NOMINATION OF THE LUMI-CELL™ ER HIGH-THROUGHPUT SYSTEM FOR SCREENING ESTROGEN-LIKE CHEMICALS FOR VALIDATION STUDIES

DRAFT EVALUATION

NICEATM

August 16, 2004

TABLE OF CONTENTS

Page

EXECUTIVE SUMMARY i

1.0 INTRODUCTION 1

1.1 XDS Nomination 1

1.2 SACATM Review (March 10-11, 2004) 4

1.3 NICEATM *Federal Register* Notice 4

1.4 XDS Pre-validation Background Review Document 5

2.0 EVALUATION OF THE ABILITY OF THE LUMI-CELL™ ER BIOASSAY TO DETECT SUBSTANCES WITH ER AGONISM AND ANTAGONISM ACTIVITY 6

2.1 To What Extent Does the Nomination and Proposed Test Method Address the ICCVAM Prioritization Criteria? 6

2.2 Do the LUMI-CELL™ Pre-Validation Agonist and Antagonist Studies Adhere to the Recommendations of the *ICCVAM Evaluation of In Vitro Test Methods for Detecting Potential Endocrine Disruptors* (NIH Publ. No. 03-4503), Especially Those Regarding Essential Test Method Components (Previously Known as Minimum Procedural Standards) and Recommended Validation Substances? 7

2.2.1 Essential Test Method Components 7

2.2.2 ICCVAM Recommended Validation Substances 9

2.3 Does LUMI-CELL™ Show Adequate Performance (Reliability and Accuracy) During Pre-Validation to Warrant Consideration for Validation Studies? 10

2.3.1 Reliability (Repeatability and Intra- and Inter-laboratory Reproducibility) of the LUMI-CELL™ ER Bioassay for Detecting ER Agonist Activity 10

2.3.2 The Accuracy of the LUMI-CELL™ ER Bioassay for Detecting ER Agonist Activity 11

2.3.3 Reliability (Repeatability and Intra- and Inter-laboratory Reproducibility) of the LUMI-CELL™ ER Bioassay for Detecting ER Antagonist Activity 14

2.3.4 The Accuracy of the LUMI-CELL™ ER Bioassay for Detecting ER Antagonist Activity 15

2.4 Does the BRD Adequately Provide the Information Requested in the Outline Provided in the *ICCVAM Guidelines for the*

Nomination and Submission of New, Revised, and Alternative Test Methods (NIH Publ. No. 03-4508)?.....18

3.0 NICEATM RECOMMENDATIONS: 20

EXECUTIVE SUMMARY

On January 22, 2004, NICEATM received a letter from Dr. George Clark of Xenobiotic Detection Systems (XDS) nominating a cell based transcriptional method (trademarked as LUMI-CELL™) for validation studies. The test method evaluates the endocrine disruptor activity of chemicals by measuring whether and to what extent the chemical induces or blocks transcription at the estrogen receptor (ER). The nomination requested that NICEATM and ICCVAM aid in and manage the cross-laboratory validation studies needed to formally evaluate the reliability and accuracy of the LUMICELL ™ ER bioassay for its proposed use as a regulatory test method for detecting chemicals with *in vitro* estrogenic agonist and antagonist activity.

On April 21, 2004, NICEATM authored a *Federal Register* (*FR*) Notice (Vol. 69, No. 77, p. 21564), entitled "In Vitro Endocrine Disruptor Test Methods: Request for Comments and Nominations." The *FR* :

- identified *in vitro* endocrine disruptor screening methods that do not require the use of animal tissues as an ICCVAM priority for validation studies;
- indicated the availability of published ICCVAM recommendations[1] for standardization and validation of *in vitro* endocrine-disruptor estrogen and androgen receptor binding and transcriptional activation assays; and
- invited the nomination for validation studies of *in vitro* test methods that meet the recommendations and for which there are standardized test method protocols, pre-validation data, and proposed validation study designs.

NICEATM received a pre-validation background review document (BRD) from XDS on April 23, 2004, and a revised BRD on June 21, 2004. In accordance with the ICCVAM nomination process, NICEATM conducted a pre-screen evaluation of the revised BRD and proposal to determine the extent that the proposed nomination addresses the ICCVAM prioritization criteria, ICCVAM submission guidelines, and ICCVAM recommendations for

[1] ICCVAM Evaluation of *In Vitro* Test Methods For Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays. (2003). NIH Publication No. 03-4503. http://iccvam.niehs.nih.gov/methods/endocrine.htm

standardization and validation of *in vitro* endocrine disruptor test methods. The performance of the test method based on pre-validation data was also reviewed to determine if this performance warrants consideration for further validation. The revised BRD is the focus of the NICEATM pre-screen evaluation.

The four areas considered in evaluating the pre-validation information provided by XDS in their background review document (BRD) and the extent to which the criteria are met are as follows:

1. To what extent does the nomination and proposed test method address the ICCVAM prioritization criteria?

The LUMI-CELL™ ER bioassay meets all of the ICCVAM prioritization criteria. The test method:

- is applicable to the needs of the US Environmental Protection Agency (EPA) for a high throughput screening system to evaluate substances for their potential estrogen disruptor activity, and may also be applicable to the US Food and Drug Administration, Department of Agriculture, Department of Defense, and Department of Homeland Security, since methodologies are being developed to screen feed and food for potential estrogen disruptor chemicals.
- is warranted, based on the worldwide concern about the association between exposure to endocrine disruptors and adverse health effects in human and wildlife populations.
- is warranted, based on it potential to refine, reduce, or replace animal use
- is warranted, based on its demonstrated ability to detect estrogenic activity at extremely low levels (i.e., some six to seven magnitudes lower than that induced by β-estradiol, the endogenous estrogen).
- is warranted, based on its relatively low cost per substances tested ($350) and the relatively quick study duration (two days)

2. Do the LUMI-CELL™ pre-validation agonist and antagonist studies adhere to

the recommendations of the *ICCVAM Evaluation of In Vitro Test Methods for Detecting Potential Endocrine Disruptors* (NIH Publ. No. 03-4503), especially those regarding essential test method components (called minimum procedural standards in this document) and recommended validation substances?

Essential Test Method Components: With a few exceptions, the agonist and antagonist protocols for the LUMI-CELL™ ER bioassay incorporates the recommended essential test method components for both agonist and antagonist studies. These exceptions do not appear to adversely impact on the performance (accuracy and reliability) of the assay. Examples of exceptions include the preferential use of dimethylsulfoxide (DMSO), rather than water or ethanol (95 to 100%) as the preferred solvent; using 40 pg and not the recommended maximum test substance concentration of 1 mM for agonism and antagonism assays; and incorporating qualitative rather than quantitative measures of cytotoxicity in the assay.

ICCVAM Recommended Validation Substances: For the validation of ER TA agonist assays, ICCVAM recommended 78 substances (35 positive/presumed positive, 43 negative/presumed negative). The BRD provided data on 108 substances, 56 of which were included in the ICCVAM recommended validation list (29 classified by ICCVAM as positive/presumed positives by ICCVAM, 27 classified by ICCVAM as negatives/presumed negatives for ER TA activity). This number of substances is considered sufficient for the pre-validation of the agonist version of the LUMI-CELL™ ER bioassay.

3. Does LUMI-CELL™ show adequate performance (reliability and accuracy) during pre-validation to warrant consideration for validation studies?

Reliability (Repeatability and Intra- and Inter-laboratory Reproducibility) of the LUMI-CELL™ ER Bioassay for Detecting ER Agonist Activity: In their BRD, XDS provided coefficient of variation (CV) data for LUMI-CELL™ agonist test results with respect to what they classified as well-to-well variability[2] within an experiment for 12 ICCVAM

[2] In LUMI-CELL™, a substance is tested at up to 11 concentrations, with each concentration tested in triplicate wells on a 96-well plate. To evaluate well-to-well variability, XDS determined the CV for the EC50 values

recommended positive reference substances and plate-to-plate (plate = experiment; minimum of three independent experiments) for 33 ICCVAM recommended validation substances reported as positive in LUMI-CELL™. An evaluation of interlaboratory agonist reproducibility has not been conducted; this evaluation would be conducted as part of a multi-laboratory validation effort. XDS did not use coded chemicals in the collection of these data. The mean and median CV values for within experiment EC50 values for the 12 ICCVAM recommended positive reference substances was 28 and 29%, respectively. The mean and median CV values for plate-to-plate (i.e., experiment-to-experiment) EC50 values for 33 ICCVAM recommended reference substances that induced a positive response in LUMI-CELL™ was 45 and 38%, respectively. These levels of repeatability and intralaboratory reproducibility are considered adequate for screening assays by NICEATM.

Accuracy of the LUMI-CELL™ ER Bioassay for Detecting ER Agonist Activity: There is no agreed-upon animal or human data set to serve as a reference for determining the accuracy of *in vitro* test methods for identifying substances with estrogen activity *in vivo*. As an alternative, the compilation of published mammalian cell *in vitro* ER TA results, as summarized in Appendix D of the ICCVAM report was compared with the LUMI-CELL™ ER bioassay test results reported in Appendix D of the XDS BRD. Fifty-six of the 78 substances recommended by ICCVAM for the validation of *in vitro* TA test methods were tested for agonist activity by XDS in the LUMI-CELL™ ER Bioassay. Based on the LUMI-CELL™ agonism test results, the concordance was 0.82, the sensitivity was 1.00, the specificity was 0.66, the false negative rate was 0, and the false positive rate was 0.34. The high "false positive" rate was due to ten of 29 ICCVAM recommended ER negative substances producing a positive or weak positive ER agonist response in LUMI-CELL™. However, due to the mechanistic basis of this test system, false positives are highly unlikely. These ten substances most likely have very weak transcriptional activity that is producing the weak positive response. Compared to the EC50 value for estradiol, all ten substances exhibited EC50 values that were six to seven fold orders of magnitude weaker. For these ten false positive substances, ICCVAM did not have supporting negative ER TA data for seven

(i.e., the concentration that induces a half-maximal agonist response) calculated using the first, the second, or the third sets of wells.

substances, and had single test data only for two substances. Only one substance, atrazine, had been reported as negative for ER TA activity in three studies. Thus, it is entirely possible that all ten of these substances are capable of producing weak ER transcriptional activation and that that increased TA activity represents "true" positives for the type and distribution of estrogen receptors in this test system. Furthermore, these responses may indicate that this test system is capable of detecting ER activity over a broad dynamic range, including very weak activity. Nonetheless, such results will need confirmation in a multi-laboratory validation study and, if possible, in other transcriptional assays with comparable receptor composition and sensitivity. Finally, the quantitative nature of the response will likely need to be considered when using this data for weight-of-evidence decisions in the EPA's Tier 1 Endocrine Disruptor Screening Program, with possibly less weight given to very weak acting substances, especially those that do not demonstrate an *in vivo* effect at established limit doses.

Another approach to evaluating the performance of the LUMI-CELL™ ER Bioassay, in terms of the ICCVAM recommended validation substances, is to compare the relative quantitative agonist activity of substances reported as positive in both data sets. Due to the lack of EC50 data for many of the substances recommended in the ICCVAM report, this analysis was limited to nine substances with ER TA activity. The regression correlations (r^2) for EC50 values and relative rankings were 0.607 ($p = 0.013$) and 0.903 ($p<0.001$), respectively. Thus, the relative ER TA activities of these nine agonist substances are significantly correlated between the LUMI-CELL™ ER bioassay and the data summarized in the ICCVAM report.

Reliability (Repeatability and Intra- and Inter-laboratory Reproducibility) of the LUMI-CELL™ ER Bioassay for Detecting ER Antagonist Activity: XDS did not provide CV data for LUMI-CELL™ antagonist test results with respect to well-to-well variability within an experiment but did provide plate-to-plate (plate = experiment; minimum of three experiments conducted on different days) for eight ICCVAM recommended substances reported as positive in LUMI-CELL™. An evaluation of interlaboratory antagonist reproducibility has not been conducted; this evaluation would be conducted as part of a multi-laboratory

validation effort. The mean and median CV values for plate-to-plate (i.e., experiment-to-experiment) IC50[3] values for eight ICCVAM recommended reference substances that induced a positive antagonist response in LUMI-CELL™ was 24 and 25%, respectively. This level of intralaboratory reproducibility is considered adequate by NICEATM for screening assays.

The Accuracy of the LUMI-CELL™ ER Bioassay for Detecting ER Antagonist Activity: Sixteen of the 78 substances recommended by ICCVAM for the validation of *in vitro* TA test methods were tested for antagonist activity by XDS in the LUMI-CELL™ ER bioassay. In their list of 78 recommended substances, ICCVAM identified eight substances with demonstrated antagonist activity, three with anticipated antagonist activity, 10 with demonstrated negative antagonist activity, and 57 with anticipated negative antagonist activity. Of the 16 substances listed by XDS as being tested for antagonist activity in the LUMI-CELL™ ER bioassay, ICCVAM had classified eight as positive for ER antagonist activity and eight without ER antagonist activity. Based on the LUMI-CELL™ antagonism test results, the concordance was 0.50, the sensitivity was 1.00, the specificity was 0, the false negative rate was 0, and the false positive rate was 1.00. All eight ICCVAM validation substances presumed to be ER antagonists induced a positive or weak positive antagonist response in LUMI-CELL™. However, ICCVAM did not have supporting ER antagonism data for six of these substances. Only eight ICCVAM validation substances with known or predicted ER antagonist activity were tested by XDS in the LUMI-CELL™ ER bioassay. However, the list of validation substances recommended by ICCVAM only contains 11 ER antagonist substances (eight with supporting data, three without *in vitro* ER TA antagonist supporting data). Due to the limited number of antagonists tested by XDS and the limited number of studies reported by ICCVAM with quantitative data, a comparative analysis of potency could not be conducted. While additional LUMI-CELL™ ER antagonist data would be useful in clarifying the performance of this assay for identifying substances with antagonist activity, the lack of such studies is not considered to be a significant detriment to conducting cross laboratory validation studies.

[3] The concentration of the test substance calculated to inhibit the estrogenic activity of a specified concentration of the reference estrogen by 50%.

4. Does the BRD adequately provide the information requested in the outline provided in the *ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods* (NIH Publ. No. 03-4508)?

The XDS BRD adheres to the recommended outline and provides nearly all of the requested information. However, additional information should be provided if the BRD is to be released beyond ICCVAM. The lack of this information did not adversely impact on the evaluation of Criterions 1 through 3.

NICEATM Recommendations: Based on the data provided in the XDS BRD on the LUMI-CELL™ ER bioassay, NICEATM recommends to the EDWG that:

- LUMI-CELL™ be considered as a high priority for validation studies as an *in vitro* test method for the detection of test substances with ER agonist and antagonist activity.
- To facilitate independent and timely standardization and validation studies, NICEATM should manage the needed studies by exercising a validation coordination option in its support contract. Such studies should include coordination and collaboration with ECVAM and JCVAM, and ideally include one laboratory in each of the three respective geographic regions supported by these three Centers.
- During finalization of their BRD and in preparation for the interlaboratory validation study, XDS conduct additional antagonist studies to more comprehensively demonstrate the suitability of LUMI-CELL™ as an assay for the detection of substances with ER antagonist activity.

.

1.0 INTRODUCTION

1.1 XDS Nomination

On January 22, 2004, NICEATM received a letter from Dr. George Clark of Xenobiotic Detection Systems (XDS) nominating for validation a cell based transcriptional method (trademarked as LUMI-CELL™) for the evaluation of the endocrine disruptor activity of chemicals for the estrogen receptor (ER). In its nomination, Dr. Clark stated that the LUMI-CELL™ ER Bioassay was a standardized test procedure in a stably transfected recombinant cell line that was sensitive, robust, and reproducible in detecting estrogen active chemicals, and summarized the extent to which this *in vitro* test method met each of the ICCVAM prioritization criteria (ICCVAM, 2003[4]). The ICCVAM prioritization criteria and the extent to which these criteria were stated to be met by the LUMI-CELL™ ER Bioassay are:

- ***The Extent To Which The Proposed Test Method Is Applicable To Regulatory Testing Needs***

  "The LUMI-CELL™ ER bioassay will meet the need for a high throughput screening (HTPS) system of chemicals for their potential estrogen disruptor activity. The US Environmental Protection Agency (EPA) identified a need for this technology in the Endocrine Disruptor Steering and Testing Advisory Committee (EDSTAC) recommendations in order to meet a mandate of the Food Quality Protection Act of 1996 and the Safe Drinking Water Act of 1996. This test method is also in response to Federal Register Notice (Vol. 66, No. 57/Friday, March 23, 2001) as a HTPS method for estrogen active compounds".

- ***The Extent To Which The Proposed Test Method Is Applicable To Multiple Agencies/Programs***

  "The LUMI-CELL™ ER bioassay technology may also be applicable to the

[4] ICCVAM. 2003. ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods. NIH Publication No: 03-4508. Research Triangle Park, North Carolina: NIEHS (http://iccvam.niehs.nih.gov/docs/guidelines/subguide.htm)

US Food and Drug Administration, Department of Agriculture, Department of Defense, and Department of Homeland Security, since methodologies are being developed to screen feed and food for potential estrogen disruptor chemicals. Both food and feed are a potential source for exposure to EDCs".

- ***The Extent To Which The Proposed Test Method Is Warranted, Based On The Extent Of Expected Use Or Application And Impact On Human, Animal, Or Ecological Health***

"The association of exposure to EDCs and adverse health effects in human and wildlife populations has led to worldwide concern. Some of the health effects that have led to this concern include global increases in testicular cancer, regional declines in sperm counts, altered sex ratios in wildlife populations, increases in the incidence of breast cancer and endometriosis, and accelerated puberty in females that are expected to result from exposure to chemicals that adversely affect steroid hormone action".

- ***The Potential For The Proposed Test Method, Compared To Current Test Methods Accepted By Regulatory Agencies, To Refine, Reduce, or Replace Animal Use***

"There are no currently accepted methods that are being used to screen for EDCs but some have been proposed and are in the process of validation by the EPA. Most of these methods require substantial use of animals to evaluate endocrine disruptor activity. The LUMI-CELL™ ER bioassay method would allow for a rapid process to screen and set priorities on testing chemicals for disruption of estrogenic activity in other animal models. This would consequently result in a significant reduction in animal use in the screening process".

- ***The Potential For The Proposed Test Method To Provide Improved Prediction Of Adverse Health Or Environmental Effects, Compared To Current Test Methods Accepted By Regulatory Agencies***

  "There are no current methods approved for the detection of ECDs by any federal agency. However, the LUMI-CELL™ ER bioassay shows tremendous potential for prediction of adverse health and environmental effects. This is shown by the very high correlation between agonist response data collected using our test method and the historical data available in the database developed by NICEATM on these compounds. The LUMI-CELL™ ER bioassay is sensitive enough to allow for an extremely low detection limit (ppq), which should be lower than federal regulations are likely to mandate. Unlike ELISA detection limits which have a lower limit of >1 ppb. The LUMI-CELL™ ER bioassay will give a measure of bioavailability, being a biological system itself.

- ***The Extent To Which The Test Method Provides Other Advantages (e.g., Reduced Cost And Time To Perform) Compared To Current Methods***

  "The LUMICELL ™ ER bioassay is an extremely rapid in vitro method that can evaluate the estrogenic activity of chemicals within two days. The method also provides relative activity of a chemical to the standard, beta-estradiol, and provides dose response activity of the chemical. The standardized protocol developed allows for a very robust system with low variability and high sensitivity. The cost of the LUMI-CELL™ ER bioassay is a few hundred dollars per chemical, which is substantially less than any animal base method. The LUMI-CELL™ ER bioassay is a transcriptionally based assay capable of testing for antagonistic responses of EDCs, which is not possible using binding assays".

In the XDS letter, Dr. Clark requested that NICEATM and ICCVAM aid in and manage the cross-laboratory validation studies needed to formally evaluate the reliability and accuracy of the LUMI-CELL ™ ER bioassay and its use as a regulatory test method for detecting chemicals with estrogenic agonist and antagonist activity. Dr. Clark stated that "the pre-validation and method development steps for this test method are essentially complete and data on the screening of 120 chemicals for estrogenic agonist activity can be made available to NICEATM and ICCVAM." Further, Dr. Clark proposed that XDS "act as the primary laboratory providing training and technical support to other participating laboratories."

1.2 SACATM Review (March 10-11, 2004)

NICEATM and ICCVAM presented for consideration two nominated *in vitro* endocrine disruptor test methods, one of which was the XDS LUMI-CELL ™ ER bioassay, to the Scientific Advisory Committee on Alternative Toxicological Methods (SACTAM) on March 10-11, 2004. The SACATM was supportive of the nominations and raised no objections to these assays being evaluated by NICEATM and considered by the EDWG and ICCVAM for future validation studies.

1.3 NICEATM *Federal Register* Notice

On April 21, 2004, NICEATM sponsored a *Federal Register* (*FR*) Notice (Vol. 69, No. 77, p. 21564), entitled "In Vitro Endocrine Disruptor Test Methods: Request for Comments and Nominations." This *FR* Notice stated that:

- ICCVAM and the SACATM had identified *in vitro* endocrine disruptor screening methods as a priority for validation.
- ICCVAM had published guidelines for development of *in vitro* endocrine-disruptor estrogen and androgen receptor binding and transcriptional activation assays. In these guidelines, ICCVAM recommended that priority be given to assays that
    1. do not require the use of animal tissue as the receptor source, but rather use recombinant-derived proteins, and

2. do not use radioactive materials.

- On behalf of ICCVAM, NICEATM invited the nomination for validation studies of *in vitro* test methods that meet these recommendations and for which there are standardized test method protocols, pre-validation data, and proposed validation study designs.
- At this time, ICCVAM had received nominations for two *in vitro* endocrine-disruptor screening methods (one was the nomination from XDS) purported to meet these recommendations.
- ICCVAM will consider nominations and comments received in response to this notice and develop recommended priorities for proposed evaluation and validation studies of endocrine disruptor screening methods.
- Prior to the initiation of such studies, the proposed validation studies would be evaluated for adherence to relevant recommendations in the report: ''ICCVAM Evaluation of *In Vitro* Test Methods for Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays'' (NIH Publication No. 03–4503; http://iccvam.niehs.nih.gov/methods/endocrine.htm) by the ICCVAM Endocrine Disruptor Working Group (EDWG) and NICEATM.

NICEATM did not receive any comments on the XDS nomination in response to this *FR* Notice.

1.4 XDS Pre-validation Background Review Document

On April 23, 2004, NICEATM received a pre-validation background review document (BRD) from XDS. A request for clarification of the structure of the appendices was submitted to XDS on May 12, 2004, with comments and questions submitted on May 28, 2004. In response to these comments and questions, XDS submitted a revised BRD on June 21, 2004. This revised BRD is the focus of this evaluation by NICEATM.

2.0 EVALUATION OF THE ABILITY OF THE LUMI-CELL™ ER BIOASSAY TO DETECT SUBSTANCES WITH ER AGONISM AND ANTAGONISM ACTIVITY

Four criteria were considered in evaluating the XDS pre-validation information provided in their BRD:

1. To what extent does the nomination and proposed test method address the ICCVAM prioritization criteria?

2. Do the LUMI-CELL™ pre-validation agonist and antagonist studies adhere to the recommendations of the *ICCVAM Evaluation of In Vitro Test Methods for Detecting Potential Endocrine Disruptors* (NIH Publ. No. 03-4503, http://iccvam.niehs.nih.gov/methods/endocrine.htm), especially those regarding essential test method components (previously known as minimum procedural standards) and recommended validation substances?

3. Does LUMI-CELL™ show adequate performance (reliability and accuracy) during pre-validation to warrant consideration for validation studies?

4. Does the BRD adequately provide the information requested in the outline provided in the *ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods* (NIH Publ. No. 03-4508)?

2.1 To What Extent Does the Nomination and Proposed Test Method Address the ICCVAM Prioritization Criteria?

The LUMI-CELL™ ER bioassay meets all of the ICCVAM prioritization criteria. The test method:

- is applicable to the needs of the US Environmental Protection Agency (EPA) for a high throughput screening system to evaluate substances for their

potential estrogen disruptor activity, and may also be applicable to the US Food and Drug Administration, Department of Agriculture, Department of Defense, and Department of Homeland Security, since methodologies are being developed to screen feed and food for potential estrogen disruptor chemicals.

- is warranted, based on the worldwide concern about the association between exposure to endocrine disruptors and adverse health effects in human and wildlife populations.
- is warranted, based on it potential to refine, reduce, or replace animal use
- is warranted, based on its demonstrated ability to detect estrogenic activity at extremely low levels (i.e., some six to seven magnitudes lower than that induced by β-estradiol, the endogenous estrogen).
- is warranted, based on its relatively low cost per substances tested ($350) and the relatively quick study duration (two days)

2.2 Do the LUMI-CELL™ Pre-Validation Agonist and Antagonist Studies Adhere to the Recommendations of the *ICCVAM Evaluation of In Vitro Test Methods for Detecting Potential Endocrine Disruptors* (NIH Publ. No. 03-4503), Especially Those Regarding Essential Test Method Components (Previously Known as Minimum Procedural Standards) and Recommended Validation Substances?

The ICCVAM recommendations in regard to essential test method components and substances to be used in the validation of ER transcriptional activation (TA) assays are described in Sections 4.1 and 4.2, respectively, of the ICCVAM report.

2.2.1 Essential Test Method Components

The ER TA section in the ICCVAM report contained essential test method component recommendations in regard to:

- the reference estrogen and associated TA response
- preparation of test substances and the volume of the administered solvent
- the concentration range of test substances that should be tested
- solvent and positive controls

- the number of within-test replicates
- methods for data analysis
- the need for Good Laboratory Practice (GLP) compliance
- study acceptance criteria
- interpretation of results
- repeat studies
- the study report

The agonist and antagonist protocols for the LUMI-CELL™ ER bioassay incorporates the recommended essential test method components for both agonist and antagonist studies, with few exceptions, and these exceptions do not appear to adversely impact on the performance (accuracy and reliability) of the assay. Examples of exceptions include the following:

ICCVAM Report Section 4.1.2 (Preparation of Test Substances and Volume of Administered Solvent): The report indicates that the preferred solvent is water, ethanol (95-100%), or dimethylsulfoxide (DMSO), in that order. Members of the ICCVAM Expert Panel stated that water or ethanol (95 to 100%) were preferred to DMSO because some substances, when dissolved in DMSO, might exhibit reduced agonist activity. In the LUMI-CELL™ ER Bioassay, DMSO is the solvent of choice. Based on the performance of the assay (see **Section 2.2** of this BRD), the use of DMSO does not appear to have impacted on the performance of this assay.

ICCVAM Report Section 4.1.3 (Concentration Range of the Test Substances): In the absence of solubility or cytotoxicity constraints, the recommended maximum test substance concentration (i.e., the limit dose) for agonism and antagonism assays should be 1 mM for negative test substances. However, as the LUMI-CELL™ ER bioassay was developed originally to test complex mixtures, the approach XDS uses is to test to a maximum concentration of 40 pg. For many, but not all, single chemicals evaluated by XDS that were negative for estrogenic activity, this level exceeds the recommended 1 mM limit concentration (*note: this information is provided in the data appendices to the XDS BRD*).

The ICCVAM report states that an evaluation of cell cytotoxicity should be included in each study, and only those dose levels not associated with toxicity greater than 10% of the concurrent solvent control considered in the analysis of the data. In the LUMI-CELL™ ER bioassay, XDS evaluates several measures of cytotoxicity. The first is a visual inspection of the cells. If the cells morphology is abnormal, or there appears to be some cell death (i.e., some cells have become detached), or if the cells are no longer attached at all and have been washed away in the PBS rinse, the data from those wells are not used. The second method of assessing cell toxicity is to use, for substances that are negative in the agonist assay, two positive response assays. This is accomplished by mixing the highest concentration and $1/10^{th}$ of the highest concentration of the test substance tested with the EC50[5] concentration of β-estradiol (*note: there is discordance between the BRD and the correspondence from XDS in how toxicity is evaluated – the information provided here is based on clarification from XDS*). If toxicity is absent, one or both of these sets of wells should result in an positive response for the reference estrogen (*note: this viability assay may be of limited use if the substances being evaluated are ER antagonists*). These approaches appear to be useful but less quantitative than what was recommended by the ICCVAM Expert Panel.

2.2.2 ICCVAM Recommended Validation Substances

To facilitate the validation of *in vitro* ER TA assays, ICCVAM provided a list of 78 recommended substances (35 substances were classified as positive or presumed positive and 43 substances were classified as presumed negative for ER TA agonist activity). It was recommended further that, at a minimum, 53 of these substances should be tested for agonist activity (34 substances were classified as positive or presumed positive, 19 substances were classified as presumed negative). Data on 108 substances were provided in the XDS BRD. Of the 108 substances, 29 were substances classified as positive or presumed positives by ICCVAM and 27 were substances classified by ICCVAM as presumed negatives for ER TA activity (i.e., for a total of 56 of 78 recommended substances). The remaining 22 of the 78 ICCVAM recommended substances were not tested due to a lack of availability, cost considerations, or because they were controlled substances for which XDS did not have a

[5] The concentration that is calculated to induce a response that is 50% of the maximally induced agonist response by that substance.

license. The 52 other substances tested by XDS were those not recommended by ICCVAM. For the purpose of evaluating the performance of the LUMI-CELL™ ER bioassay as a screen for the detection of substances with ER agonist activity, the number of ICCVAM recommended substances tested by XDS was deemed adequate.

2.3 Does LUMI-CELL™ Show Adequate Performance (Reliability and Accuracy) During Pre-Validation to Warrant Consideration for Validation Studies?

2.3.1 Reliability (Repeatability and Intra- and Inter-laboratory Reproducibility) of the LUMI-CELL™ ER Bioassay for Detecting ER Agonist Activity

In their BRD, XDS provided coefficient of variation (CV) data for LUMI-CELL™ agonist test results with respect to well-to-well variability[6] within an experiment for 12 ICCVAM recommended positive reference substances and plate-to-plate (plate = experiment; minimum of three independent experiments) for 33 ICCVAM recommended validation substances reported as positive in LUMI-CELL™. An evaluation of interlaboratory agonist reproducibility has not been conducted; this evaluation would be conducted as part of a multi-laboratory validation effort. XDS did not use coded chemicals in the collection of these data.

Test Method Repeatability: The mean and median CV values for within experiment EC50 values for the 12 ICCVAM recommended agonists were 28 and 29%, respectively. This level of repeatability is considered adequate by NICEATM for screening assays.

Test Method Intralaboratory Reproducibility: The mean and median CV values for plate-to-plate (i.e., experiment-to-experiment) EC50 values for 33 ICCVAM recommended reference substances that induced a positive response in LUMI-CELL™ was 45 and 38%, respectively. This level of intralaboratory reproducibility is considered adequate by NICEATM for screening assays.

[6] In LUMI-CELL™, a substance is tested at up to 11 concentrations, with each concentration tested in triplicate wells on a 96-well plate. To evaluate well-to-well variability, XDS determined the CV for the EC50 values calculated using the first, the second, or the third sets of wells.

582

583 2.3.2 The Accuracy of the LUMI-CELL™ ER Bioassay for Detecting ER Agonist Activity

584 There is no agreed-upon animal or human data set to serve as a reference for determining the
585 accuracy of *in vitro* test methods for identifying substances with estrogen activity *in vivo*. As
586 an alternative, the compilation of published mammalian cell *in vitro* ER TA results, as
587 summarized in Appendix D of the ICCVAM report was compared with the LUMI-CELL™
588 ER bioassay test results reported in Appendix D of the XDS BRD. One difficulty in using
589 the ICCVAM compilation as a reference data base is the lack of agreement among published
590 studies regarding the positive or negative responses of a number of the substances
591 recommended by ICCVAM for *in vitro* ER TA validation studies. This lack of agreement
592 among laboratories is largely due to the diversity of test methods and the varied decision
593 criteria developed by different investigators to evaluate ER TA activity. Another concern
594 with using the list of ICCVAM recommended validation substances is that the classification
595 of some substances is based on a single test in a single laboratory using a system that may not
596 have been well-defined or was based on theory rather than experimentally obtained data.

597

598 *Evaluation of Concordance:* Fifty-six of the 78 substances recommended by ICCVAM for
599 the validation of *in vitro* TA test methods were tested for agonist activity by XDS in the
600 LUMI-CELL™ ER Bioassay. ICCVAM has classified 29 of these 56 substances as positive
601 or presumed positive[7] and 27 as negative or presumed negative for *in vitro* ER TA activity.
602 The results obtained by XDS for the 56 substances tested in LUMI-CELL™ are as follows:

603 • Positive in LUMI-CELL™ and ICCVAM Positive 25 substances
604 • Weak Positive[8] in LUMI-CELL™ and ICCVAM Positive 2 substances
605 • Negative in LUMI-CELL™ and ICCVAM Positive 0 substances
606 • Positive in LUMI-CELL™ and ICCVAM Negative 9 substances
607 • Weak Positive in LUMI-CELL™ and ICCVAM Negative 1 substances
608 • Negative in LUMI-CELL™ and ICCVAM Negative 19 substances[9]

609

[7] Two of these substances are well-known ER antagonist reported as positive in some ER agonist assays.
[8] XDS classifies substances as positive even if the nature of the agonist response is such that an EC50 cannot be calculated. NICEATM has designated these substances as weak positives.
[9] This number includes two well-known ER antagonists (tamoxifen and 4-hydroxytamoxifen) that are listed in the ICCVAM report as being positive in some agonist assays.

Using these data, the concordance, sensitivity, specificity, positive and negative predictivity, and false negative and false positive rates for the LUMI-CELL™ ER bioassay were calculated (see **Table 1**). Substances classified as weak positives were included in the analysis of accuracy.

	ICCVAM Classification			*total*
results		+	–	
	+	27	10	*37*
	–	0	19	*19*
total		*27*	*29*	*56*

Concordance	*= 0.82*		
Sensitivity	*= 1.00*	*False negative rate*	*= 0.00*
Specificity	*= 0.66*	*False positive rate*	*= 0.34*
Positive predictivity	*= 0.73*	*Negative predictivity*	*= 1.00*

The LUMI-CELL™ ER bioassay correctly identified all 27 ICCVAM recommended ER positive agonists tested by XDS. Among the 29 (including the two antagonists) ICCVAM recommended ER negative substances, ten induced a positive agonist TA response in LUMI-CELL™. Compared to the EC50 value for estradiol, all nine of these "false positive" substances exhibited EC50 values that were six to seven fold orders of magnitude weaker. The nine false positive substances included:

- 4-Androstene (ICCVAM reported as reported as presumed negative for ER agonist activity and as a strong androgen receptor [AR] agonist)
- Atrazine (ICCVAM reported as negative in three of three different ER agonist assays)
- 2-sec-Butylphenol (ICCVAM reported as presumed negative for ER agonist activity)
- Corticosterone (ICCVAM reported as negative in one ER agonist study and as binding weakly to the AR)

- Linuron (ICCVAM reported as negative in one ER agonist study and as a weak AR agonist and antagonist)
- Medroxyprogesterone acetate (ICCVAM reported as presumed negative for ER agonist activity and as a weak AR agonist)
- Morin (ICCVAM reported as presumed negative for ER agonist activity but as binding weakly to the ER)
- Phenolphthalin (ICCVAM reported as presumed negative for ER agonist activity)
- Spironolactone (ICCVAM reported as presumed negative for ER agonist activity and as an AR agonist and antagonist)
- L-Thyroxine (ICCVAM reported as expected to be negative for ER agonist activity)

Of the ten ICCVAM recommended negative ER TA substances reported as positive for agonist activity in LUMI-CELL™, ICCVAM did not have supporting negative ER TA data for seven substances, and had single test data only for two substances. Only one substance, atrazine, had been reported as negative for ER TA activity in multiple (three) studies. However, due to the mechanistic basis of this test system, false positives are highly unlikely. These ten substances most likely have very weak transcriptional activity that is producing the weak positive response. Thus, it is entirely possible that all ten of these substances are capable of producing weak ER transcriptional activation and that that increased TA activity represents "true" positives for the type and distribution of estrogen receptors in this test system. Furthermore, these responses may indicate that this test system is capable of detecting ER activity over a broad dynamic range, including very weak activity. Nonetheless, such results will need confirmation in a multi-laboratory validation study and, if possible, in other transcriptional assays with comparable receptor composition and sensitivity. Finally, the quantitative nature of the response will likely need to be considered when using this data for weight-of-evidence decisions in the EPA's Tier 1 Endocrine Disruptor Screening Program, with possibly less weight given to very weak acting substances, especially those that do not demonstrate an *in vivo* effect at established limit doses.

Evaluation of Comparative Activity: Another approach to evaluating the performance of the LUMI-CELL™ ER Bioassay, in terms of the ICCVAM recommended validation substances, is to compare the relative agonist activity of substances reported as positive in both data sets. Due to the lack of EC50 data for many of the substances recommended in the ICCVAM report, this analysis was limited to nine substances with ER TA activity. **Table 2** presents the EC50 values for these substances obtained in LUMI-CELL™ and the median EC50 values reported by ICCVAM (*note: the EC50 values reported by ICCVAM were generated by varied test methods and protocols; where multiple studies were conducted for the same substance, the median value was used*). Also presented in **Table 2** are the relative rankings (from most to least potent) for the nine substances. The regression correlations (r^2) for EC50 values and relative rankings were 0.607 ($p = 0.013$) and 0.903 ($p<0.001$), respectively. Thus, the relative ER TA activities of these nine agonist substances are significantly correlated between the LUMI-CELL™ ER bioassay and the data summarized in the ICCVAM report.

Table 2. Correlation Between Positive LUMI-CELL™ and Positive ICCVAM Substances with Agonist Activity

Substance	ICCVAM*		LUMI-CELL™	
	Median EC50 Value (μM)	Ranking	EC50 Value (μM)	Ranking
Diethylstilbestrol	0.000019	1	0.0000000311	1
Estrone	0.0032	3	0.00000061	2
17a-Estradiol	0.0001	2	0.00000316	3
Coumestrol	0.015	4	0.000043	4
n-Nonylphenol	0.085	6	0.000236	5
Genistein	0.062	5	0.00079	6
Bisphenol A	0.4	8	0.00107	7
Daidzein	0.29	7	0.0026	8
Methoxychlor	8.85	9	0.00353	9

* The ICCVAM EC50 data are generated by different investigators using different test ER TA test methods

2.3.3 Reliability (Repeatability and Intra- and Inter-laboratory Reproducibility) of the LUMI-CELL™ ER Bioassay for Detecting ER Antagonist Activity

XDS did not provide CV data for LUMI-CELL™ antagonist test results with respect to well-to-well variability within an experiment but did provide plate-to-plate (plate = experiment;

minimum of three experiments conducted on different days) for eight ICCVAM recommended substances reported as positive in LUMI-CELL™. An evaluation of interlaboratory antagonist reproducibility has not been conducted; this evaluation would be conducted as part of a multi-laboratory validation effort.

Test Method Intralaboratory Reproducibility: The mean and median CV values for plate-to-plate (i.e., experiment-to-experiment) IC50 values for eight ICCVAM recommended reference substances that induced a positive antagonist response in LUMI-CELL™ was 24 and 25%, respectively. This level of intralaboratory reproducibility is considered adequate.

2.3.4 The Accuracy of the LUMI-CELL™ ER Bioassay for Detecting ER Antagonist Activity

The discussion in **Section 2.2.2** about approaches for evaluating the accuracy of the agonist version of the LUMI-CELL™ ER bioassay are relevant also to approaches for evaluating the accuracy of the antagonist version of the same assay.

Evaluation of Concordance: Sixteen of the 78 substances recommended by ICCVAM for the validation of *in vitro* TA test methods were tested for antagonist activity by XDS in the LUMI-CELL™ ER bioassay. In their list of 78 recommended substances, ICCVAM identified eight substances with demonstrated antagonist activity, three with anticipated antagonist activity, 10 with demonstrated negative antagonist activity, and 57 with anticipated negative antagonist activity. Of the 16 substances listed by XDS as being tested for antagonist activity in the LUMI-CELL™ ER bioassay, ICCVAM had classified eight as positive for ER antagonist activity and eight without ER antagonist activity. The results obtained by XDS for these 16 substances are as follows:

- Positive in LUMI-CELL™ and ICCVAM Positive 6 substances
- Weak Positive[10] in LUMI-CELL™ and ICCVAM Positive 2 substances
- Negative in LUMI-CELL™ and ICCVAM Positive 0 substances
- Positive in LUMI-CELL™ and ICCVAM Negative 3 substances

[10] XDS classifies substances as positive even if the nature of the antagonist response is such that an IC50 cannot be calculated. NICEATM has designated these substances as weak positives.

- Weak Positive in LUMI-CELL™ and ICCVAM Negative 5 substances
- Negative in LUMI-CELL™ and ICCVAM Negative 0 substances

Using these antagonist data, the concordance, sensitivity, specificity, positive and negative predictivity, and false negative and false positive rates for the LUMI-CELL™ ER bioassay were calculated (see **Table 3**). Substances classified as weak positives were included in the analysis of accuracy.

	ICCVAM Classification			*total*
results		+	–	
	+	8	8	*16*
	–	0	0	*0*
total		*8*	*8*	*16*

Concordance = *0.50*
Sensitivity = *1.00* *False negative rate* = *0.00*
Specificity = *0.00* *False positive rate* = *1.00*
Positive predictivity = *0.50* *Negative predictivity* = *not calculated*

The LUMI-CELL™ ER bioassay correctly identified all eight ICCVAM recommended ER antagonist tested by XDS. Among the eight ICCVAM recommended ER TA validation substances presumed to be without antagonist activity, all eight induced a positive or weak positive antagonist ER response in LUMI-CELL™. The eight "false positive" substances included:

- Bisphenol A (ICCVAM reported as negative for ER antagonism activity in two of two antagonism studies)
- Corticostrone (ICCVAM reported as presumed negative for ER antagonism activity and as binding weakly to the AR)
- Daidzen (ICCVAM reported as negative for ER antagonist activity in two of two antagonism studies and as binding weakly to the AR)

- Diethylstilbestrol (ICCVAM reported as presumed negative for ER antagonism activity and as strong ER agonist)
- 17α-ethynyl estradiol (ICCVAM reported as presumed negative for ER antagonism activity and as a strong ER agonist)
- Medroxyprogesterone acetate (ICCVAM reported as presumed negative for ER antagonism activity and as a weak AR agonist)
- Spironolactone (ICCVAM reported as presumed negative for ER antagonism activity and as an AR agonist and antagonist)
- Vinclozolin (ICCVAM reported as presumed negative for ER antagonism activity and as an AR agonist and antagonist)

Thus, of the eight ICCVAM recommended negative antagonists reported as positive for antagonist activity in LUMI-CELL™, ICCVAM did not have supporting ER antagonism data for six substances; the other two substances were reported negative in two of two ER antagonist studies. Daidzein was a weak antagonist in LUMI-CELL™ (i.e., reduced the agonist activity of the reference estrogen but and IC50 could not be calculated).

Only eight ICCVAM recommended validation substances with known or predicted ER antagonist activity were tested by XDS in the LUMI-CELL™ ER bioassay. However, the list of validation substances recommended by ICCVAM contains only 11 ER antagonist substances (eight with supporting data, three without *in vitro* ER TA antagonist supporting data).

Evaluation of Comparative Activity: Another approach to evaluating the performance of the LUMI-CELL™ ER bioassay for detecting antagonist activity, in terms of the ICCVAM recommended validation substances, is to compare the relative antagonist activity of substances reported as positive in both data sets. However, due to the limited number of antagonists tested by XDS and the limited number of studies reported by ICCVAM with quantitative data, this type of analysis could not be conducted.

Thus, while additional LUMI-CELL™ ER antagonist data would be useful in clarifying the performance of this assay for identifying substances with antagonist activity, the lack of such studies is not considered to be a significant detriment to conducting cross laboratory validation studies.

2.4 Does the BRD Adequately Provide the Information Requested in the Outline Provided in the *ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods* (NIH Publ. No. 03-4508)?

The XDS BRD adheres to the recommended outline and provides nearly all of the requested information. However, additional information should be provided if the BRD is to be released beyond ICCVAM. The lack of this information did not adversely impact on the evaluation of Criterions 1 through 3. Examples of additional information or clarifications that are needed include:

1. The information (or at least subsets of information) provided in the CD should be included in the BRD.
2. In the Table of Contents, Appendices B-K should be identified and paginated, and a lists of figures and tables and their locations should be included.
3. Lists of abbreviations should be in alphabetic order.
4. Figure numbers should be sequential within the main body and within each Appendix.
5. Information is needed on the nature of the ER receptor in BG1Luc4E2 cell line (subsequent communication from XDS indicated that ERα was the primary active form but that ERβ was also responsive in these cells).
6. More explanation is needed in the Appendices for some of the column headings and for some of the symbols used in the various columns.
7. The approaches used by XDS to assess viability in the LUMI-CELL™ ER bioassay and the way the results are presented in the various tables and appendices requires clarification.
8. XDS has developed a LUMI-CELL™ historical control database for the solvent controls, for the reference standard, 17β-estradiol, and for concurrent

positive control chemicals. Although the relevant data appears to be the subject of Appendix J (QC Charts), this information needs to be summarized in **Section 7.3** of the BRD.

9. Appendix D-F. More information is needed on the source of the values for the plate-to-plate and well-to-well CV values presented in these Appendices.
10. The criteria for an acceptable assay or for a positive result should be clarified.
11. A more comprehensive protocol (than the one provided) for both the agonist and antagonist versions of LUMI-CELL™ is needed in Appendix A.

3.0 NICEATM RECOMMENDATIONS:

Based on the data provided in the XDS BRD on the LUMI-CELL™ ER bioassay, NICEATM recommends to the EDWG that:

- LUMI-CELL™ be considered as a high priority for validation studies as an *in vitro* test method for the detection of test substances with ER agonist and antagonist activity.
- To facilitate independent and timely standardization and validation studies, NICEATM should manage the needed studies by exercising a validation coordination option in its support contract. Such studies should include coordination and collaboration with ECVAM and JCVAM, and ideally include one laboratory in each of the three respective geographic regions supported by these three Centers.
- During finalization of their BRD and in preparation for the interlaboratory validation study, XDS conduct additional antagonist studies to more comprehensively demonstrate the suitability of LUMI-CELL™ as an assay for the detection of substances with ER antagonist activity.