vitro***[4] tests provide additional information concerning a judgment of carcinogenicity of a chemical."

- **Neurotoxicity** (Federal Register, 1992; 16 CFR part 1500 (VI)(C)(4) – "Evidence of Neurotoxicity Derived From Studies in Animals". . .
  - (a) "Neurotoxicity endpoints are studied using different test methodologies designed either to **screen or investigate a mechanism of action of neurotoxicity**, or to gather additional data."
  - (b) "Categories of neurotoxicity studies".
    - (iv) "Biochemical and **endocrinological studies** may include determination of . . . (6) anterior pituitary hormones, *e.g.*, follicle stimulating hormone, thyrotropic hormone, hypothalamic control of pituitary secretions."
    - (vi) "***In vitro*** neurotoxicity studies may be used to support the animal studies. However, they are not considered adequate by themselves to classify neurotoxicants."
  - (c) "Classification of neurotoxicity evidence derived from studies in animals."
    - "The confidence in evidence of neurotoxicity derived from animal studies increases (becomes convincing) with: (1) an increase in the number of responding species, strains, dose-levels, experiments, severity and multiplicity of effects; (2) the observation of a dose-response relationship,

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[2] Labeling Requirements for Art Materials Presenting Chronic Hazards; Guidelines for Determining Chronic Toxicity of Products Subject to the FHSA; Supplementary Definition of "Toxic Under the Federal Hazardous Substances Act (Federal Register, 1992).
[3] *In vivo* study – A study that is conducted with a living organism in its intact state (i.e. rat or mouse study).
[4] *In vitro* study – A study that is conducted using organism components that have been removed from their normal biological surroundings (i.e. tissue culture study).

consistency and reproducibility of results, and specificity and strength of the association; (3) supportive ***in vitro*** and other studies; and (4) an increase in statistical significance of neurotoxic effects over controls."

- **Reproductive and Developmental Toxicity** (Federal Register, 1992; 16 CFR part 1500 (VI)(D)(3) – "Identification of Developmental and Reproductive Toxicity Hazards from Studies in Animals"
    - "Positive findings for supplemental endpoints such as estrous cycle abnormalities, and hormone evaluations (**estrogen**, progesterone, FSH, LH) increase the evidence for hazard identification."

3. How the LUMI-CELL® Assay Can Support or Modify Decisions Regarding Chronic Toxicants

The principal testing component of the *in vitro* LUMI-CELL® assay is a human female ovarian adenocarcinoma cell line (BG-1) that endogenously expresses[5] both forms of the ER (ERα ~90% and ERβ ~10%). These ERs are structurally and functionally the same as those expressed *in vivo* in humans in tissues such as ovarian stromal cells, breast, and endometrium (ERα; females), the hypothalamus (ERα; males and females), efferent ducts in the testes (ERα; males) and the kidney, brain, bone, heart, lungs, intestinal mucosa, prostate, and endothelial cells (ERβ; males and females).

Since the BG-1 cell line is derived from a female reproductive organ, the assay results may be used as additional information to support decisions regarding the determination of whether a chemical is a female reproductive (chronic) toxicant.

Since the BG-1 cell line also has functional ERs, and different isoforms of the ER (either α or β) are found in other body tissues, the assay results can also be used as additional information to support determinations of whether a chemical is a chronic toxicant for other organ systems (male reproduction; neurological) or mechanisms (development).

ERs also figure prominently in the development of, or are associated with certain types of breast, ovarian, endometrial, colon, and prostate cancer (Deroo and Korach, 2006). Excessive ER activation by estrogen is one widely accepted mechanism for the development of breast cancer. The estrogen/ER complex activates a cell division cascade, which results in the proliferation of mammary cells. The increase in mammary cell division and DNA replication increases the potential for replication errors, which yields mutations that disrupt cell regulatory machinery and leads to unrestrained cell division. Theoretically, a chemical ER activator could induce the same effects as estrogen if sufficiently potent.

ER inactivation also can influence the development of cancer. Both colon and prostate cancers have reduced ERβ expression when compared to normal tissues, and ERβ is lost in advanced stages of the cancers. This suggests that ERβ plays a protective role in these cancers. Theoretically, a chemical ERβ inactivator could induce the same effects as the loss of ERβ mediated by these two cancer types.

[5] Endogenous expression – Development or origination (of receptors) naturally from the BG-1 cell line.

Because ER activity affects these many diverse tissue and disease processes, classification of a substance as a carcinogen, neurotoxicant, reproductive, or developmental toxicant (chronic toxicant), could therefore, involve information related to ER activation or inactivation.

D. Current Estrogenicity or Estrogen Receptor Activation or Inactivation Tests

The LUMI-CELL® assay is currently the only validated protocol for measuring **both** the agonistic or antagonistic effect that a chemical substance can have on estrogenicity or ERs. Five other validated and unvalidated assays assess some of the similar or related endpoints and are used by government and industry to assess estrogenic endpoints. These are described in Appendix B.

II. History and Background of the LUMI-CELL® Assay, an Alternative Test for Estrogen Receptor Activation/Inactivation

A. History

In 2000, the EPA nominated four *in vitro* test types for review by ICCVAM. These four types of tests [*in vitro* ER binding; *in vitro* androgen receptor (AR) binding; *in vitro* ER transcriptional activation (TA); and *in vitro* AR TA] were designed for determining substances that could potentially disrupt endocrine activity. The EPA also requested that ICCVAM develop validated performance standards for outlining an acceptable endocrine disruption test.

In 2002, NICEATM drafted background review documents (BRD) that detailed each of the four test methods. An independent international expert panel reviewed the BRD information (detailed descriptions of 137 test assays) in a public meeting, and concluded that none of the *in vitro* ER- or AR-based assays were validated adequately.

In 2003, ICCVAM published "ICCVAM Evaluation of *In Vitro* Test Methods for Detecting Potential Endocrine Disruptors," which was based on the panel's and the public's suggestions and comments. This document included a list of reference substances and essential test method components useful for validating the four types of assays. It also suggested that future method performance standards be based on validated test methods using the recommended reference substances and test method components.

In 2004, Xenobiotic Detection Systems, Inc., (XDS) nominated the LUMI-CELL® BG1Luc4E2 ER TA test assay for interlaboratory validation. ICCVAM and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) recommended that this assay be given a high priority for validation based on five criteria: (1) applicability to agency programs for identifying endocrine disruptors; (2) adequate use in the program and a sufficient impact on human, animal, and ecological health; (3) a reduction in the need to use animal models for testing; (4) a sufficient potential for assessing impacts to health when compared to other currently used assays; and (5) additional advantages, such as processing time and cost when compared to other methods. NICEATM, Japan Center for the Validation of Alternative Methods (JaCVAM), and the European Center for the Validation of Alternative Methods (ECVAM)

subsequently coordinated an international validation study that was overseen by a scientific Study Management Team (SMT). The SMT was later joined by a representative from the Korean Center for the Validation of Alternative Methods (KoCVAM). Validation of the LUMI-CELL® assay occurred in four phases:

- Phase 1 – NICEATM, ECVAM, and JaCVAM selected laboratories to validate the assay. The laboratories reviewed the protocols and demonstrated method proficiency by satisfactorily completing 10 agonist and 10 antagonist replicate tests.
- Phase 2 – The protocols were evaluated and refined at all labs until reproducible results were obtained. Twelve coded (blinded) reference substances, chosen by ICCVAM to standardize and validate the method, were assayed at each lab in three replicate tests.
- Phase 3 – The optimized protocols were used to complete interlaboratory studies between the same three labs. Forty-one coded chemicals were tested with no replication using the optimized methods.
- Phase 4 – The optimized protocols were used to test 25 coded chemicals with no replication at the XDS (United States) laboratory.

The reliability and accuracy of the validation results were later drafted into a BRD by NICEATM and the ICCVAM Interagency Endocrine Disruptor Working Group (EDWG). The BRD also provided recommendations regarding the assay's usefulness, its limitations, performance standards, and possible future studies to improve the assay. The BRD was released for public comment following completion.

In March 2011, a second public, independent, international science review panel was convened by NICEATM. The panel reviewed the draft BRD for completeness and the extent that data supported ICCVAM recommendations. Additional consideration was given to validation, acceptance criteria, and performance standards.

Following the international science review, the draft BRD was revised to consider the conclusions and recommendations of the international panel, public comments, and those received from SACATM. The BRD was then finalized and forwarded to other federal agencies for consideration and acceptance decisions, as required by the ICCVAM Authorization Act (42 U.S.C. 2851-3). The Organisation for Economic Co-operation and Development (OECD) Test Guidelines Programme was also sent a copy for consideration and adoption as an international testing protocol.

B. Background on the Lumi-Cell® Assay

The LUMI-CELL® assay is an *in vitro* test method developed to assess the potential of a test substance to activate or inactivate ERs that are present on human ovarian adenocarcinoma BG-1 cells. The basic principle underlying the LUMI-CELL® assay is that substances that bind to and activate or inactivate ERs will alter the expression of a gene (*luc*), which catalyzes light production from luciferin,[6] resulting in an increase (agonist) or decrease (antagonist) of luminescence, as measured on a luminometer. The increase or decrease in luminosity

[6] Luciferin – A molecule that emits light following enzyme-catalyzed breakdown by luciferase.

(concentration-response curve) is compared to criteria for concurrent positive and negative controls and concurrent cytotoxicity estimates to determine if results are acceptable (controls fall within certain levels, minimal cell cytotoxcity), and to obtain an objective measurement of ER activity.

The LUMI-CELL® assay is the first "endocrine disruptor" test method evaluated and recommended by ICCVAM. Some of the advantages of this test method include:

- The assay does not use animals.
- The assay uses a human cell line.
- The cell line expresses a large number of both ERα and ERβ.
- The assay can identify both ER agonists and antagonists.
- The assay measures the biological response resulting from specific ER binding.
- The assay is highly responsive to estrogens and has low background noise.
- The assay provides concentration-response information.
- The assay costs 10 times less than current ER agonist assays (for example, OECD TG 455) with a comparable time to completion (4 versus 3 days, respectively).
- Currently, the LUMI-CELL® assay is the only *in vitro* estrogen receptor assay that has undergone validation for ER agonists and antagonists.

C. Validation and Performance

Specifically, the LUMI-CELL® assay was validated for its reliability and its ability to identify *in vitro* ER agonists (activators) and antagonists (inactivators). In order to do this, the test substance classification (positive or negative) determined by the LUMI-CELL® assay was compared to a reference substance that had been previously classified by a weight-of-evidence approach by ICCVAM (a "consensus classification[7]").

The accuracy of the LUMI-CELL® assay was also compared to the EPA OPPTS 890.1300/OECD TG 455 assay and other assays in order to determine its relevance.

Validation results of the LUMI-CELL® assay can be seen in Appendix A.

III. ICCVAM Recommendations and Independent Peer-Review Panel Conclusions

1. ICCVAM recommended that the LUMI-CELL® assay was accurate and reliable enough to "support its use to screen substances for *in vitro* ER agonist and/or antagonist activity" and that the accuracy of the assay was "at least equivalent to that of the current ER TA test method included in regulatory testing guidance (EPA OPPTS 890.1300)."

[7] Consensus classification – Classification of a test substance as positive (POS) or negative (NEG) for ER TA activity (agonist or antagonist). Classification was based on a review of the published information and fulfilling certain criteria (positive in >50% of published information = POS; negative in all published information (n > 2 studies) = NEG; positive in ≤ 50% of published material or positive in only study conducted = presumed positive (PP); negative in the only study conducted = presumed negative (PN); PP or PN depending on other information including mechanism of action).

2. ICCVAM further recommended that when using the LUMI-CELL® assay to screen substances, characterizing the assay, or describing the assay limitations, the protocols provided in the ICCVAM NICEATM report be used.

3. Even though ICCVAM considers the LUMI-CELL® assay to be validated as is, ICCVAM recommended that a variety of future studies could potentially improve the usefulness and applicability of the test method. These considerations could be roughly divided into: (1) comparative validation studies, and (2) assay refinement studies.

   Comparative Validation Studies

   A. Additional comparative validation studies could be conducted to determine whether the LUMI-CELL® assay could replace the In Vitro Estrogen Receptor Saturation Binding and Competitive Binding Assay (rat uterine cytosol assay).
   B. Additional studies could be conducted to determine if the LUMI-CELL® assay could be combined with other methods (*e.g.*, in vitro metabolic activation) to replace the Short-Term In-Vivo Uterotrophic Bioassay.

   Assay Refinement Studies

   A. Additional studies could be performed to further characterize the ratio of ERα and ERβ in the BG-1 cell line and the extent that differing receptor subtype ratios affect the performance of the LUMI-CELL® assay.
   B. Additional studies could be performed to determine if testing volatile substances was feasible (*e.g.*, using a CO_2-permeable film to seal off the test plate).
   C. Additional studies could be performed to determine if a solubilization vehicle other than dimethyl sulfoxide (DMSO) could be used.
   D. Additional studies could be performed to determine if and how the LUMI-CELL® assay could incorporate metabolic activation of test substances into the protocol.
   E. Additional studies could be conducted to increase the number of antagonist substances tested. This would characterize better the usefulness of the LUMI-CELL® assay as a screen for ER antagonist activity.

4. ICCVAM further recommended that all data generated from assay refinement and comparative validation studies be provided to ICCVAM so that they can further "characterize the usefulness and limitations of the BG1LucER TA test method as a screening test to identify substances with ER agonist or antagonist activity."

5. ICCVAM also recommended that performance standards developed by NICEATM and the Endocrine Disruptor Working Group (EDWG) be used by laboratories with no experience in order to demonstrate their technical proficiency at performing the LUMI-CELL® assay.

IV. Discussion by CPSC Staff

Staff agrees with the five ICCVAM recommendations and the Independent Peer-Review Panel conclusions. The information presented in the summary and BRD documents provide sufficient detail for CPSC staff to evaluate the assay utility and performance as described.

As described, a variety of other methods are available for assessing ER activity (activation or inactivation). CPSC staff agrees that some of these methods are made superfluous by the LUMI-CELL® assay for screening and prioritization of chemicals for further study.

Interlaboratory validation of the LUMI-CELL® assay was based on the comparison of after-analysis substance classifications, not actual or normalized data. Although this technique does not address the actual variability of the data produced within and between laboratories, it does compare the ultimate "decision point," which is appropriate for the assay's purported use as a screening and prioritization tool.

The quantitative data generated by the LUMI-CELL® assay may be most useful for CPSC staff because it provides a relative level of ER activation or inactivation by a compound. Relatively good association of LUMI-CELL® assay results to published quantitative reference values supports the notion that the quantitative aspect of the assay may be useful and close to that generated by other methods.

CSPC staff agrees with both the Panel's and ICCVAM's recommendation to continue to accrue data because the dataset for ER antagonist testing is relatively sparse.

In 1984, the CPSC adopted a policy to reduce the number of animals tested and to minimize the pain and suffering associated with testing (49 FR 22522). In addition, the use of laboratory animals was recommended in a tiered and sequential approach to testing. In a tiered-testing strategy, the test substance is tested *in vivo* if the appropriate hazard determination cannot be made from physicochemical characteristics, expert opinion, prior human experience, or prior animal testing. Under the FHSA, the determination of whether a substance is a "chronic toxicant" is based upon a weight-of-evidence approach.

Therefore, the LUMI-CELL® assay would fit into a weight-of-evidence evaluation under the FHSA. CPSC staff agrees with the ICCVAM Panel that the NICEATM analyses on the ability of the LUMI-CELL® assay to determine substances that activate or antagonize human ERs are based on sound science and are scientifically valid for the proposed uses.

V. Recommendation by CPSC Staff

Staff recommends that the Executive Director of CPSC accept all five of the ICCVAM recommendations for the LUMI-CELL® assay as a scientifically sound and validated *in vitro* test method. Specifically, these recommendations are:

1. that the assay is accurate and reliable enough to "support its use to screen substances for *in vitro* ER agonist and/or antagonist activity" and that the accuracy of the assay is "at least equivalent to that of the current ER TA test method included in regulatory testing guidance (EPA OPPTS 890.1300)";

2. that the protocols provided in the ICCVAM NICEATM report be performed when using the LUMI-CELL® assay to screen substances, characterize the assay, or describe the assay limitations;

3. that a variety of future studies could potentially improve the usefulness and applicability of the test method;

4. that all data generated from assay refinement and comparative validation studies be provided to ICCVAM so that they can further "characterize the usefulness and limitations of the BG1LucER TA test method as a screening test to identify substances with ER agonist or antagonist activity"; and

5. that performance standards developed by NICEATM and the EDWG be used by laboratories with no experience in order to demonstrate their technical proficiency at performing the LUMI-CELL® assay.

Cautionary labeling of a consumer product regarding the hazards associated with that product is required by the FHSA. In order to determine the appropriate labeling to use, product-associated substances (chemicals) first must be investigated toxicologically. Data from *in vivo* or alternative-to-animal test models (*i.e., in vitro* testing) may be used during this process to decide whether the substance is "toxic" or a "chronic hazard" under the FHSA. The CPSC encourages the development and use of alternative-to-animal test models because these minimize the number of animals used and reduce the pain or suffering associated with animal testing.

Thus, staff recommends that the Executive Director of CPSC accept the ICCVAM recommendations because information from the LUMI-CELL® assay may be invaluable when determining whether a compound is a chronic toxicant in a weight-of-evidence approach. The assay may also provide supporting information that reduces the need to use a full complement of test animals to determine whether a chemical or substance is a chronic toxicant.

Following the Executive Director's decision, staff will draft a letter to ICCVAM, indicating the Executive Director's actions with regard to the ICCVAM recommendations. The ICCVAM website (http://iccvam.niehs.nih.gov/home.htm) will link to the CPSC website, where we will post our acceptance or nonacceptance of the recommendations. In the section of the ICCVAM website, News Updates (http://iccvam.niehs.nih.gov), there will be an announcement of the

Executive Director's action on the acceptance or nonacceptance of the ICCVAM recommendations. Once ICCVAM receives responses from all the agencies, it will publish a *Federal Register* notice announcing all of the agencies' responses.

VI. Options

The Executive Director can:

1. Accept the ICCVAM recommendations and instruct staff to draft a letter to ICCVAM indicating acceptance of its recommendations; or

2. Reject the ICCVAM recommendations and instruct staff to draft a letter to ICCVAM indicating rejection of its recommendations.

VII. Decision

X Accept the ICCVAM recommendations and instruct staff to draft a letter to ICCVAM indicating acceptance.

_____ Reject the ICCVAM recommendations and instruct staff to draft a letter to ICCVAM indicating rejection.

Kenneth R. Hinson

Signature
Kenneth R. Hinson
Executive Director

7/23/12

Date

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Appendix A.

Validation Results for the LUMI-CELL® Assay

1. LUMI-CELL® assay accuracy for agonist substances

The accuracy of the LUMI-CELL® assay for activation of the ER (agonist activity) was tested with 28 chemicals that activate the ER and 7 chemicals that do not. Testing yielded a concordance[8] of 97 percent (34/35), sensitivity[9] of 96 percent (27/28), specificity[10] of 100 percent (7/7), false positive rate[11] of 0 percent (0/7), and false negative rate[12] of 4 percent (1/28).

Data on 26 agonist reference chemicals were available to evaluate the concordance of the LUMI-CELL® assay with the only other validated ER TA available (EPA OPPTS 890.1300/ OECD TG 455). The 2 test methods had identical accuracy, with a concordance of 96 percent (25/26), sensitivity of 95 percent (21/22), specificity of 100 percent (4/4), false positive rate of 0 percent (0/4), and false negative rate of 5 percent (1/22).

EC_{50}[13] data on 26 chemicals generated from the LUMI-CELL® assay and from other ER TA test methods were compared. Values were input into a regression analysis to determine if the LUMI-CELL® assay values and other ER TA test method values were correlated. Although EC_{50} values sometimes differed by an order of magnitude, the correlation coefficient (R^2) was high (0.839), suggesting that the LUMI-CELL® assay results might also be adequate for a quantitative estimate of a substance's estrogenic agonist activity.

IC_{50}[14] data on 3 chemicals generated from the LUMI-CELL® assay and other ER transactivation test methods were also compared. As with EC_{50} values, these data were input into a regression analysis to assess whether or not the results for the methods were similar (correlated). IC_{50} values differed only by approximately 2-fold and the correlation coefficient (R^2) was high (0.95) between methods, suggesting that the LUMI-CELL® assay results might also be adequate for a quantitative estimate of a substance's estrogenic antagonist activity. This conclusion, however, is qualified by the fact that only 3 substances were available for comparison.

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[8] Concordance – "The proportion of all substances tested that are correctly classified as positive or negative. It is a measure of test method performance and is often used interchangeably with 'accuracy.'"

[9] Sensitivity – "The proportion of all positive substances that are classified correctly as positive in a test method. It is a measure of test method accuracy."

[10] Specificity – "The proportion of all negative substances that are classified correctly as negative in a test method. It is a measure of test method accuracy."

[11] False positive – "An inactive substance incorrectly identified as positive by a test method."

[12] False negative – "An active substance incorrectly identified as negative by a test method."

[13] "The half-maximal effective concentration of an agonist test substance (concentration required to induce 50 percent of the maximum possible response)."

[14] "The half-maximal inhibitory concentration of an antagonist (concentration that causes 50 percent inhibition of the measured response)."

2. LUMI-CELL® assay accuracy for antagonist substances

The accuracy of the LUMI-CELL® assay for inactivation of the ER (antagonist activity) was tested with 3 chemicals that activate the ER and 22 chemicals that do not. Testing yielded a concordance of 100 percent (25/25), sensitivity of 100 percent (3/3), specificity of 100 percent (22/22), false positive rate of 0 percent (0/22), and false negative rate of 0 percent (0/3).

No additional validated ER TA assays exist for antagonists, so direct comparisons (of concordance, specificity, false positive rate and false negative rate) to other methods were not able to be performed.

IC_{50}[15] data on three chemicals generated from the LUMI-CELL® assay and other ER transactivation test methods were also compared. As with EC_{50} values, these data were input into a regression analysis to assess whether or not the results for the methods were similar (correlated). IC_{50} values differed only by approximately 2-fold and the correlation coefficient (R^2) was high (0.95) between methods, suggesting that the LUMI-CELL® assay results might also be adequate for a quantitative estimate of a substance's estrogenic antagonist activity. This conclusion, however, is qualified by the fact that only 3 substances were available for comparison.

3. LUMI-CELL® assay concordance with other assays that measure "endocrine disruption"

Quantitative results for the LUMI-CELL® assay (EC_{50} and IC_{50}) were compared to median values reported from other relevant test methods (ER binding assays and uterotrophic assays). The LUMI-CELL® assay had a 97 percent (33/34 chemicals) concordance with ER binding data, and a 92 percent (12/13 chemicals) concordance with the *in vivo* uterotrophic assay. The high concordance between assay results suggests that the LUMI-CELL® assay might be a viable alternative to performing ER binding assays for both ER agonists and antagonists.

4. LUMI-CELL® assay intra-laboratory reproducibility

Adequate test reproducibility (multiple tests of the same chemical within one laboratory) is critical for ensuring that data produced within the same laboratory is accurate.

For the LUMI-CELL® assay, reproducibility was first assessed by comparing converted relative light unit (RLU; luminescence) data for the dimethyl sulfoxide (DMSO) control (agonist and antagonist tests) and E2 control (antagonist test) wells for all plates tested within each laboratory during the validation.

Plate RLU values were not compared directly for these assessments because they were not normalized and can vary between tests and over time. Instead, the within plate variability (CV) for each plate was compared for the DMSO agonist and antagonist and E2 antagonist control

[15] "The half-maximal inhibitory concentration of an antagonist (concentration that causes 50% inhibition of the measured response)."

wells. The within-DMSO agonist plate CV values for all of the labs ranged from 1 to 43%, but had low overall mean within-plate CVs of 6–8 percent, depending on the lab. Only 6 out of 218 agonist test plates that passed acceptance criteria actually had within-plate CVs greater than 20 percent, suggesting that within lab reproducibility was good. The within-DMSO antagonist plate CV values for all of the labs ranged from 1 to 52 percent but had low overall mean within-plate CVs of 6–9 percent, depending on the lab. Only 8 out of 194 antagonist test plates that passed acceptance criteria actually had within-plate CVs greater than 20 percent, suggesting that within lab reproducibility was good. The within-E2 antagonist plate CV values for all of the labs ranged from 9 to 19 percent, suggesting that within-E2 plate variability was low.

Reproducibility was also assessed by considering all data from Phase 2 testing, in which 12 substances were tested and classified in at least three independent experiments within each lab. Classifications (positive and negative agonist or antagonist) for each substance/plate were compared within each lab to estimate reproducibility.

Within each laboratory there was 100 percent agreement as to the classification for each of the 3 repeat tests for agonists and antagonists. This essentially meant that if a substance was classified positive (or negative) on the first replicate, the second and third replicates also ended up being classified positive (or negative). This high level of agreement in within-lab classification suggested that within lab reproducibility was good.

5. LUMI-CELL® assay inter-laboratory reproducibility

Adequate test reproducibility between laboratories (single test of the same chemical with multiple laboratories) is also critical for ensuring that substance classifications are accurate.

In order to determine inter-laboratory reproducibility, LUMI-CELL® assay results for the 12 Phase 2 substances (three replicates of each) were used to classify each of the test substances as either positive or negative for ER activation or inactivation. The classifications for each substance (not the raw or normalized data) were then compared between labs.

Between the laboratories there was 67 percent (8/12) agreement for the classification of agonists and 100 percent (12/12) agreement for the classification of chemicals tested for antagonist activity. Three of the discordant results for the chemicals in agonist testing were hypothesized to have resulted from contamination of the testing stocks (in one lab) after initial range finding experiments.

LUMI-CELL® assay results for the 41 Phase 3 substances (only one test was performed for each substance) were also used to classify each of the test substances as either positive or negative for ER activation or inactivation. As in Phase 2 testing, the classifications for each substance (not the raw or normalized data) were then compared between labs. In contrast to Phase 2 chemicals, 5 chemicals in Phase 3 agonist testing had results that were inadequate for determining a classification. All three laboratories agreed on 30 of the 36 (83%) remaining chemical classifications when considering agonist activity. Four of the discordant chemical classifications for agonist testing were suggested to have resulted from differences in estimates of solubility and test chemical concentrations chosen for testing by each lab. Misinterpretation of range-finding

data was the primary reason for using incorrect test chemical concentrations in comprehensive testing.

When considering antagonist classifications, 38 out of 41 classifications (93%) were in agreement. The 3 discordant classifications cannot be attributed to the same issues that affected agonist testing of Phase 3 chemicals.

Overall, if only chemicals with conclusive classifications are considered, 35 out of 36 classifications (97%) were similar between the three labs.

Appendix B.

Assays that Measure Estrogenicity or Estrogen Receptor Activation or Inactivation

1. **The Stably Tranfected Transactivation Assay (STTA)** assesses ER agonist activity of test substances. This test has been developed by the Japanese Chemicals Evaluation and Research Institute (CERI), and has been adopted by the U.S. Environmental Protection Agency (EPA) as an Endocrine Disruptor Screening Program (EDSP) Test Guideline titled, OPPTS 890.1300: Estrogen Receptor Transcriptional Activation (Human Cell Line (HeLa-9903). This test protocol is also adopted by OECD as OECD TG 455, "Stably Transfected Human Estrogen Receptor-α Transcriptional Activation Assay for Detection of Estrogenic Agonist-Activity of Chemicals." This test protocol is intended for use as part of a full screening battery for EDSP and specifically addresses activation of an estrogen-regulated gene following an agonist binding to human (h)ERα in the HeLa-9903 cell line. For example, if a chemical ER agonist binds to the hERα receptor, the receptor/agonist complex will move to the nucleus and bind to specific DNA response elements which activates a luciferase reporter gene, which results in the increase in production of the enzyme luciferase. Luciferase converts luciferin (which has been added to the wells of the test plate) into a bioluminescent product which is quantitatively measured with a luminometer. Increased agonist activation of the hERα, therefore, results in increased luminescence. Unlike the LUMI-CELL® assay, this protocol only measures **agonist** activity on the hERα receptor. Antagonist activity is currently being validated.

2. **The *In-Vitro* Estrogen Receptor Saturation Binding and Competitive Binding Assay** assesses the ability of a test substance to bind to the hormone-binding domain (HBD) of an estrogen receptor mixture obtained from rat (**r**) uteri (primarily ERα and secondarily ERβ). It has been incorporated into the EPA's EDSP program and is intended for use as part of a full screening battery. This assay determines whether the chemical substance can bind to the ER receptor (but not necessarily activate it). For example, if a chemical substance binds to the rER, it will competitively displace or replace a coincubated radiolabeled ER agonist (in this assay it is 17β-estradiol). If the chemical substance binds to the ER receptor, therefore, less 17β-estradiol radiolabel will remain in the final measurement tube. Unlike, the LUMI-CELL® assay, this protocol only measures whether the test substance will **bind** to the **r**ER, not if it can activate or inactivate the receptor. A human ERα prepared as a recombinant protein may replace use of the rat uterine ER once successfully validated in the future.

3. **The Short-Term *In-Vivo* Uterotrophic Bioassay** in female rodents provides information on estrogenicity of a substance by measuring the increase in wet and dry uterine weight following chemical exposure. It is included in OECD testing (TG 440) and also as an *in vivo* test in the EPA EDSP screening battery. If a chemical substance acts as an estrogenic agonist in the rat following oral or subcutaneous exposure, uterine weight will increase through enhanced water retention and uterine tissue growth. This test is specific for estrogen **agonists** only and has not been validated for anti-estrogens. Anti-estrogenicity is, however, commonly tested in this system and a guidance document for this test is available through OECD (OECD Report of the Validation of the Rodent

Uterotrophic Bioassay: Phase 2 - Testing of Potent and Weak Oestrogen Agonists by Multiple Laboratories No. 66OECD (2007a). Guidance Document on the Uterotrophic Bioassay - Procedure to Test for Antioestrogenicity. OECD Series on Testing and Assessment. No. 71.).

4. **Rat in-vivo pubertal female assay** provides information on the effects of a substance on the hypothalamic-pituitary-gonadal (HPG) axis (pubertal development) in intact female young/peripubertal rats. The assay identifies substances that have the potential for acting as **estrogen receptor mediators** and thyroid hormone mediators. In the assay, young female rats are dosed with the substance and then assessed for vaginal opening (an estrogen-dependent event) and the production of thyroid hormones. Substances that decrease or antagonize estrogen-related events will result in a delay in vaginal opening. Substances that alter the thyroid or associated organs will result in a change in production of thyroid hormones. This test has been validated by the US EPA (2007) using ER agonists, an ER antagonist, a chemical that induces metabolism of thyroid hormones or alters thyroid synthesis, and other chemicals. The rat pubertal female assay is currently included as an *in vivo* Tier 1 test in the EPA EDSP screening battery.

5. **The Short-term In-Vivo Fish Reproduction Assay** provides information on the estrogenic and androgenic effects of water soluble chemicals on the HPG axis in sexually mature male and spawning female fish. Specifically, it looks at two biomarkers of effect; changes in vitellogenin production and secondary sexual characteristics. The assay also determines the daily quantitative fecundity (ability to reproduce) of the exposed fish and can also involve interpretation of the gonadal histopathology. Estrogenic compounds can stimulate the male fish liver to synthesize and secret vitellogenin into the plasma. Vitellogenin is normally undetectable in the circulating plasma of male fish. Anti-estrogenic compounds can inhibit the synthesis and release of vitellogenin into the plasma of female fish, which normally produce large amounts of vitellogenin when spawning. Androgenic compounds can also induce the development of quantifiable male secondary sex characteristics in female fish. Anti-androgenic chemicals can impair the development of male secondary sex characteristics in male fish. This test has been validated by the US EPA (2007) using an estrogen receptor agonist and androgenic receptor antagonists. The fish reproduction assay is currently included in OECD testing (TG 229) and also as an *in vivo* Tier 1 test in the EPA EDSP screening battery.