ICCVAM Test Method Evaluation Report
The LUMI-CELL® ER (BG1Luc ER TA) Test Method: An *In Vitro* Assay for Identifying Human Estrogen Receptor Agonist and Antagonist Activity of Chemicals

Interagency Coordinating Committee on the Validation of Alternative Methods

National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

National Institute of Environmental Health Sciences
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U.S. Public Health Service
Department of Health and Human Services

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National Toxicology Program
P.O. Box 12233
Research Triangle Park, NC 27709

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List of Abbreviations and Acronyms

AR	Androgen receptor
BRD	Background review document
CASRN	CAS Registry Number® (a trademark of the American Chemical Society)
CERI	Chemicals Evaluation and Research Institute, Japan
CV	Coefficient of variation
DMSO	Dimethyl sulfoxide
EAC	Endocrine-active compound
EC_{50}	Half–maximal effective concentration
ECVAM	European Centre for the Validation of Alternative Methods
ED	Endocrine disruptor
EDSP	Endocrine Disruptor Screening Program (U.S. EPA)
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee (U.S. EPA)
EDWG	ICCVAM Interagency Endocrine Disruptor Working Group
EPA	U.S. Environmental Protection Agency
ER	Estrogen receptor
FDA	U.S. Food and Drug Administration
FR	*Federal Register*
FW	Formula weight
GLP	Good Laboratory Practice
I	Inadequate
IC_{50}	Half-maximal inhibitory concentration
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ILS	Integrated Laboratory Systems, Inc.
ISO	International Organization for Standardization
JaCVAM	Japanese Center for the Validation of Alternative Methods
KoCVAM	Korean Center for the Validation of Alternative Methods
M	Molar
Max	Maximum
MeSH®	Medical Subject Headings (U.S. National Library of Medicine)
N	Number; negative
NEG	Negative
NICEATM	National Toxicology Program Center for the Evaluation of Alternative Toxicological Methods

NIEHS	U.S. National Institute of Environmental Health Sciences
NIH	U.S. National Institutes of Health
NT	Not tested
NTP	U.S. National Toxicology Program (U.S. NIH)
OECD	Organisation for Economic Co-operation and Development
OPPTS	Office of Prevention, Pesticides and Toxic Substances (U.S. EPA)
PN	Presumed negative
POS	Positive
PP	Presumed positive
RLU	Relative light unit
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
SD	Standard deviation
SMT	Study Management Team
STTA	Stably transfected human estrogen receptor-α transcriptional activation
TA	Transcriptional activation
TG	Test Guideline
U.S.C.	United States Code
XDS	Xenobiotic Detection Systems, Inc.

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Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM): Agency Representatives

Agency for Toxic Substances and Disease Registry
* Moiz Mumtaz, PhD
  Edward Murray, PhD
  Eric Sampson, PhD
  Bruce Fowler, PhD (to June 2011)

Consumer Product Safety Commission
* Joanna Matheson, PhD (Vice-Chair)
+ Kristina Hatlelid, PhD
  Marilyn L. Wind, PhD (Chair, to July 2010)

Department of Agriculture
* Jodie Kulpa-Eddy, DVM (Chair)
+ Elizabeth Goldentyer, DVM

Department of Defense
* Patrick Mason, PhD
+ Terry Besch, DVM, MS, DACLAM, DACVPM
+ Patty Decot
  David Honey, PhD (to July 2011)
  Robert E. Foster, PhD (to Aug. 2010)
  Harry Salem, PhD (to Aug. 2010)
  Peter J. Schultheiss, DVM, DACLAM (to Aug. 2010)

Department of Energy
* Michael Kuperberg, PhD
  Marvin Stodolsky, PhD (retired 2011)

Department of the Interior
* Barnett A. Rattner, PhD
  Sarah Gerould, PhD (to Feb. 2009)

Department of Transportation
+ Steve Hwang, PhD
  George Cushmac, PhD (retired 2010)

Environmental Protection Agency
Office of Pesticide Programs
* John R. "Jack" Fowle III, PhD, DABT
+ Vicki Dellarco, PhD
  Christine Augustyniak, PhD (*U.S. Coordinator, OECD Test Guidelines Program*) (to Sept. 2011)
  Tina Levine, PhD (to Sept. 2011)
  Deborah McCall (to Sept. 2011)

Office of Pollution Prevention and Toxics
  Jerry Smrchek, PhD (*U.S. Coordinator, OECD Test Guidelines Program*) (to July 2009)

Environmental Protection Agency (cont.)
Office of Research and Development
  Stephanie Padilla, PhD (to July 2009)
  Julian Preston, PhD (to July 2009)
  Suzanne McMaster, PhD (to Dec. 2008)

Office of Science Coordination and Policy
  Karen Hamernik, PhD (to July 2009)

Food and Drug Administration
Office of the Commissioner
* Suzanne Fitzpatrick, PhD, DABT

Center for Biologics Evaluation and Research
  Ying Huang, PhD
  Richard McFarland, PhD, MD

Center for Devices and Radiological Health
  Vasant G. Malshet, PhD, DABT

Center for Drug Evaluation and Research
+ Abigail C. Jacobs, PhD
  Paul C. Brown, PhD

Center for Food Safety and Applied Nutrition
  Robert Bronaugh, PhD
  David G. Hattan, PhD
  Diego Rua, PhD
  Neil Wilcox, DVM, MPH (to May 2011)

Center for Veterinary Medicine
  M. Cecilia Aguila, DVM
  Li You, PhD
  Devaraya Jagannath, PhD (to April 2011)

National Center for Toxicological Research
  Paul Howard, PhD
  Donna L. Mendrick, PhD
  William T. Allaben, PhD (to Jan. 2009)

Office of Regulatory Affairs
  Lawrence D'Hoostelaere, PhD

National Cancer Institute
* T. Kevin Howcroft, PhD
+ Chand Khanna, DVM, PhD
  Alan Poland, MD (to Oct. 2008)

continued

* Principal agency representative
+ Alternate principal agency representative

National Institute of Environmental Health Sciences
- * William S. Stokes, DVM, DACLAM
- \+ Warren Casey, PhD, DABT
- Rajendra S. Chhabra, PhD, DABT
- Jerrold J. Heindel, PhD
- Raymond R. Tice, PhD (to June 2010)

National Institute for Occupational Safety and Health
- * Paul Nicolaysen, VMD

National Institutes of Health
- * Margaret D. Snyder, PhD

National Library of Medicine
- * Pertti (Bert) Hakkinen, PhD
- \+ Jeanne C. Goshorn, MS

Occupational Safety and Health Administration
- * Surender Ahir, PhD

* Principal agency representative
\+ Alternate principal agency representative

Acknowledgements

ICCVAM Interagency Endocrine Disruptor Working Group (EDWG)

U.S. Consumer Product Safety Commission
Kent Carlson, PhD

U.S. Department of the Interior
Catherine Richter, PhD
Donald Tillitt, PhD

U.S. Environmental Protection Agency
Don Bergfelt, PhD
John R. "Jack" Fowle, III, PhD, DABT
Jesudoss Rowland

U.S. Food and Drug Administration
Center for Devices and Radiological Health
Thomas Umbreit, PhD

Center for Drug Evaluation and Research
Jeffrey Bray, PhD
Paul C. Brown, PhD
Karen L. Davis-Bruno, PhD
Ross Filice, MD
Abigail (Abby) Jacobs, PhD
Leslie McKinney, PhD

Center for Food Safety and Nutrition
Michael Bolger, PhD, DABT
David G. Hattan, PhD (Chair)

Center for Veterinary Medicine
M. Cecilia Aguila, DVM
Charles Eirkson, PhD
Kevin Gaido, PhD
Devaraya Jagannath, PhD
Annette McCarthy, PhD
Li You, PhD

National Center for Toxicological Research
Kenneth Delclos, PhD
Huixiao Hong, PhD
Jon Wilkes, PhD

Office of the Commissioner
Suzanne Fitzpatrick, PhD, DABT

National Institute of Environmental Health Sciences
Warren Casey, PhD, DABT
Jerrold Heindel, PhD
William Stokes, DVM, DACLAM
Julius Thigpen, PhD

Occupational Safety and Health Administration
Surender Ahir, PhD

European Centre for the Validation of Alternative Methods – Liaison
Susanne Bremer, PhD
Elise Grignard, PhD
Valerie Zuang, PhD

Japanese Center for the Validation of Alternative Methods – Liaison
Hajime Kojima, PhD
Atsushi Ono, PhD

Study Management Team

NICEATM

William Stokes, DVM, DACLAM (NIEHS)
Director, NICEATM

Warren Casey, PhD (NIEHS)
Deputy Director, NICEATM

Ray Tice, PhD (NIEHS)
Deputy Director, NICEATM
(On team until June 2005)

David Allen, PhD (ILS, Inc.)
Principal Investigator

Jeff Charles, PhD (ILS, Inc.)
Principal Investigator
(On team until April 2007)

Frank Deal, MS (ILS, Inc., until March 2011)
Staff Toxicologist

Patricia Ceger, MS (ILS, Inc.)
Project Coordinator

ECVAM

Susanne Bremer, PhD

Elise Grignard, PhD

Thomas Hartung, PhD
Head of ECVAM
(On team until April 2008)

Miriam Jacobs, PhD
(On team until August 2007)

JaCVAM

Hajime Kojima, PhD
Director, JaCVAM

Atsushi Ono, PhD
National Institute of Health Sciences
(On team until January 2007)

KoCVAM

Soon Young Han, PhD
Director, KoCVAM

Participants in the BG1Luc ER TA Validation Study

NICEATM gratefully acknowledges the generous contributions of the individuals who directly participated in the NICEATM/ECVAM/JaCVAM/KoCVAM validation study. Their time and efforts are greatly appreciated.

Xenobiotic Detection Systems, Inc. (XDS)
(Lead Laboratory)

George Clark, PhD – Study Director from 2010

John Gordon, PhD – Study Director until 2008

Jun Liu, PhD – Study Director until 2009

Ruth McMillan, PhD – Technical Lead from 2010

Andrew Chu – Laboratory Manager until 2009

Ying Gu – Lead Chemist

ECVAM (In Vitro Methods Unit)
(Participating Laboratory)

Miriam Jacobs, PhD – Study Director until 2008

Patricia Pazos, PhD – Study Director from 2009

Jan de Lange – Technical Lead

Hiyoshi Corporation
(Participating Laboratory)

Masafumi Nakamura – Study Director

Hiyorhi Handa – Technical Lead

Clevita Eldrida Caesar Mascalenhas – Translator

NIEHS (Chemistry Resources Group)

Cynthia Smith, PhD – Branch Chief

Beby Jayaram, PhD – Chemist

Statistical Consultant

Joseph Haseman, PhD

2011 LUMI-CELL® (BG1Luc ER TA) Test Method Independent Scientific Peer Review Panel (March 29 – 30, 2011)

A. John Bailer, PhD
Distinguished Professor and Chair
Department of Statistics
Miami University
Oxford, Ohio

Christopher Borgert, PhD
President and Principal Scientist
Applied Pharmacology and Toxicology, Inc.
Gainesville, FL

Grantley Charles, PhD, DABT
Senior Scientist, Toxicology
Drug Safety Evaluation
Allergan Inc.
Irvine, CA

Daniel Desaulniers, PhD
Research Scientist
Health Canada
Hazard Identification Division
Ottawa, ON, Canada

J. Charles Eldridge, PhD
Professor of Physiology & Pharmacology
Wake Forest University Health Sciences
Winston-Salem, NC

William R. Kelce, PhD
Principal
Exponent®
Cary, NC

Hyung Kim, PhD
Associate Professor
College of Pharmacy, Pusan National University
Busan, Republic of Korea

Steven L. Levine, PhD
Science Fellow and Product Safety Manager
Monsanto Company
St. Louis, MO

Alberto Mantovani, DVM
Head of Food and Veterinary Toxicology Unit
Italian National Health Institute
(ISS - Istituto Superiore di Sanità)
Rome, Italy

Ellen Mihaich, PhD, DABT
President and Principal Scientist
Environmental and Regulatory Resources, LLC
Durham, NC

Hiroshi Ono, PhD
Scientific Advisor
Hatano Research Institute, Food and Drug Safety Center
Kanagawa, Japan

John G. Vandenbergh, PhD
Professor Emeritus
Department of Biology
North Carolina State University
Raleigh, NC

Sherry L. Ward, PhD, MBA
Consultant
BioTred Solutions
New Market, MD

Marc Weimer, PhD
Biostatistician
German Cancer Research Center
Department of Biostatistics
Heidelberg, Germany

James Wittliff, PhD, MD *hc*, FACB
Professor, Department of Biochemistry & Molecular Biology
Director, Hormone Receptor Laboratory
Director, Institute for Molecular Diversity & Drug Design
University of Louisville
Louisville, KY

James Yager, PhD
Professor and Director of Toxicology
Johns Hopkins University Bloomberg School of Public Health
Baltimore, MD

National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

National Institute of Environmental Health Sciences
William Stokes, DVM, DACLAM
Director (NICEATM); Project Officer

Warren Casey, PhD, DABT
Deputy Director

Deborah McCarley
Special Assistant; Assistant Project Officer

NICEATM Support Contract Staff (Integrated Laboratory Systems [ILS], Inc.)
David Allen, PhD
Patricia Ceger, MS
Frank Deal, MS (until March 2011)
Jonathan Hamm, PhD
Linda Litchfield
Steven Morefield, MD
Michael Paris
Catherine Sprankle
Linda Wilson

Statistical Consultant for ILS, Inc.
Joseph Haseman, PhD

Preface

Endocrine-active compounds (EACs) are both naturally occurring and synthetic substances. Some may, depending on the dose, interfere with the normal function of hormones in the endocrine system. Public health concerns have resulted largely from studies indicating that animal populations exposed to high levels of these substances, sometimes referred to as endocrine disruptors (EDs), have an increased incidence of reproductive and developmental abnormalities (EPA 1997; NRC 1999). In response to growing concerns about possible adverse health effects in humans exposed to such substances, the U.S. Congress enacted relevant provisions to safeguard public health in the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 301 et seq.); the Food Quality Protection Act (7 U.S.C. 136); and the 1996 Amendments to the Safe Drinking Water Act (110 Stat 1613). The U.S. Environmental Protection Agency (EPA) was required to develop and validate a screening and testing program to identify substances with endocrine-disrupting activity. The EPA subsequently established the Endocrine Disruptor Screening Program (EDSP) and initiated efforts to standardize and validate test methods for inclusion in the EDSP (66 FR 23022). Validation is necessary to assess the usefulness and limitations of a test method for a specific proposed purpose and to characterize the extent to which test methods are sufficiently accurate and reproducible for their intended use (ICCVAM 1997).

In April 2000, the EPA nominated four types of *in vitro* test methods for detecting substances with potential endocrine-disrupting activity for review by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). These included *in vitro* estrogen receptor (ER) and androgen receptor (AR) binding and ER and AR transcriptional activation (TA) test methods. The EPA also asked ICCVAM to develop performance standards that could be used to define acceptable *in vitro* ER and AR binding and TA assays. It was envisioned that these standards would be based on the performance of adequately validated *in vitro* ER- and AR-based assays.

In 2002, the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) prepared background review documents (BRDs) that included all available information on each of the four types of test methods (ICCVAM 2002d, 2002a, 2002c, 2002b). In a public meeting, an independent international expert panel (Panel) reviewed the information on the 137 assays described in the BRDs and concluded that there were no adequately validated *in vitro* ER- or AR-based test methods (ICCVAM 2002e). Based on recommendations from the Panel, ICCVAM published the *ICCVAM Evaluation of In Vitro Test Methods for Detecting Potential Endocrine Disruptors*, which included a list of reference substances that should be used to validate each of the four types of *in vitro* test methods (ICCVAM 2003a). It also identified essential test method components that should be included in each of the standardized test method protocols used for future validation studies. ICCVAM recommended that future performance standards for these methods be based on test methods that have undergone adequate validation studies using the recommended accuracy chemicals and essential test method components.

In January 2004, Xenobiotic Detection Systems, Inc. (XDS; Durham, NC), nominated the LUMI-CELL® BG1Luc4E2 ER TA test method (BG1Luc ER TA test method) for an interlaboratory validation study. ICCVAM and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) recommended that the BG1Luc ER TA test method be considered a high priority for interlaboratory validation studies due to the lack of adequately validated test methods and the regulatory and public health need for such test methods. NICEATM subsequently led and coordinated an international validation study with its counterparts in Japan (JaCVAM) and Europe (ECVAM), using laboratories sponsored by each validation organization. NICEATM organized a validation Study Management Team (SMT) to oversee the scientific aspects of the

validation study and coordinate the day-to-day activities among the participating laboratories. A representative from the recently established Korean Center for the Validation of Alternative Methods (KoCVAM) joined the SMT in 2010.

ICCVAM reviewed the validation status of the BG1Luc ER TA test method for identification of substances with ER agonist or antagonist activity. NICEATM and the ICCVAM Interagency Endocrine Disruptor Working Group (EDWG) prepared a draft BRD that provided a comprehensive description and the data from the validation study used to assess the accuracy and reliability of the BG1Luc ER TA test method.

NICEATM convened an independent international scientific peer review panel (Panel) that met in public on March 29–30, 2011. The Panel was charged with reviewing the draft BRD for completeness, assessing the extent that established validation and acceptance criteria were adequately addressed, and determining the extent to which the data and information supported draft ICCVAM test method recommendations on the usefulness and limitations of the BG1Luc ER TA test method. The Panel also evaluated the proposed performance standards. The Panel included expert scientists nominated by ECVAM, JaCVAM, and KoCVAM.

ICCVAM considered the conclusions and recommendations of the Panel, along with comments from the public and SACATM, and then finalized the BRD and test method recommendations, which are provided in this test method evaluation report. As required by the ICCVAM Authorization Act (42 U.S.C. 285*l*-3), ICCVAM forwarded this report and recommendations to Federal agencies for their consideration and acceptance decisions where appropriate. The BG1Luc ER TA test method protocol and performance standards were also forwarded to the Organisation for Economic Co-operation and Development (OECD) Test Guidelines Programme for consideration and adoption as international testing guidelines.

We gratefully acknowledge the organizations and scientists who generated and provided data and information for this document, especially the staff at the participating validation laboratories: XDS, Inc., in Durham, North Carolina; Hiyoshi Corporation in Japan; and the In Vitro Methods Unit at ECVAM in Italy. We would also like to recognize the efforts of the individuals who contributed to its preparation, review, and revision. We thank Dr. David Hattan (U.S. Food and Drug Administration) for serving as Chair of the EDWG, as well as the members of the EDWG and ICCVAM representatives who subsequently reviewed and provided comments throughout the process leading to this test method evaluation report. We also want to thank Dr. Warren Casey, Deputy Director of NICEATM, for his excellent leadership and extensive efforts on this project.

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RADM William S. Stokes, DVM, DACLAM
Rear Admiral/Assistant Surgeon General, U.S. Public Health Service
Director, NICEATM
Executive Director, ICCVAM

Jodie A. Kulpa-Eddy, DVM
VS-NCIE-Agricultural Select Agent Program
U.S. Department of Agriculture
Chair, ICCVAM

Executive Summary

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) has completed its evaluation of the validation status of the LUMI-CELL® BG1Luc4E2 estrogen receptor (ER) transcriptional activation (TA) test method (hereafter BG1Luc ER TA test method) as a screening test to identify substances with *in vitro* ER agonist and antagonist activity. The BG1Luc ER TA test method uses BG-1 cells, a human ovarian adenocarcinoma cell line that is stably transfected with an estrogen-responsive luminescence (luciferase reporter) gene, to measure whether and how much a substance induces (agonist) or inhibits (antagonist) TA activity via ER-mediated pathways. Such substances could interfere with the normal function of hormones in the endocrine system (i.e., endocrine disruptors), which may lead to abnormal growth, development, or reproduction.

This test method evaluation report provides ICCVAM's recommendations for the BG1Luc ER TA test method based on the results of an international validation study and the demonstrated validity (usefulness and limitations). The report also includes (1) recommendations for future studies, (2) performance standards to evaluate functionally and mechanistically similar test methods, (3) protocols recommended by ICCVAM for future data collection and evaluation of the BG1Luc ER TA test method, and (4) a final background review document (BRD) describing the validation status of this test method.

In 2004, Xenobiotic Detection Systems, Inc. (XDS; Durham, NC), nominated the LUMI-CELL ER test method to ICCVAM for an interlaboratory validation study. ICCVAM and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) recommended that the BG1Luc ER TA test method be considered a high priority for interlaboratory validation studies based on the lack of adequately validated test methods and the regulatory and public health need for such test methods.

When the BG1Luc ER TA validation study was initiated, no *in vitro* ER TA test methods were considered adequately valid for regulatory use. Today, only one *in vitro* ER TA test method is considered adequately validated by national and international agencies, the Organisation for Economic Co-operation and Development (OECD) Stably Transfected Human Estrogen Receptor-α Transcriptional Activation (STTA) Assay for the Detection of Estrogenic Agonist-Activity, described in OECD Chemicals Test Guideline (TG) 455 (OECD 2009). Validated by the Chemicals Evaluation and Research Institute (CERI, Japan), this method has been adopted by the U.S. Environmental Protection Agency (EPA) as OPPTS 890.1300: Estrogen Receptor Transcriptional Activation (Human Cell Line [HeLa-9903]) (EPA 2009).

After recommendation by ICCVAM and SACATM, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) led and coordinated an international validation study with its counterparts in Europe (the European Centre for the Validation of Alternative Methods [ECVAM]) and Japan (the Japanese Center for the Evaluation of Alternative Methods [JaCVAM]) to assess the accuracy and reliability of the BG1Luc ER TA test method for the qualitative detection of substances with *in vitro* ER agonist or antagonist activity. The BG1Luc ER TA test method was evaluated using laboratories in the United States (XDS), Europe (ECVAM), and Japan (Hiyoshi Corporation).

The validation study proceeded in four phases. During Phase 1, each of the three participating centers (NICEATM, ECVAM, and JaCVAM) selected validation laboratories. The protocols were reviewed, and the laboratories demonstrated proficiency with the test method by successfully completing 10 replicate agonist and 10 replicate antagonist tests. In Phases 2 through 4, the protocols were evaluated and refined, and 78 ICCVAM reference substances that

should be used to standardize and validate *in vitro* ER and androgen receptor binding and TA test methods were tested.

After this study was completed, NICEATM, ICCVAM, and the ICCVAM Interagency Endocrine Disruptor Working Group (EDWG) prepared a draft BRD and draft test method recommendations. The drafts were provided to an independent international scientific peer review panel (hereafter Panel) and to the public for comment. The Panel met in public session on March 29–30, 2011, to discuss its peer review of the ICCVAM draft BRD and to provide conclusions and recommendations regarding the validation status of the BG1Luc ER TA test method. The Panel also reviewed how well the information contained in the draft BRD supported ICCVAM's draft test method recommendations.

In finalizing this test method evaluation report and the BRD, which is included here as an appendix, ICCVAM considered (1) the conclusions and recommendations of the Panel, (2) comments from SACATM, and (3) public comments.

ICCVAM Recommendations: Test Method Usefulness and Limitations
ICCVAM concludes that the accuracy and reliability of the BG1Luc ER TA test method support its use to screen substances for *in vitro* ER agonist and/or antagonist activity. This determination is based on an evaluation of data from the validation study and the corresponding accuracy and reliability. ICCVAM concludes that the accuracy of this assay is at least equivalent to that of the current ER TA test method included in regulatory testing guidance (EPA OPPTS 890.1300) (EPA 2009).

ICCVAM Recommendations: BG1Luc ER TA Test Method Protocol
For use of the BG1Luc ER TA test method to screen substances for *in vitro* ER agonist and/or antagonist activity, ICCVAM recommends using the ICCVAM BG1Luc ER TA protocols (included here as **Appendices B1** and **B2**). All future studies intended to further characterize the usefulness and limitations of the BG1Luc ER TA test method should use these protocols.

ICCVAM Recommendations: Future Studies
ICCVAM considers the BG1Luc ER TA test method to be valid as described. However, ICCVAM recommends the following for interested parties to further characterize and potentially improve the usefulness and applicability of the BG1Luc ER TA test method:

- Additional validation studies may be performed to determine whether the BG1Luc ER TA test method or other similar assays could replace the rat uterine cytosol ER binding assay.
- Further work may be carried out to determine if the BG1Luc ER TA test method could be combined with other methods (to include *in vitro* metabolic activation) in a weight-of-evidence approach to replace the uterotrophic bioassay.
- Additional studies/evaluations may be conducted to more completely characterize the ratio of ERα and ERβ in the BG-1 cell line and the extent to which these receptor subtypes contribute to the overall performance of the BG1Luc ER TA test method.
- Additional studies/evaluations may be conducted to determine the feasibility of testing volatile substances using CO_2-permeable plastic film or other methods to seal the test plates.
- Additional studies/evaluations may be conducted to determine if substances that are not soluble in dimethyl sulfoxide (DMSO) could be tested in another vehicle that would more adequately dissolve the substance in culture media.
- Additional studies may be conducted to account for metabolic activation that could expand the utility of this and other ER TA test methods.
- As ER antagonists are identified, additional studies/evaluations may be conducted to expand the database of positive substances tested and thereby better characterize the

usefulness and limitations of the BG1Luc ER TA test method as a screening test to identify substances with ER antagonist activity.

ICCVAM encourages users to provide to ICCVAM all data that are generated from future studies. These data could be used to further characterize the usefulness and limitations of the BG1Luc ER TA test method as a screening test to identify substances with ER agonist or antagonist activity.

Validation Status of the BG1Luc ER TA Test Method

ICCVAM evaluated the BG1Luc ER TA test method for its ability to correctly identify *in vitro* ER agonists and antagonists. For this analysis, test substance classification (positive or negative for ER agonist/antagonist activity) obtained during the validation study was compared to the ICCVAM reference classification of the same substance, which was based on a preponderance of available data.

The BG1Luc ER TA test method accuracy was evaluated based on several different analyses, but the primary evaluation was based on two comparisons: (1) the extent to which the result of the test method corresponds to the ICCVAM reference classification for each substance and (2) the accuracy of the BG1Luc ER TA test method compared to that of the EPA OPPTS 890.1300/OECD TG 455 (EPA 2009; OECD 2009)[1] assay.

Test Method Accuracy – Agonist Assay

Thirty-five substances (28 positive, 7 negative) were used to evaluate the accuracy of the BG1Luc ER TA agonist assay. The consensus classification obtained from all BG1Luc ER TA tests for these 35 substances yielded the following statistics: concordance of 97% (34/35), sensitivity of 96% (27/28), specificity of 100% (7/7), a false positive rate of 0% (0/7), and a false negative rate of 4% (1/28). Similar results were obtained when the results from each laboratory were used instead of the consensus classification.

EPA OPPTS 890.1300/OECD TG 455 is the only test guideline published by a U.S. regulatory agency for generating ER TA data. Therefore, BG1Luc ER TA test method concordance with EPA OPPTS 890.1300/OECD TG 455 was also evaluated using the 26 reference substances for which data are available from both BG1Luc ER TA and EPA OPPTS 890.1300/OECD TG 455 assays. Accuracy statistics for the two test methods were identical: concordance of 96% (25/26), sensitivity of 95% (21/22), specificity of 100% (4/4), a false positive rate of 0% (0/4), and a false negative rate of 5% (1/22).

Test Method Accuracy – Antagonist Assay

To evaluate the accuracy of the BG1Luc ER TA antagonist assay, 25 substances (3 positive, 22 negative) were used. The consensus classification obtained from all BG1Luc ER TA tests for these 25 substances yielded the following statistics: concordance of 100% (25/25), sensitivity of 100% (3/3), specificity of 100% (22/22), a false positive rate of 0% (0/22), and a false negative rate of 0% (0/3). Similar results were obtained when the results from each laboratory were used instead of the consensus classification.

Because there currently is no valid EPA OPPTS 890.1300/OECD TG 455 antagonist protocol, no comparison with the BG1Luc ER TA antagonist results was conducted.

Concordance with Other Endocrine Disruptor Assays

Although the primary goal of the BG1Luc ER TA test method is to provide a qualitative assessment of estrogenic/anti-estrogenic activity, quantitative measures of activity are usually obtained for positive results. The values obtained from BG1Luc ER TA test results (half-maximal

[1] The EPA OPPTS 890.1300/OECD TG 455 (OECD 2009) assay uses the hERα-HeLa-9903 human cervical cancer cell line to detect estrogen agonist activity mediated through human ER alpha (hERα).

effective concentration [EC_{50}] and half-maximal inhibitory concentration [IC_{50}]), were compared to median values from other ER TA test methods reported in the literature. This comparison found a high correlation. There was 97% (33/34) concordance between the BG1Luc ER TA test method and ER binding data. The only discordant substance (medroxyprogesterone acetate) was positive in the BG1Luc ER TA test method and negative based on ER binding data. Similarly, based on a comparison with available data in the *in vivo* uterotrophic assay, there was 92% (12/13) concordance between the BG1Luc ER TA test method and ER binding data. The only discordant substance (butylbenzyl phthalate) was positive in the BG1Luc ER TA test method and negative based on uterotrophic data.

Test Method Reliability

Intralaboratory reproducibility (whether multiple tests of the same substance at a single laboratory produce the same results) of the BG1Luc ER TA agonist and antagonist test methods was assessed by comparing (1) reference standard and control results for all plates tested within each laboratory during the course of the validation study and (2) results from Phase 2 testing, during which 12 substances were tested in at least three independent experiments in each of the three laboratories. Intralaboratory agreement for agonist and antagonist classification was determined for the 12 substances that were tested at least three times at each laboratory.

In the agonist testing, mean induction in each laboratory ranged from 4.6 to 7.8 fold, and 17β-estradiol (E2) reference standard EC_{50} values ranged from 8.0×10^{-12} to 1.2×10^{-11} M. There was 100% agreement within each laboratory for each of the three repeat tests, although the agonist classifications for some of the 12 test substances differed among the different laboratories.

In the antagonist testing, mean reduction ranged from 8.0 to 9.9 fold, and raloxifene reference standard IC_{50} values ranged from 1.1×10^{-9} to 1.3×10^{-9} M. There was 100% agreement within each laboratory for each of the three repeat tests, although the antagonist classifications for some of the 12 test substances differed among the different laboratories.

Interlaboratory reproducibility (whether tests of a single substance run at different laboratories produce the same results) of the BG1Luc ER TA agonist and antagonist test methods was determined for the 12 substances that were tested at least three times for agonist and antagonist activity during Phase 2 at each of the three laboratories. The three laboratories agreed on 67% (8/12) of the substances tested for agonist activity and on 100% (12/12) of the substances tested for antagonist activity.

Interlaboratory reproducibility was also determined for 41 substances that were tested once for agonist and antagonist activity during Phase 3 testing at each of the three laboratories. Five of the 41 substances produced inadequate results for agonist activity and could not be considered in the evaluation. Among the 36 remaining substances that produced a definitive test result in at least two laboratories, there was 100% agreement. All 41 substances produced definitive results for antagonist activity. The three laboratories agreed on 93% (38/41) of these substances.

ICCVAM Recommendations: Performance Standards

Based on the results of this study, NICEATM and the EDWG developed performance standards applicable to methods that are functionally and mechanistically similar to the BG1Luc ER TA test method. These performance standards can also be used by laboratories with no experience with the BG1Luc ER TA test method to demonstrate technical proficiency.

Essential Test Method Components

In order to be considered functionally and mechanistically similar to the BG1Luc ER TA test method, a modified ER TA test method protocol must include the following components to ensure that the same biological effect is being measured:

- The test method should be based on a cell line that endogenously expresses ER.
- Reference standards, controls, and test substances should be dissolved in a solvent that mixes well with cell culture media at concentrations that are noncytotoxic and that do not otherwise interfere with the test system.
- The maximum test substance concentration should be 1 mM for ER TA agonist testing and 10 µM for ER TA antagonist testing unless otherwise limited by solubility, cytotoxicity, or other mechanisms that interfere with assay performance.
- At least seven concentrations spaced at logarithmic (log_{10}) intervals, up to the limit concentration, should be tested.
- An evaluation of cytotoxicity should be included, and only data from concentrations at or above 80% viability should be used for data analyses.
- A reference estrogen and a reference anti-estrogen should be used to demonstrate the adequacy of the test method for detecting ER TA agonist and antagonist activity.
- The ability of the reference estrogen to induce ER TA activity and the ability of the reference anti-estrogen to inhibit ER TA activity should be demonstrated by generating a full concentration–response curve in each experiment that provides a minimum threefold estrogenic induction and a minimum threefold anti-estrogenic reduction.
- A set of concurrent controls should be included. For agonist assays, this would include the vehicle control and a weak agonist. For antagonist assays, this would include the vehicle control, weak antagonist, and reference estrogen.
- Test substances that are positive for ER agonist activity should have a concentration–response curve consisting of a baseline, followed by a positive slope, with a response peak of at least 20% of the average maximal value of the reference estrogen response.
- Test substances are negative for agonist activity if all data points are below 20% of the average maximal value of the reference estrogen response.
- Test substances that are positive for ER antagonist activity should have a concentration–response curve consisting of a baseline, followed by a negative slope, with a response decrease to at least 80% of the average maximal value of the reference estrogen response.
- Test substances are negative for ER antagonist activity if all data points are above 80% of the average maximal value of the reference estrogen response.

Test method protocols should incorporate the essential components listed above. Modifications should be detailed and scientifically justified, and the modified test method should perform as well as or better than the BG1Luc ER TA test method.

Reference Substances

ICCVAM recommends for test method validation a subset of those substances that were definitively classified as positive or negative for ER TA activity in the scientific literature and that were tested in the BG1Luc ER TA validation study. The reference substances include a range of chemical and product classes commonly associated with endocrine disruption.

Test Method Accuracy and Reliability

When evaluated using this minimum list of recommended reference substances, a proposed ER TA test method should have accuracy (i.e., sensitivity, specificity, false positive rates, and false negative rates) and reliability characteristics equal to or better than those of the BG1Luc ER TA test method. Any misclassified reference substances should be addressed in terms of the test

method's ability to accurately classify other substances with similar potencies and from the same chemical/product classes.

Using the Performance Standards

Test method developers are encouraged to consult directly with ICCVAM before using these performance standards to conduct a validation study for a proposed test method. Developers are also encouraged to submit results of validation studies to ICCVAM for an evaluation of the validation status. Upon completing its evaluation in accordance with the ICCVAM Authorization Act (42 U.S.C. 285*l*-3), ICCVAM will forward recommendations to ICCVAM agencies regarding the usefulness and limitations of the test method.

ICCVAM Consideration of the Independent Peer Review Panel Report and Other Comments

The ICCVAM evaluation process incorporates scientific peer review and a high level of transparency. The evaluation process for the BG1Luc ER TA test method included a public review meeting by an independent scientific peer review panel, multiple opportunities for public comments, and comments from SACATM. ICCVAM and the EDWG considered the Panel report, SACATM comments, and all public comments before finalizing the ICCVAM test method evaluation report and final BRD for the BG1Luc ER TA test method.

1.0 Introduction

In vitro estrogen receptor (ER) transcriptional activation (TA) assays are designed to identify agonist or antagonist substances that might interfere with estrogen activity *in vivo*. Unlike receptor binding assays, TA assays can distinguish between agonist and antagonist activity. The BG1Luc ER TA test method utilizes an ER-responsive reporter gene (*luc*) in the human ovarian adenocarcinoma cell line BG-1 to detect substances with *in vitro* ER agonist or antagonist activity. ER-mediated transcription of the *luc* gene results in the production of luciferase, the activity of which is quantified using a luminometer. A concentration–response curve can be established to provide qualitative and quantitative information regarding the *in vitro* estrogenic activity of a test substance (Rogers and Denison 2000).

The Federal Food, Drug, and Cosmetic Act; the Food Quality Protection Act; and the Safe Drinking Water Act all aim to identify potential endocrine disruptors and thereby protect humans and animals (7 U.S.C. 136; 21 U.S.C. 301 et seq.; 110 Stat 1613). The U.S. Environmental Protection Agency (EPA) was specifically required to "develop a screening program, using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen, or such other endocrine effect as the Administrator may designate" (21 U.S.C. 346a[p][1]). In 1996, the EPA formed the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), a committee of scientists and stakeholders that was charged by the EPA to provide recommendations on how to implement its Endocrine Disruptor Screening Program (EDSP). The EDSP is described in detail at http://www.epa.gov/scipoly/oscpendo/.

The EPA accepted EDSTAC's recommendations for a two-tier screening program as proposed in the *Federal Register* (63 FR 71542). The purpose of Tier 1, which consists of *in vivo* and *in vitro* test methods, is to identify the potential of chemicals to interact with the estrogen, androgen, or thyroid hormonal systems. Tier 1 currently includes EPA OPPTS 890.1300: Estrogen Receptor Transcriptional Activation (Human Cell Line [HeLa-9903]) (EPA 2009). EPA OPPTS 890.1300 is an ER TA test method validated for the detection of *in vitro* ER agonists.

In 2004, Xenobiotic Detection Systems, Inc. (XDS), nominated their LUMI-CELL® ER test method (hereafter BG1Luc ER TA test method) to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) for validation. ICCVAM and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) recommended that the BG1Luc ER TA test method should be considered a high priority for interlaboratory validation studies based on the lack of adequately validated test methods and the regulatory and public health need for such test methods.

The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) led and coordinated an international validation study with its counterparts in Europe (the European Centre for the Validation of Alternative Methods [ECVAM]) and Japan (the Japanese Center for the Evaluation of Alternative Methods [JaCVAM]) using laboratories sponsored by each validation organization. NICEATM organized a Study Management Team (SMT) to oversee the scientific aspects of the validation study and coordinate the day-to-day activities among the participating laboratories (XDS, ECVAM, and Hiyoshi). A representative from the recently established Korean Center for the Validation of Alternative Methods (KoCVAM) joined the SMT in 2010.

The validation study proceeded in four phases. During Phase 1, each of the three participating centers (ICCVAM, ECVAM, and JaCVAM) selected validation laboratories. The protocols were reviewed, and the laboratories demonstrated proficiency with the test method by successfully completing 10 replicate agonist and 10 replicate antagonist tests. In Phases 2 through 4, the

protocols were evaluated and refined, and 78 ICCVAM reference substances that should be used to standardize and validate *in vitro* ER and AR binding and TA test methods were tested.

Based on the results of this study, ICCVAM reviewed the validation status of the BG1Luc ER TA test method for identification of substances with *in vitro* ER agonist or antagonist activity. NICEATM and the ICCVAM Interagency Endocrine Disruptor Working Group (EDWG) prepared a draft background review document (BRD) that provides a comprehensive description and the data from the validation study used to assess the accuracy and reliability of the BG1Luc ER TA test method.

On January 24, 2011, ICCVAM announced the availability of the draft BRD to the public and a public Panel meeting to review the validation status of the BG1Luc ER TA test method as a screening test to identify *in vitro* ER agonists and antagonists (76 FR 4113[2]). All of the information provided to the Panel, including the draft BRD, ICCVAM draft test method recommendations, and all public comments received before the Panel meeting, were made publicly available via the NICEATM–ICCVAM website (http://iccvam.niehs.nih.gov/).

The public Panel meeting was held on March 29–30, 2011. The Panel evaluated (1) the extent to which the draft BRD addressed established validation and acceptance criteria and (2) the extent to which the draft BRD supported ICCVAM's draft test method recommendations. Interested stakeholders from the public were provided opportunities to comment at the Panel meeting. After considering all public comments, the Panel agreed with the ICCVAM draft recommendation that the BG1Luc ER TA test method can be used as a screening test to identify substances with *in vitro* ER agonist and antagonist activity. On May 18, 2011, ICCVAM posted a report of the Panel's recommendations[3] (see **Appendix D**) on the NICEATM–ICCVAM website for public review and comment (announced in 76 FR 28781).

ICCVAM provided SACATM with the draft BRD and test method recommendations, the Panel report, and all public comments for discussion at their meeting on June 16–17, 2011, where public stakeholders were given another opportunity to comment.

ICCVAM and the EDWG considered the SACATM comments, the Panel report, and all public comments before finalizing ICCVAM test method recommendations for use of the BG1Luc ER TA test method as a screening test to identify substances with *in vitro* ER agonist and antagonist activity. ICCVAM's recommendations (see **Section 2.0**) and the final BRD (see **Appendix C**) are incorporated in this test method evaluation report. As required by the ICCVAM Authorization Act of 2000 (42 U.S.C. 285*l*-3), ICCVAM will forward this report and its recommendations to U.S. Federal agencies for consideration. Federal agencies must respond to ICCVAM within 180 days after receiving ICCVAM test method recommendations. ICCVAM recommendations are available to the public on the NICEATM–ICCVAM website (http://iccvam.niehs.nih.gov/), and agency responses will also be made available as they are received.

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[2] *Federal Register* notices published by NICEATM–ICCVAM during evaluation of the BG1Luc ER TA test method are available in Appendix E and from the NICEATM–ICCVAM website (http://iccvam.niehs.nih.gov/).

[3] http://iccvam.niehs.nih.gov/docs/endo_docs/EDPRPRept2011.pdf

2.0 ICCVAM Recommendations: Usefulness and Limitations of the BG1Luc ER TA Test Method

2.1 Background and Introduction

ICCVAM has completed its evaluation of the validation status of the BG1Luc ER TA test method, an *in vitro* method proposed to identify potential agonist or antagonist substances that might interfere with normal estrogen activity. NICEATM and ICCVAM prepared a comprehensive BRD that includes the data and information available to characterize the validity of this proposed use of the BG1Luc ER TA test method. The information included in the BRD (**Appendix C**) is based on an international validation study that utilized 78 reference substances that should be used to standardize and validate *in vitro* ER and androgen receptor (AR) binding and TA test methods. Based on the results of this study, ICCVAM developed these draft test method recommendations on the usefulness and limitations of the BG1Luc ER TA test method for identifying potential ER agonists or antagonists. ICCVAM also developed draft recommendations for standardized test method protocols, future studies, and performance standards.

2.2 ICCVAM Recommendations: Test Method Usefulness and Limitations

2.2.1 Evaluation as a Screening Test to Identify Substances with Estrogen Receptor Agonist Activity

ICCVAM concludes that the BG1Luc ER TA test method can be used as a screening test to identify substances with *in vitro* ER agonist activity. This recommendation is based on an evaluation of available validation study data and corresponding accuracy and reliability. ICCVAM concludes that the accuracy of this assay is at least equivalent to that of EPA OPPTS 890.1300, part of the EDSP Tier 1 screening battery. The supporting accuracy analysis used 35 ICCVAM reference substances, which produced the following definitive results in agonist testing when compared with existing reference data from other *in vitro* ER TA assays:

- Concordance of 97% (34/35)
- Sensitivity of 96% (27/28)
- Specificity of 100% (7/7)
- False positive rate of 0% (0/7)
- False negative rate of 4% (1/28)

Only L-thyroxine was false negative in the BG1Luc ER TA test method when compared to the ICCVAM reference classification. This reference substance is classified as positive (2/3) based on two reports of positive agonist activity and one report of no agonist activity. The two positive results were in GH3 cells (rat pituitary adenoma) and HeLa cells (human cervical carcinoma), whereas MCF-7 cells (human breast adenocarcinoma) showed no estrogenic response when exposed to L-thyroxine. These results indicate a possible tissue-specific response to L-thyroxine, which may explain the lack of ER agonist activity observed in this experiment with BG-1 cells (human ovarian carcinoma).

During Phase 1, 12 substances were tested in each of the three laboratories (XDS, ECVAM, and Hiyoshi) to evaluate intralaboratory reproducibility. Although the classifications for some of the test substances differed among the laboratories, there was 100% agreement within each laboratory for each of the three repeat tests. When results were compared *across* laboratories for these 12 substances, all three laboratories agreed on 67% (8/12) of the substances. An additional 36 substances tested for agonist activity once in each laboratory produced a definitive result in at

least two laboratories. There was 100% agreement among the laboratories for 83% (30/36) of these substances.

Only one *in vitro* ER TA test method is currently accepted to assess ERα agonist activity of test substances. This test method was validated by the Chemicals Evaluation and Research Institute (CERI) and is described in Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 455: the Stably Transfected Human Estrogen Receptor-α Transcriptional Activation (STTA) Assay for the Detection of Estrogenic Agonist-Activity (OECD 2009). Adopted by the EPA as OPPTS 890.1300: Estrogen Receptor Transcriptional Activation (Human Cell Line [HeLa-9903]) (EPA 2009), it is considered adequately validated by national and international regulatory agencies.

Because the BG1Luc ER TA test method is another STTA assay that could be considered for regulatory use, a comparison of test method accuracy between these two test methods was conducted based on a list of ICCVAM-recommended agonist reference substances for which definitive classifications have been produced in both methods. These results show identical levels of accuracy when both methods tested the same agonist reference chemicals: concordance of 95% (24/25), sensitivity of 95% (21/22), and specificity of 100% (4/4). Overall, these data indicate that the BG1Luc ER TA test method is equivalent to the EPA OPPTS 890.1300/OECD TG 455 method for assessing ERα agonist activity.

Based on these results, the BG1Luc ER TA agonist test method can be applied to a wide range of substances, provided they (1) can be dissolved in dimethyl sulfoxide (DMSO), (2) do not react with DMSO or the cell culture medium, and (3) are not toxic to the cells. Although this method may apply to mixtures, none was evaluated in this validation study. Volatile substances may yield acceptable results if CO_2-permeable plastic film is used to seal the test plates, but no volatile substances were evaluated in this validation study. Although relatively few are known, substances with endogenous luminescence or that naturally inhibit luciferase activity cannot be used in this or any other luciferase-based test method. The demonstrated performance of the BG1Luc ER TA agonist test method suggests that data generated with this test method could be routinely considered for prioritization of substances for further testing.

Independent Peer Review Panel Conclusions and Recommendations

The Panel concluded that the available data and test method performance support the ICCVAM draft recommendation that the BG1Luc ER TA test method can be used as a screening test to identify substances with *in vitro* ER agonist activity. However, the Panel emphasized that, because there has been no clear regulatory guidance on how ER TA test methods will be used in the EPA EDSP Program, the use of the BG1Luc ER TA test method in the overall strategy of hazard identification or safety assessment of endocrine-disruptive chemicals is unclear.

2.2.2 Evaluation as a Screening Test to Identify Substances with Estrogen Receptor Antagonist Activity

Based on an evaluation of available data and corresponding performance (accuracy and reliability), ICCVAM recommends that the BG1Luc ER TA test method can be used as a screening test to identify substances with ER antagonist activity. The accuracy analysis, conducted with 25 reference substances, produced the following definitive results in antagonist testing:

- Accuracy of 100% (25/25)
- Sensitivity of 100% (3/3)
- Specificity of 100% (22/22)
- False positive rate of 0% (0/22)

- False negative rate of 0% (0/3)

Intralaboratory reproducibility of the BG1Luc ER TA agonist and antagonist test methods was assessed by comparing (1) reference standard and control results for all plates tested within each laboratory during the course of the validation study and (2) results from Phases 2a and 2b testing, during which 12 substances were tested in at least three independent experiments in each of three laboratories. Although the classifications for some of the test substances differed among the laboratories, there was 100% agreement within each laboratory for each of the three repeat tests.

When results were compared *across* laboratories for these 12 substances, there was 100% agreement among the three laboratories for all 12 substances. An additional 41 substances tested once in each laboratory for antagonist activity during Phase 3 produced a definitive result in at least two laboratories. There was 100% agreement among the laboratories for 93% (38/41) of the 41 substances.

Based on these results, the limitations of the BG1Luc ER TA antagonist test method appear to be the same as those identified for the agonist test method described above. Although the validation database is somewhat limited in number (n = 25), the demonstrated performance of the BG1Luc ER TA antagonist test method suggests that data generated with this test method could be routinely considered for prioritization of substances for further testing. This is further supported by the fact that so few ER antagonists have been definitively identified, and all three tested in the BG1Luc ER TA antagonist test method were correctly identified.

Independent Peer Review Panel Conclusions and Recommendations
The Panel concluded that the available data and test method performance support the ICCVAM draft recommendation that the BG1Luc ER TA test method can be used as a screening test to identify substances with *in vitro* ER antagonist activity. The Panel further concluded that, based upon support of the ICCVAM draft recommendation, the BG1Luc ER TA test method could be considered as a replacement for the currently accepted ER TA assay (EPA OPPTS 890.1300/OECD TG 455) and the rat uterine cytosol binding assays. However, the Panel noted that additional analysis may be necessary to further support this recommendation, particularly regarding the rat uterine cytosol ER binding assay.

2.3 ICCVAM Recommendations: Test Method Protocol for the BG1Luc ER TA Test Method

For use of the BG1Luc ER TA test method as a screening test to identify substances with *in vitro* ER agonist or antagonist activity, ICCVAM recommends using the ICCVAM BG1Luc ER TA agonist and antagonist test method protocols (**Appendix B**). In addition, all future studies intended to further characterize the usefulness and limitations of the BG1Luc ER TA agonist and antagonist test methods should be conducted using these recommended protocols.

Independent Peer Review Panel Conclusions and Recommendations
The Panel concluded that the BG1Luc ER TA test method protocols are complete and adequate in detail for a laboratory to conduct the study (see **Appendix D**). The Panel noted several advantages provided by this assay over the currently accepted test method (EPA OPPTS 890.1300/OECD TG 455). The BG1Luc ER TA test method:

- Has more detailed and complete test method protocols than those provided in EPA OPPTS 890.1300/OECD TG 455
- Is validated for testing up to 1 mM per EPA requirements. EPA OPPTS 890.1300/OECD TG 455 is only validated up to a limit dose of 10 μM.

- Has a more restrictive set of classification criteria for determination of a positive response, which will reduce the number of false positive results, resulting in fewer follow-up tests conducted in animal studies
- Can detect substances with *in vitro* anti-estrogenic activity
- Endogenously expresses both hERα and hERβ, whereas the HeLa-9903 cell line used in EPA OPPTS 890.1300/OECD TG 455 was transfected only with hERα

2.4 ICCVAM Recommendations: Future Studies for the BG1Luc ER TA Test Method

ICCVAM promotes the scientific validation and regulatory acceptance of new methods that reduce, refine, or replace animal use where scientifically feasible. The rat uterine cytosol ER binding assay, currently listed as part of the EDSP Tier 1 screening battery, requires the use of animals as a source of ERs. Results from the BG1Luc ER TA test method were examined for concordance with published reports of ER binding for 34 reference substances. There was 97% (33/34) concordance between the BG1Luc ER TA test method and ER binding data from the literature, and 100% sensitivity (no false negatives). In light of the excellent degree of agreement between ER binding and BG1Luc ER TA data, it appears that evaluating results from BG1Luc ER TA agonist and antagonist testing may provide a viable alternative to conducting ER binding studies. This cannot currently be accomplished with EPA OPPTS 890.1300/OECD TG 455 due to the inability of this method to assess ER antagonist activity. ICCVAM recommends that additional validation studies could be performed to determine whether or not the BG1Luc ER TA method could replace the rat uterine cytosol ER binding assay.

Results from the BG1Luc ER TA test method were examined for concordance with published data from the uterotrophic bioassay (n = 13 reference substances), which is currently listed as part of the EDSP Tier 1 screening battery. There was 92% (12/13) concordance between the BG1Luc ER TA test method and the uterotrophic bioassay data, and 100% specificity (no false negatives). These data indicate that the BG1Luc ER TA agonist test method has very good agreement with the *in vivo* results obtained with the uterotrophic bioassay. Accordingly, ICCVAM recommends that further work be carried out to determine if the BG1Luc ER TA test method could be used in combination with other methods (to include *in vitro* metabolic activation) in a weight-of-evidence approach to replace the uterotrophic bioassay.

To further characterize the BG1Luc ER TA test method, ICCVAM identified additional studies that may be considered by interested parties:

- Additional studies/evaluations may be conducted to more completely characterize the ratio of ERα and ERβ in the BG-1 cell line and the extent to which these receptor subtypes contribute to the overall performance of the BG1Luc ER TA test method.
- Additional studies/evaluations may be conducted to determine the feasibility of testing volatile substances using CO_2-permeable plastic film or other methods to seal the test plates.
- Additional studies/evaluations may be conducted to determine if substances that are not soluble in DMSO could be tested in another vehicle that would more adequately solubilize the substance in culture media.
- As ER antagonists are identified, additional studies/evaluations may be conducted to expand the database of positive substances tested and thereby better characterize the usefulness and limitations of the BG1Luc ER TA test method as a screening test to identify substances with ER antagonist activity.
- ICCVAM encourages users to provide all data that are generated from future studies to ICCVAM so that they may be used to further characterize the usefulness and limitations of the BG1Luc ER TA test method as a screening test to identify substances with *in vitro* ER agonist or antagonist activity.

Independent Peer Review Panel Conclusions and Recommendations

The Panel concluded that the available data support the draft ICCVAM-recommended future studies. The Panel encouraged additional studies and evaluations to assess the utility of the current visual assessment of cytotoxicity evaluation for chemicals, as well as efforts to identify a quantitative cytotoxicity method. The Panel also recommended future studies to account for metabolic activation that could expand the utility of this and other ER TA methods. The Panel further recommended an effort to expand the reference substance list and associated BG1Luc ER TA database with additional negative agonist and positive antagonist test substances as they are identified.

2.5 ICCVAM Recommendations: Performance Standards for the BG1Luc ER TA Test Method

ICCVAM has developed test method performance standards so that modified versions of the BG1Luc ER TA test method that are mechanistically and functionally similar can be effectively and efficiently evaluated for their validity by national and international validation organizations (e.g., ICCVAM, ECVAM, and JaCVAM) or other organizations. The ICCVAM-recommended BG1Luc ER TA agonist and antagonist test method protocols are the key references used to establish these performance standards.

Independent Peer Review Panel Conclusions and Recommendations

The Panel concluded that the draft ICCVAM performance standards are adequate, but they proposed modifications that could expand the performance standards' applicability. The Panel suggested that the specific tissue source, type, and species used for the cell system in ER TA test methods may not be critical but recommended that the appropriate cellular machinery be included. The Panel also recommended that, ideally, more negatives should be included. They recognized, however, that data on such substances are not currently available. The Panel also suggested that reference substance classification be based upon reports that have been ranked with a method that focuses on the reliability of the published data (e.g., Klimisch criteria) (Klimisch et al. 1997).

Classification of reference substances was based on the following published guidance from ICCVAM (ICCVAM 2003a, 2006):

- A substance was classified as "positive" if it was reported as positive in >50% of referenced ER TA studies.
- A substance was classified as "presumed positive" if it was positive in 50% or less of referenced ER TA studies.

Prior to the BG1Luc ER TA test method validation study, L-thyroxine was classified as positive because two of three literature citations described estrogenic activity for this compound. Because the BG-1 validation study will be considered a published study, and L-thyroxine was negative in the study, the updated database will reflect that this compound is reported as positive in two of four studies (50%), changing its classification from positive to presumed positive per the guidelines given above. Because only those compounds with definitive classifications (positive or negative) are used as reference substances, L-thyroxine will not be used as a reference substance in future studies.

3.0 Validation Status for Use of the BG1Luc ER TA Test Method as a Screening Test to Identify *In Vitro* ER Agonists and Antagonists

The ICCVAM BRD (see **Appendix C**) provides a comprehensive review of the current validation status of the BG1Luc ER TA test method, including its accuracy and reliability, the substances tested, the rationale for the standardized test method protocol used for the validation study, and all available data supporting its validity. This section provides a brief description and summary of the validation status of the BG1Luc ER TA test method.

3.1 Test Method Description

The BG1Luc ER TA test method uses an ER-responsive reporter gene (*luc*) in the human ovarian adenocarcinoma cell line BG-1 to detect substances with *in vitro* ER agonist or antagonist activity. ER-mediated transcription of the *luc* gene results in the production of luciferase, the activity of which is quantified using a luminometer. A concentration–response curve can be established to provide qualitative and quantitative information regarding the *in vitro* estrogenic activity of a test substance.

3.2 General Test Method Procedures

ICCVAM previously recommended minimum essential test method components for *in vitro* ER TA assays (ICCVAM 2003a), and these components are incorporated into the ICCVAM-recommended BG1Luc ER TA protocols (see **Appendices B1** and **B2**). These protocols include three sequential phases: solubility, range finder, and comprehensive testing. During solubility testing, the maximum test substance concentration that is soluble in 100% DMSO is established in order to set the starting concentration for range finder testing. The test substance concentration range to be included in comprehensive testing is established during range finder testing. Results from comprehensive testing are used to determine the extent to which a test substance influences ER-mediated luciferase transcription as a correlate to *in vitro* ER TA activity. These data can then be used to classify a test substance based on its *in vitro* ER agonist or antagonist activity.

3.3 Validation Database

The validation database used to evaluate the BG1Luc ER TA test method is based upon the list of 78 substances that ICCVAM recommended for use in validation studies for *in vitro* ER and AR binding and TA test methods (ICCVAM 2003a, 2006). The purpose of this list is to ensure that the usefulness and limitations of *in vitro* ER and AR binding and TA assays can be adequately characterized across a broad range of chemical classes and responses. These substances were selected based on information contained in the ICCVAM BRDs for ER and AR binding and TA test methods (ICCVAM 2002d, 2002a, 2002c, 2002b), as well as information obtained from publications reviewed or published after completion of the ICCVAM BRDs. The complete list of substances and their respective reference classifications for agonist and antagonist activity based on available reference data is provided in Section 3-2 of the BG1Luc ER TA BRD (**Appendix C**).

Only those substances that could be definitively classified as positive (POS) or negative (NEG) were used to assess accuracy, resulting in 48 unique substances used to assess accuracy. (Substances classified as presumed positive [PP] or presumed negative [PN] were not considered when evaluating test method accuracy.) Separate lists were generated for evaluating accuracy based on agonist (42 substances: 33 positive, 9 negative) activity and antagonist (25 substances: 3 positive, 22 negative) activity. Nineteen substances appeared on both reference lists. The 42 reference substances used to assess accuracy based on ER agonist activity are provided in **Table 3-1**, and the 25 reference substances used to assess accuracy based on ER antagonist

activity are provided in **Table 3-2**. These tables also include the BG1Luc ER TA results from each of the participating laboratories.

3.4 Test Method Accuracy

Thirty-five substances (28 positive, 7 negative) had definitive results and were used to evaluate test method accuracy for ER agonist activity. The remaining seven (17%) of the 42 substances used to evaluate test method accuracy had inadequate (I) testing results and were therefore excluded from the analysis. Data are classified as inadequate if, because of major qualitative or quantitative limitations, they cannot be interpreted as valid for showing either the presence or absence of agonist activity. The following seven substances had inadequate BG1Luc ER TA agonist test method data:

- Clomiphene citrate
- *p,p'*-DDE
- 5∝-Dihydrotestosterone
- Flutamide
- Procymidone
- Resveratrol
- Tamoxifen

It should be emphasized that the "inadequate" classification is usually a result of poor data quality and would normally require retesting. However, the classification system was revised after testing to include positive, negative, and inadequate classifications. Retesting of these substances was therefore not possible.

These seven substances (clomiphene citrate, *p,p'*-DDE, 5∝-dihydrotestosterone, flutamide, procymidone, resveratrol, and tamoxifen) represent eight chemical classes (two cyclic hydrocarbons, and one each of an amide, amine, carboxylic acid, halogenated hydrocarbon, heterocyclic compound, polycyclic compound, and steroid) and five product classes (four pharmaceuticals and one each of a fungicide, natural product, pesticide intermediate, and veterinary agent). The diversity of chemical and product classes indicates that no one category or class is overrepresented with inadequate data.

Table 3-1 42 ICCVAM-Recommended Substances Used to Evaluate ER Agonist Accuracy

Substance	CASRN	Classification[a]				
		ICCVAM Consensus	BG1Luc ER TA Consensus[b]	XDS	ECVAM	Hiyoshi
17∝-Estradiol	57-91-0	POS	POS	POS (1/1)	POS (3/3)	POS (2/2)
17∝-Ethinyl estradiol	57-63-6	POS	POS	POS (3/3)	POS (3/3)	POS (3/3)
17ß-Estradiol	50-28-2	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
19-Nortestosterone	434-22-0	POS	POS	POS (1/1)	NT	NT
4-Cumylphenol	599-64-4	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
4-*tert*-Octylphenol	140-66-9	POS	POS	I (1/1)	POS (1/1)	POS (2/2)
5∝-Dihydrotestosterone	521-18-6	POS	I	I (1/1)	I (1/1)	POS (1/1)
Apigenin	520-36-5	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
Atrazine	1912-24-9	NEG	NEG	NEG (3/3)	POS (3/3)	NEG (3/3)
Bicalutamide	90357-06-5	NEG	NEG	NEG (1/1)	NT	NT
Bisphenol A	80-05-7	POS	POS	POS (3/3)	POS (3/3)	POS (3/3)
Bisphenol B	77-40-7	POS	POS	POS (3/3)	POS (3/3)	POS (3/3)
Butylbenzyl phthalate	85-68-7	POS	POS	POS (3/3)	POS (3/3)	POS (3/3)
Chrysin	480-40-0	POS	POS	POS (2/2)	NT	NT
Clomiphene citrate	50-41-9	POS	I	I (1/1)	NEG (1/1)	POS (1/1)
Corticosterone	50-22-6	NEG	NEG	NEG (3/3)	POS (3/3)	NEG (4/4)
Coumestrol	479-13-0	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
Daidzein	486-66-8	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
Dicofol	115-32-2	POS	POS	POS (1/1)	NEG (1/1)	POS (1/1)
Diethylstilbestrol	56-53-1	POS	POS	POS (3/3)	POS (3/3)	POS (3/3)
Estrone	53-16-7	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
Ethyl paraben	120-47-8	POS	POS	I (1)	POS (1/1)	POS (1/1)
Fenarimol	60168-88-9	POS	POS	POS (1/1)	NT	NT
Flutamide	13311-84-7	NEG	I	I (1)	NT	NT
Genistein	446-72-0	POS	POS	POS (3/3)	POS (3/3)	POS (4/4)
Hydroxyflutamide	52806-53-8	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Kaempferol	520-18-3	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
Kepone	143-50-0	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
L-Thyroxine	51-48-9	POS	NEG	NEG (1/1)	NT	NT
Linuron	330-55-2	NEG	NEG	NEG (1/1)	NT	NT
meso-Hexestrol	84-16-2	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
Methyl testosterone	58-18-4	POS	POS	POS (3/3)	POS (1/1)	POS (2/2)

Substance	CASRN	Classification[a]				
		ICCVAM Consensus	BG1Luc ER TA Consensus[b]	XDS	ECVAM	Hiyoshi
Norethynodrel	68-23-5	POS	POS	POS (2/2)	POS (1/1)	POS (2/2)
o,p'-DDT	789-02-6	POS	POS	POS (3/3)	POS (3/3)	POS (3/3)
p-n-Nonylphenol	104-40-5	POS	POS	POS (3/3)	POS (3/3)	POS (3/3)
p,p'-DDE	72-55-9	POS	I	I (1/1)	I (1/1)	NEG (1/1)
p,p'- Methoxychlor	72-43-5	POS	POS	POS (1/1)	POS (1/1)	POS (2/2)
Phenobarbital	50-06-6	NEG	NEG	NEG (1/1)	NEG (1/1)	NT
Procymidone	32809-16-8	NEG	I	I (1/1)	NT	NT
Resveratrol	501-36-0	POS	I	POS (1/1)	I (1/1)	NEG (2/3)
Spironolactone	52-01-7	NEG	NEG	NEG (1/1)	NT	NT
Tamoxifen	10540-29-1	POS	I	I (1/1)	I (1/1)	POS (1/1)

Abbreviations: BG1Luc ER TA = LUMI-CELL BG1Luc4E2 ER TA test method; CASRN = CAS Registry Number (American Chemical Society); ECVAM = European Centre for the Validation of Alternative Methods; I = inadequate (positive or negative classification could not be determined because of poor-quality data); NEG = negative; NT = not tested; POS = positive; XDS = Xenobiotic Detection Systems, Inc.

[a] Number in parentheses represents test results (POS, NEG, or I) over the total number of trials that met test plate acceptance criteria.

[b] BG1Luc ER TA consensus classification represents the majority classification among the three validation laboratories.

Definitive classifications (positive or negative) were obtained for all 25 substances used to evaluate test method accuracy for ER antagonist activity, allowing all 25 substances to be used to assess antagonist accuracy.

Table 3-2 25 ICCVAM-Recommended Substances Used to Evaluate ER Antagonist Accuracy

Substance	CASRN	Classification[a]				
		ICCVAM Consensus	BG1Luc ER TA Consensus[b]	XDS	ECVAM	Hiyoshi
17α–Ethinyl estradiol	57-63-6	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
4-Hydroxytamoxifen	68047-06-3	POS	POS	POS (1/1)	I (2/2)	POS (1/1)
5α-Dihydrotestosterone	521-18-6	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Apigenin	520-36-5	NEG	NEG	NEG (3/3)	NEG (3/3)	NEG (4/4)
Bisphenol A	80-05-7	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Butylbenzyl phthalate	85-68-7	NEG	NEG	NEG (3/3)	NEG (3/3)	NEG (4/4)
Chrysin	480-40-0	NEG	NEG	NEG (1/1)	NT	NT
Coumestrol	479-13-0	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Daidzein	486-66-8	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Di-*n*-butyl phthalate	84-74-2	NEG	NEG	NEG (2/2)	NEG (1/1)	NEG (1/1)
Dicofol	115-32-2	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Diethylhexyl phthalate	117-81-7	NEG	NEG	NEG (1/1)	NEG (2/2)	NEG (1/1)
Diethylstilbestrol	56-53-1	NEG	NEG	NEG (1/1)	NEG (1/1)	POS (1/1)
Genistein	446-72-0	NEG	NEG	NEG (3/3)	NEG (3/3)	NEG (3/3)
Kaempferol	520-18-3	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Kepone	143-50-0	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Mifepristone	84371-65-3	NEG	NEG	NEG (1/1)	NT	NT
Norethynodrel	68-23-5	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
o.p'-DDT	789-02-6	NEG	NEG	NEG (3/3)	NEG (3/3)	NEG (4/4)
p-n-Nonylphenol	104-40-5	NEG	NEG	NEG (3/3)	NEG (3/3)	NEG (3/3)
p.p'-DDE	72-55-9	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Progesterone	57-83-0	NEG	NEG	NEG (3/3)	NEG (3/3)	NEG (3/3)
Raloxifene HCl	82640-04-8	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
Resveratrol	501-36-0	NEG	NEG	NEG (3/3)	NEG (3/3)	NEG (3/3)
Tamoxifen	10540-29-1	POS	POS	POS (4/4)	POS (3/3)	POS (3/3)

Abbreviations: BG1Luc ER TA = LUMI-CELL BG1Luc4E2 ER TA test method; CASRN = CAS Registry Number (American Chemical Society); ECVAM = European Centre for the Validation of Alternative Methods; I = inadequate (positive or negative classification could not be determined because of poor-quality data); NEG = negative; NT = not tested; POS = positive; XDS = Xenobiotic Detection Systems, Inc.

[a] Number in parentheses represents test results (POS, NEG, or I) over the total number of trials that met test plate acceptance criteria.

[b] BG1Luc ER TA consensus classification represents the majority classification among the three validation laboratories.

The accuracy analysis using the 35 ICCVAM reference substances that produced a definitive BG1Luc ER TA result in agonist testing indicated accuracy of 97% (34/35), sensitivity of 96% (27/28), specificity of 100% (7/7), false positive rate of 0% (0/7), and false negative rate of 4% (1/28) (**Table 3-3**). Analysis of accuracy using individual laboratory results indicated accuracy ranging from 86% (25/29) to 97% (33/34), sensitivity from 92% (23/25) to 96% (27/28),

specificity from 50% (2/4) to 100% (6/6), false positive rates from 0% (0/6) to 50% (2/4), and false negative rates from 4% (1/28) to 8% (2/25).

Table 3-3 Accuracy of the BG1Luc ER TA Agonist Data

Laboratory	N	Accuracy	Sensitivity	Specificity	False Positive Rate	False Negative Rate
Combined	35[a]	97% (34/35)	96% (27/28)	100% (7/7)	0% (0/7)	4% (1/28)
XDS	34	97% (33/34)	96% (27/28)	100% (6/6)	0% (0/6)	4% (1/28)
ECVAM	29	86% (25/29)	92% (23/25)	50% (2/4)	50% (2/4)	8% (2/25)
Hiyoshi	32	94% (30/32)	93% (27/29)	100% (3/3)	0% (0/3)	7% (2/29)

Abbreviations: ECVAM = European Centre for the Validation of Alternative Methods; N = number; XDS = Xenobiotic Detection Systems, Inc.

[a] A total of 42 substances were evaluated in the BG1Luc ER TA agonist test method. Seven substances did not produce a consensus classification and were omitted, leaving 35 substances for analysis.

The antagonist accuracy analysis indicated an overall accuracy of 100% (25/25), sensitivity of 100% (3/3), specificity of 100% (22/22), false positive rate of 0% (0/22), and false negative rate of 0% (0/3) (**Table 3-4**). Similarly, individual laboratory results indicated accuracy ranging from 96% (22/23) to 100% (25/25), sensitivity of 100% (3/3), and specificity of 95% (19/20) to 100% (22/22).

Table 3-4 Accuracy of the BG1Luc ER TA Antagonist Data

Laboratory	N	Accuracy	Sensitivity	Specificity	False Positive Rate	False Negative Rate
Combined	25	100% (25/25)	100% (3/3)	100% (22/22)	0% (0/22)	0% (0/3)
XDS	25	100% (25/25)	100% (3/3)	100% (22/22)	0% (0/22)	0% (0/3)
ECVAM	23	100% (23/23)	100% (3/3)	100% (20/20)	0% (0/20)	0% (0/3)
Hiyoshi	23	96% (22/23)	100% (3/3)	95% (19/20)	5% (1/20)	0% (0/3)

Abbreviations: ECVAM = European Centre for the Validation of Alternative Methods; N = number; XDS = Xenobiotic Detection Systems, Inc.

3.5 Test Method Reliability

Intralaboratory reproducibility of the BG1Luc ER TA agonist and antagonist test methods was assessed quantitatively by comparing the following:

- Relative light unit (RLU) values for the agonist and antagonist DMSO control and the antagonist E2 control for all plates tested within each laboratory during the course of the validation study

- Results from Phases 2a and 2b testing, during which 12 substances were tested in at least three independent experiments in each of the three laboratories

Because DMSO control RLU values are not normalized, they vary considerably between test plates and across time. Therefore, intralaboratory reproducibility was evaluated by comparing the within-plate variability of the four replicate DMSO control RLU values for all test plates that passed acceptance criteria (i.e., coefficient of variation [CV] associated with within-plate DMSO control RLU values). The range of means and CV values for within-plate DMSO control RLU values are provided in **Table 3-5**. Mean plate DMSO RLU values ranged from a low of 511 to a high of 9885, with a mean of 3749. However, within-plate variability of DMSO RLU control values between replicate DMSO wells was low. Coefficients of variation ranged from 1% to 43%, with a mean of 8%. Of the 218 agonist test plates that passed acceptance criteria, only six plates had within-plate CV values greater than 20%.

Table 3-5 Agonist Within-Plate DMSO Control Data

Laboratory	Mean and Range of DMSO Control RLU Values	Mean and Range of CV (%)	N
Combined	3749 (511-9885)	8 (1-43)	218
XDS	2800 (511-9885)	8 (1-43)	93
ECVAM	3379 (828-7306)	8 (1-33)	60
Hiyoshi	5465 (1362-9383)	6 (1-24)	65

Abbreviations: CV = coefficient of variation; DMSO = dimethyl sulfoxide; ECVAM = European Centre for the Validation of Alternative Methods; N = number of plates that passed acceptance criteria; RLU = relative light unit; XDS = Xenobiotic Detection Systems, Inc.

For the antagonist assay, although mean plate DMSO RLU values ranged from a low of 132 to a high of 8451 (mean = 3299), within-plate variability of DMSO RLU control values between replicate DMSO wells was low, with CV values ranging from 1% to 52% (mean = 8%) (**Table 3-6**). Of the 194 antagonist test plates that passed acceptance criteria, only eight plates had within-plate CV values greater than 20%.

Table 3-6 Antagonist Within-Plate DMSO Control Data

Laboratory	Mean and Range of DMSO Control RLU Values	Mean and Range of CV (%)	N
Combined	3299 (132-8451)	8 (1-52)	194
XDS	2230 (132-6860)	9 (1-52)	79
ECVAM	3622 (1352-7333)	9 (1-37)	62
Hiyoshi	4030 (1625-8451)	6 (1-20)	53

Abbreviations: CV = coefficient of variation; DMSO = dimethyl sulfoxide; ECVAM = European Centre for the Validation of Alternative Methods; N = number of plates that passed acceptance criteria; RLU = relative light unit; XDS = Xenobiotic Detection Systems, Inc.

Normalized and adjusted antagonist E2 control RLU values were used as acceptance criteria throughout the validation study. The mean, standard deviation (SD), and CV values calculated for the E2 control RLU value from all antagonist test plates that passed acceptance criteria are provided in **Table 3-7**. Mean E2 control RLU values ranged from 5793 at Hiyoshi to 9246 at ECVAM. Variability was low, with associated CV values ranging from 9% at ECVAM to 19% at XDS.

Table 3-7 Antagonist E2 Control Values

Laboratory	Mean RLU	SD	CV (%)	N
XDS	7524	1443	19	79
ECVAM	9246	805	9	62
Hiyoshi	5793	791	14	53

Abbreviations: CV = coefficient of variation; ECVAM = European Centre for the Validation of Alternative Methods; N = number of plates that passed acceptance criteria; RLU = relative light unit; SD = standard deviation; XDS = Xenobiotic Detection Systems, Inc.

Test substances are classified as positive or negative for agonist activity based on a specific set of criteria. The resulting classifications for each of the 12 substances that were tested at least three times at each laboratory were used to evaluate the extent of intralaboratory agreement (see **Table 3-8**). Although the classifications for some of the test substances differed among the laboratories, there was 100% agreement within each laboratory for each of the three repeat tests. There were no “inadequate” data generated at any laboratory during this phase of the validation study.

Table 3-8 Intralaboratory Agreement for Multiple Testing of the 12 Phase 2 Agonist Substances Tested Independently at Least Three Times at Each Laboratory

Activity per Test	XDS	ECVAM	Hiyoshi
Agreement within laboratory	12/12 (100%)	12/12 (100%)	12/12 (100%)
+++	8/12	12/12	9/12
−−−	4/12	0/12	3/12
Discordance within laboratory	0/12 (0%)	0/12 (0%)	0/12 (0%)
++−	0/12	0/12	0/12
+−−	0/12	0/12	0/12

Abbreviations: ECVAM = European Centre for the Validation of Alternative Methods; XDS = Xenobiotic Detection Systems, Inc.

+ denotes a positive test result.

- denotes a negative test result.

+++ indicates that each of three replicate tests within each laboratory had a classification as positive.

--- indicates that each of three replicate tests within each laboratory had a classification as negative.

++- indicates that in two of three replicate tests, a test substance was classified as positive. The substance was classified as negative in a third replicate test.

+-- indicates that in one of three replicate tests, the test substance was classified as positive. The substance was classified as negative in the remaining two tests.

3.6 Animal Welfare Considerations: Reduction, Refinement, and Replacement

The BG1Luc ER TA test method utilizes cultured human ovary adenocarcinoma cells that endogenously express human ER and contain an estrogen-inducible gene expression system. Except for the fetal bovine sera used as part of the cell culture media, the test method does not require the use of animals.

The BG1Luc ER TA test method is being proposed as an independent part of a weight-of-evidence approach to prioritize potentially endocrine-active substances for further testing. Therefore, like the EPA OPPTS 890.1300/OECD TG 455 method, the test does not directly reduce, refine, or replace animal use. However, regulators currently use the following three *in vivo* methods to assess the estrogenic potential of substances: (1) rat uterotrophic assay, (2) rat pubertal female assay, and (3) fish short-term reproduction assay. In addition, the "*in vitro*" rat uterine cytosol ER binding assay also requires the use of animals as a source of ER.

Results from the BG1Luc ER TA test method were examined for concordance with published reports of ER binding. There was 97% (33/34) concordance between the BG1Luc ER TA test method and ER binding data. In light of the excellent degree of agreement between ER binding and BG1Luc ER TA test method results (with no false negative results), it appears that evaluating results from BG1Luc ER TA agonist and antagonist testing may provide a viable alternative to conducting ER binding studies, which use animals as a source of ER. This cannot currently be accomplished with the only accepted ER TA method because of the inability of the EPA OPPTS 890.1300/OECD TG 455 method to assess ER antagonist activity.

Results from the BG1Luc ER TA test method were examined for concordance with published data from the uterotrophic assay. Based on a comparison with the *in vivo* uterotrophic assay classification, the 13 substances with data from the uterotrophic assay and conclusive test results in the BG1Luc ER TA agonist test method produced overall concordance of 92% (12/13). All

substances found positive in the uterotrophic assay were also positive in the BG1Luc ER TA test method. The only discordant substance, butylbenzyl phthalate, was positive for ER agonist activity in the BG1Luc ER TA agonist test method and negative in the uterotrophic assay. These data indicate that the BG1Luc ER TA agonist test method had very good agreement with the *in vivo* results obtained with the uterotrophic assay, with no false negative results.

The development of a battery of *in vitro* and *in silico* methods that can replace animal testing for detecting potential EDs is a biologically complex challenge. The experience derived from validating and using the *in vitro* BG1Luc ER TA test method is expected to contribute to our knowledge and promote progress toward this goal.

4.0 BG1Luc ER TA Test Method Performance Standards

Prior to the acceptance of a new test method for regulatory testing applications, validation studies are conducted to assess its reliability (i.e., the extent of intra- and interlaboratory reproducibility) and its relevance (i.e., the ability of the test method to correctly predict or measure the biological effect of interest) (ICCVAM 1997, 2003b; OECD 1996, 2005). The purpose of performance standards is to communicate the basis by which new proprietary and nonproprietary test methods have been determined to have sufficient accuracy and reliability for a specific testing purpose. These performance standards can then be used to evaluate the accuracy and reliability of other proposed test methods that are considered functionally and mechanistically similar to the accepted test method.

4.1 Elements of ICCVAM Performance Standards

Performance standards are based on an adequately validated test method and provide a basis for evaluating the comparability of a proposed test method that is functionally and mechanistically similar (ICCVAM 2003b). The three elements of performance standards are the following:

- Essential test method components: These consist of essential structural, functional, and procedural elements of a validated test method. They should be included in the protocol of a proposed test method that is functionally and mechanistically similar to the validated method. Essential test method components include unique characteristics of the test method, critical procedural details, and quality control measures.
- A minimum list of reference substances: Reference substances are used to assess the accuracy and reliability of a proposed functionally and mechanistically similar test method. These substances are a representative subset of those used to demonstrate the accuracy and reliability of the validated test method.
- Accuracy and reliability values: These are the standards for accuracy and reliability that the proposed test method should meet or exceed when evaluated using the minimum list of reference substances.

4.2 LUMI-CELL (BG1Luc ER TA) Test Method Performance Standards

4.2.1 Background

The BG1Luc ER TA test method uses an ER-responsive reporter gene (*luc*) in the human ovarian adenocarcinoma cell line BG-1 to detect substances with *in vitro* ER agonist or antagonist activity. The primary objective of this test method is to provide a qualitative assessment of *in vitro* estrogenic activity (i.e., whether a substance is positive or negative for estrogenic activity). Quantitative analysis is also performed to provide additional information on the estrogenic potency of test substances. For example, quantitative analysis can determine the half-maximal effective concentration (EC_{50}) or the half-maximal inhibitory concentration (IC_{50}). Separate protocols are used to identify substances that possess ER agonist or antagonist activity, although the two protocols share most major components (see **Appendices B1** and **B2**).

NICEATM coordinated and led an international validation study of the BG1Luc ER TA test method with ECVAM and JaCVAM. The study proceeded in four phases, during which 78 reference substances were tested (see **Appendix C**). Results from this validation study served as the basis for the BG1Luc ER TA test method performance standards, which are applicable for assessing the validity of methods that are functionally and mechanistically similar to the BG1Luc ER TA test method. These performance standards can also be used by naïve laboratories to demonstrate technical proficiency in performing the BG1Luc ER TA test method. The

performance standards consist of (1) essential test method components, (2) reference substances, and (3) an assessment of accuracy and reliability.

4.2.2 BG1Luc ER TA Essential Test Method Components and Other Validation Considerations

Certain principles are important in delineating the essential test method components that determine whether a modified test is functionally and mechanistically similar to the BG1Luc ER TA test method. *In vitro* ER TA assays are designed to identify substances that might interfere with estrogenic homeostasis *in vivo*. The interaction of estrogens with cellular ERs initiates a cascade of events. A number of *in vitro* endpoints can be used to assess ER–ligand interactions, including receptor binding, cellular proliferation, and transcriptional activation (reporter gene). Unlike receptor binding assays, TA assays can identify whether ligand–receptor association potentiates (agonist) or inhibits (antagonist) estrogenic signaling (Davenport and Russell 1996).

In the BG1Luc ER TA test method, ER-mediated transcription of the *luc* gene results in the production of luciferase, the activity of which is quantified using a luminometer. A concentration–response curve can be established to provide qualitative and quantitative information regarding the *in vitro* estrogenic activity of a test substance.

4.2.2.1 Essential Test Method Components

ICCVAM previously recommended minimum essential test method components for *in vitro* ER TA test method protocols (ICCVAM 2003a). These components were incorporated into the BG1Luc ER TA test method protocols during a protocol standardization study. During the protocol standardization study, protocols were developed for use in the international validation study (see **Appendices B1** and **B2**). During the multiphase validation study, the protocols were refined, ultimately resulting in optimized protocols for agonist and antagonist testing. In order to be considered functionally and mechanistically similar to the BG1Luc ER TA test method, a modified ER TA test method protocol must include the following components, which are based on the optimized test method protocols, to ensure that the same biological effect is being measured. If any of these criteria are not met, then these performance standards cannot be used for validation of the modified test method.

Cell Line

The BG1Luc ER TA test method is based on a human ovarian adenocarcinoma cell line that endogenously expresses ERα (90%) and ERβ (10%) (Pujol et al. 1998) and uses a stably transfected luciferase-based reporter gene system. Other cell lines that endogenously express human ERs and are stably transfected with a reporter gene system may be appropriate for validation using these performance standards.

Solvent

Reference standards, controls, and test substances should be dissolved in a solvent (e.g., 1% DMSO) that is miscible with cell culture media at concentrations that are not cytotoxic and that do not otherwise interfere with the test system.

Limit Concentration and Cytotoxicity

The maximum test substance concentration should be 1 mM for ER TA agonist testing and 10 µM for ER TA antagonist testing unless otherwise limited by solubility, cytotoxicity, or other mechanisms that interfere with assay performance. A minimum of seven concentrations spaced at logarithmic (log10) intervals, up to the limit concentration, should be tested. An evaluation of cytotoxicity and how it is applied to the test method should be included in each study. Any

concentration of test substance that reduces viability by greater than 20% should not be considered in the analysis of the data.

Reference Standards

A reference estrogen (e.g., 17β-estradiol [E2]) and a reference anti-estrogen (e.g., raloxifene HCl) should be used as reference standards to demonstrate the adequacy of the test method for detecting ER TA agonist and antagonist activity, respectively. The ability of the reference estrogen to induce ER TA activity and the reference anti-estrogen to inhibit ER TA activity should be demonstrated by generating a full concentration–response curve in each experiment. At a minimum, the E2 reference standard should provide a threefold induction relative to the solvent control. For antagonist testing, a minimum threefold reduction in the reference anti-estrogenic standard response (e.g., raloxifene HCl) should be demonstrated.

Controls

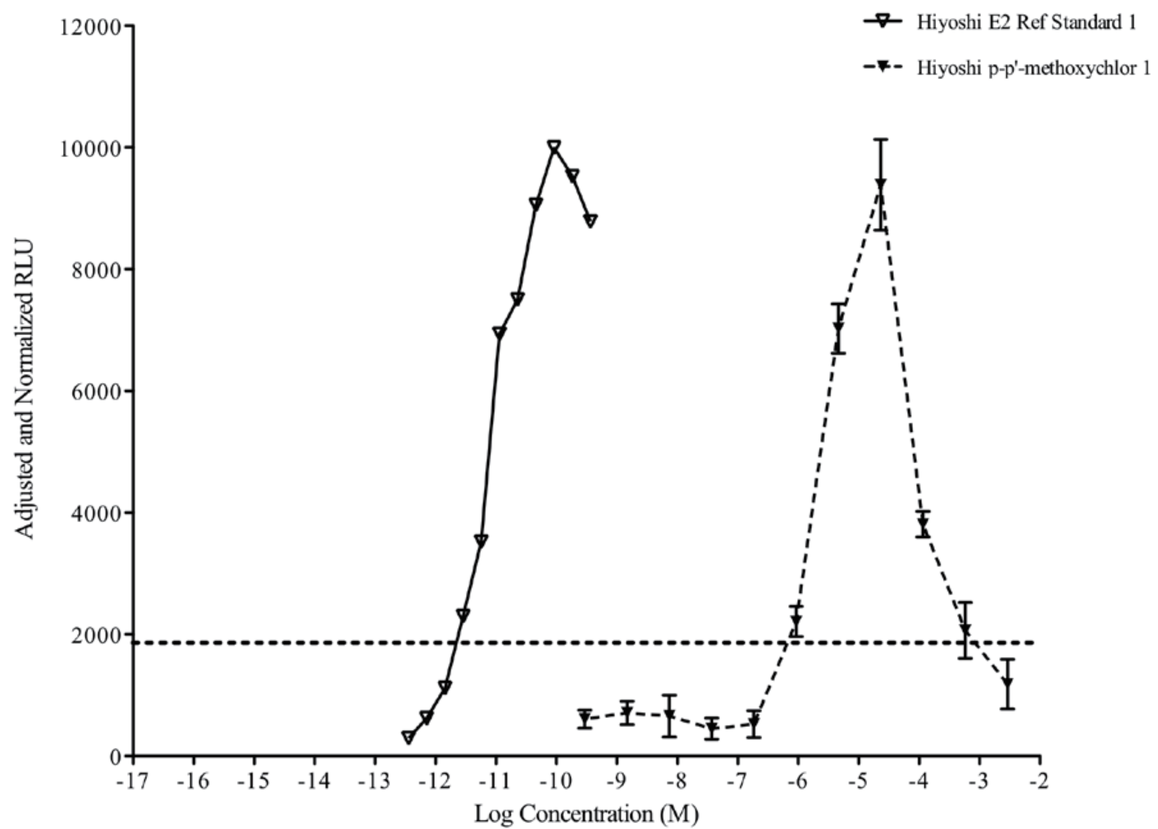
A set of concurrent controls (i.e., solvent, cell culture media) should be included in each experiment to provide a measure of ER TA activity in the absence of reference standards or test substances. A weak positive agonist control (e.g., *p.p'*-methoxychlor) with an EC_{50} five to six orders of magnitude higher than the reference estrogen should be included in each ER TA agonist study to demonstrate that the test method is functioning properly and is sufficiently sensitive to detect weak ER agonists. A weak positive antagonist control (e.g., tamoxifen) that demonstrates ER TA antagonist activity slightly below the 10 µM limit concentration should be included in each ER TA antagonist study to demonstrate that the test method is functioning properly and is sufficiently sensitive to detect weak ER antagonists. In addition, ER TA antagonist studies should include a concurrent control using the reference estrogen (e.g., E2) to establish a baseline level of induction (~80% of E2 maximum) against which antagonistic activity of test substances can be assessed.

Interpretation of Results

For ER TA agonist testing:

- All test substances classified as positive for ER TA agonist activity should have a concentration–response curve consisting of a baseline followed by a positive slope, concluding in a plateau or peak. In some cases, only two of these characteristics (baseline–slope or slope–peak) may be defined.
- The line defining the positive slope must contain at least three points with nonoverlapping error bars (mean ± SD). Points forming the baseline are excluded, but the linear portion of the curve may include the peak or first point of the plateau.
- A positive classification requires a response amplitude, the difference between baseline and peak, of at least 20% of the average maximal value of the reference estrogen, e.g., 2000 RLUs when the maximal response value of the reference estrogen is adjusted to 10,000 RLUs. (See **Figure 4-1** for an example of a concentration–response curve for a substance that is positive for ER TA agonist activity.)
- If possible, an EC_{50} value should be calculated for each positive substance.
- For all concentration–response curves that fail to meet the criteria for a positive response, test substances are classified as negative for agonist activity if all data points are below 20% of the maximal value for the reference estrogen, e.g., 2000 RLUs when the maximal response value of the reference estrogen is adjusted to 10,000 RLUs.

Figure 4-1 Example Concentration–Response Curve for an ER TA Agonist

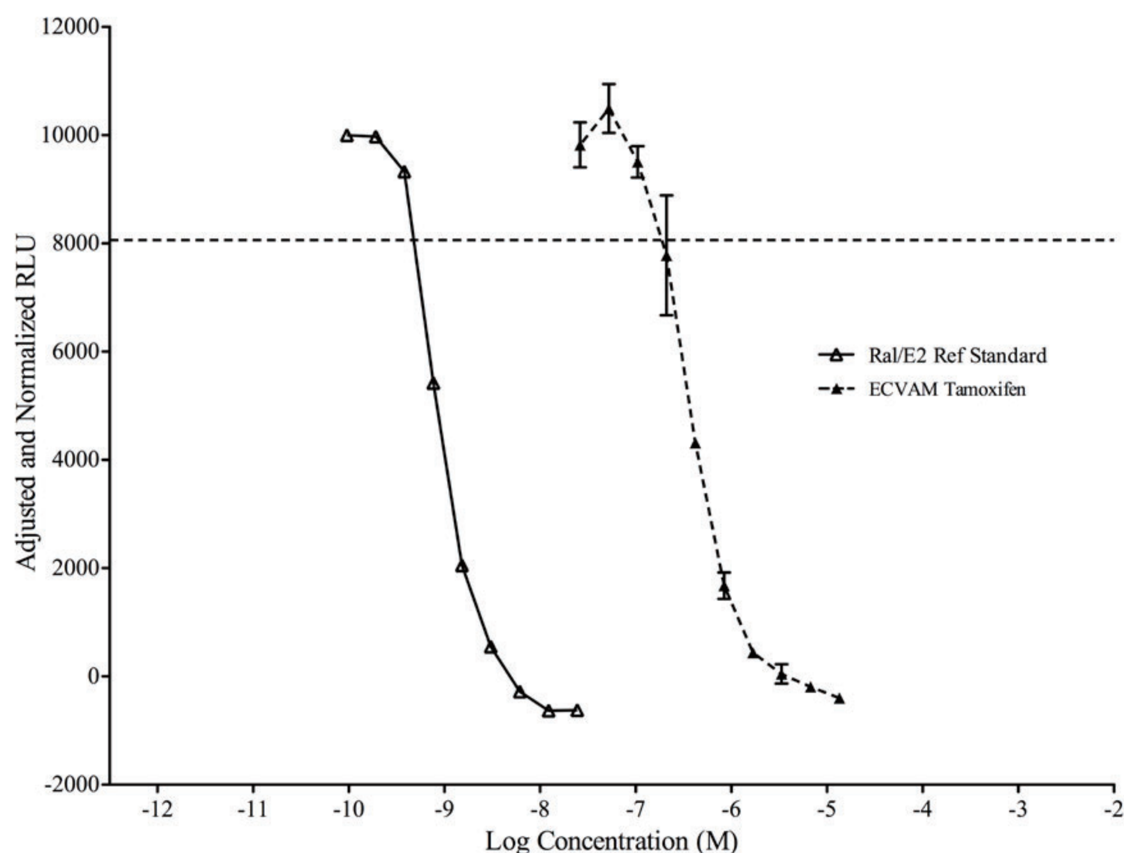


Abbreviations: E2 = 17β-estradiol; M = molar; RLU = relative light unit.
Horizontal dotted line represents 20% of the maximum response of the E2 reference standard.
Test substance shown is *p,p'*-methoxychlor.
E2 reference standard data is presented as the mean value of duplicate wells.
p,p'-Methoxychlor data are presented as the mean and SD values of three replicate wells.

For ER TA antagonist testing:

- All substances classified as positive for ER antagonist activity should have a concentration–response curve consisting of a baseline followed by a negative slope.
- The line defining the negative slope must contain at least three points with nonoverlapping error bars (representative of means ± SDs). Points forming the baseline are excluded, but the linear portion of the curve may include the first point of the plateau.
- A positive classification requires a response amplitude of less than 80% of the value for the reference estrogen. The response amplitude is defined as the difference between the baseline, established by the reference estrogen, and the bottom of the dose–response curve.
- The highest noncytotoxic concentrations of the test substance should be less than or equal to 10 µM. (See **Figure 4-2** for an example of a concentration–response curve for a substance that is positive for ER TA antagonist activity.)
- Test substances are classified as negative for ER antagonist activity if all data points are above 80% of the reference estrogen response, or 8000 RLUs.

Figure 4-2 Example Concentration–Response Curve for an ER TA Antagonist



Abbreviations: M = molar; RLU = relative light unit.
Horizontal dotted line represents 80% of the response of the 17β-estradiol reference estrogen.
Test substance shown is tamoxifen.
Ral/E2 reference standard data are presented as the mean value of duplicate wells.
Tamoxifen data are presented as the mean and SD values of three replicate wells.

Data and Reporting

The validation report should include the following information:

—Reporter Plasmid (if different than that used in BG1Luc ER TA test method)

- Type and structure of ER response elements
- Description of promoter region
- Name, identification, and source of original plasmid used to make construct
- Description and methodology used to make the transfected plasmid
- Nomenclature and genetic components comprising the reporter construct

—Cell Line

- Source and nomenclature of the cell line and protocol for its maintenance before and after transfection
- Source of cell culture media, materials, and supplies
- Passage number of subcultures used in the study
- Methods for maintaining stably transfected cell line

- Methods used to monitor the stability of the cell line used for testing
- Rationale, based on data, for deciding on the number of passages a cell line can undergo without a decrease in activity
- Details regarding selection requirements needed to maintain stable cell lines
- If known, details regarding the relative amounts of ERα and ERβ

—Test Method Conditions

- Composition of media and reagents used
- Incubation volume, duration, and temperature
- Method used to measure ER TA activity
- Methods used to evaluate data, determine response, and calculate EC_{50} or IC_{50} values

—Reference Standards, Controls, and Test Substances

- Name, chemical structure, CAS Registry Number (CASRN), purity, and supplier
- Physicochemical properties relevant to the study (e.g., solubility, pH, stability, volatility)
- Concentrations and volumes used

—Solvent

- Name, CASRN, purity, and supplier
- Justification for choice of solvent
- Information on the solubility of test substances in solvent used
- Information to demonstrate that the solvent, at the maximum volume used, is not cytotoxic and does not otherwise interfere with the study

—Criteria for an Acceptable Test

- Concurrent reference standard and control data
- Laboratory-specific historical ranges of reference standard and control data
- Definition of exclusion criteria and description of the impact of any excluded data

—Results

- Reference standard and control results
- Test substance solubility results
- Test substance cell viability results
- Calculated reference standard and test substance EC_{50} and IC_{50} values
- Graphically presented reference standard, control, and test substance results

—Discussion of Results

- Impact of solubility and cytotoxicity on test results
- Reproducibility of reference standard and control data

—Conclusion

- Classification of test substances with regard to *in vitro* ER TA agonist or antagonist activity

Other Validation Considerations

The following additional points should be considered during the validation of test methods that are functionally and mechanistically similar to the BG1Luc ER TA test method:

- Appropriate quality assurance systems (i.e., in accordance with Good Laboratory Practice guidelines (EPA 2006b, 2006a; FDA 2009; OECD 1998) are required.

- The study should be conducted according to U.S. (ICCVAM 1997) and international validation principles (OECD Guidance Document 34) (OECD 2005).

4.2.3 Reference Substances for *In Vitro* ER TA Test Methods

To ensure that a proposed *in vitro* ER TA test method possesses reliability and accuracy characteristics similar to those of the validated test method (in this case the BG1Luc ER TA test method), the proposed test method should use at least the agonist reference substances listed in **Table 4-1** and the antagonist reference substances listed in **Table 4-2**. All substances should be tested in a coded/blinded manner. When evaluated using these reference substances, the accuracy (i.e., sensitivity, specificity, false positive rates, and false negative rates) and reliability of the proposed ER TA test method should approximate those of the validated ER TA test method, as detailed in **Section 4.2.4**. Although it is not realistic to expect test methods to perform identically, discordant results should be addressed in terms of the test method's ability to accurately classify other substances with similar potencies and from the same chemical/product classes.

4.2.3.1 Criteria for Selection of Reference Substances

ICCVAM previously compiled and recommended a list of 78 substances for use in validation studies for *in vitro* ER and AR binding and TA test methods (ICCVAM 2003a, 2006). These substances were selected based on information contained in the ICCVAM BRDs for AR and ER binding and TA test methods (ICCVAM 2002d, 2002a, 2002c, 2002b), as well as information obtained from publications reviewed or published after completion of the ICCVAM BRDs. Factors and criteria considered necessary for selecting reference substances included:

- A well-defined chemical structure
- Comparatively low systemic toxicity
- Good availability from commercial sources
- A concentration–response range that could be measured or predicted by the test method
- Minimal disposal cost

Because the BG1Luc ER TA test method is used only to detect substances with *in vitro* ER TA agonist or antagonist activity, the following criteria were used to classify each reference substance with respect to ER TA agonist and antagonist activity:

- A substance was classified as POS if it was reported as positive in >50% of referenced ER TA studies.
- A substance was classified as NEG if it was reported as negative in all referenced ER TA studies (at least two studies were required for negative classification).
- A substance was classified as PP (presumed positive) if it was positive in 50% or fewer referenced ER TA studies, or if it was reported positive in the single study conducted.
- A substance was classified as PN (presumed negative) if it was reported negative in a single ER TA study.
- Substances without data were classified as PP or PN based on other available information, including their known mechanism of action or their responses in other ER assays.

Only those substances that could be definitively classified as POS or NEG were used to assess accuracy (substances classified as PP or PN were not considered when evaluating test method accuracy). Accordingly, this subset of substances was used to select the final list of reference substances listed in **Tables 4-1** and **4-2**. Recognizing that the number of available reference substances that are definitively negative for agonist activity (**Table 4-1**) or definitively positive for antagonist activity (**Table 4-2**) is limited, these lists may be updated as additional substances with these characteristics are identified. Accordingly, users should be aware that the reference substance list could be revised based on any additional studies that are conducted in the future.

ICCVAM recommends that users consult the NICEATM–ICCVAM website (http://iccvam.niehs.nih.gov/) to ensure use of the most current reference substance list.

Table 4-1 34 Reference Substances for Evaluation of ER Agonist Accuracy

Substance[a]	CASRN	ICCVAM Consensus	BG1Luc ER TA Consensus[b]	BG1Luc ER TA Mean EC_{50} (M)[c]	MeSH Chemical Class[d]	Product Class[e]
Ethyl paraben	120-47-8	POS	POS	2.48×10^{-5}	Carboxylic Acid, Phenol	Pharmaceutical, Preservative
Fenarimol	60168-88-9	POS	POS	4.59×10^{-6}	Heterocyclic Compound, Pyrimidine	Fungicide
Kaempferol	520-18-3	POS	POS	3.99×10^{-6}	Flavonoid, Heterocyclic Compound	Natural Product
Methyl testosterone	58-18-4	POS	POS	3.29×10^{-6}	Steroid	Pharmaceutical, Veterinary Agent
Chrysin	480-40-0	POS	POS	3.20×10^{-6}	Flavonoid, Heterocyclic Compound	Natural Product
p-n-Nonylphenol	104-40-5	POS	POS	3.06×10^{-6}	Phenol	Chemical Intermediate
Dicofol	115-32-2	POS	POS	2.22×10^{-6}	Hydrocarbon (Cyclic), Hydrocarbon (Halogenated)	Pesticide
Butylbenzyl phthalate	85-68-7	POS	POS	1.98×10^{-6}	Carboxylic Acid, Ester, Phthalic Acid	Plasticizer, Industrial Chemical
p,p'-Methoxychlor	72-43-5	POS	POS	1.92×10^{-6}	Hydrocarbon (Halogenated)	Pesticide, Veterinary Agent
Apigenin	520-36-5	POS	POS	1.85×10^{-6}	Heterocyclic Compound	Dye, Natural Product, Pharmaceutical Intermediate
19-Nortestosterone	434-22-0	POS	POS	1.80×10^{-6}	Steroid	Pharmaceutical, Veterinary Agent
Daidzein	486-66-8	POS	POS	8.71×10^{-7}	Flavonoid, Heterocyclic Compound	Natural Product
Bisphenol A	80-05-7	POS	POS	5.33×10^{-7}	Phenol	Chemical Intermediate, Flame Retardant, Fungicide
Kepone	143-50-0	POS	POS	4.91×10^{-7}	Hydrocarbon (Halogenated)	Pesticide
o,p'-DDT	789-02-6	POS	POS	3.94×10^{-7}	Hydrocarbon (Halogenated)	Pesticide
4-Cumylphenol	599-64-4	POS	POS	3.20×10^{-7}	Phenol	Chemical Intermediate
Genistein	446-72-0	POS	POS	2.71×10^{-7}	Flavonoid, Heterocyclic Compound	Natural Product, Pharmaceutical

Substance[a]	CASRN	ICCVAM Consensus	BG1Luc ER TA Consensus[b]	BG1Luc ER TA Mean EC_{50} (M)[c]	MeSH Chemical Class[d]	Product Class[e]
Bisphenol B	77-40-7	POS	POS	1.67×10^{-7}	Phenol	Chemical Intermediate, Flame Retardant, Fungicide
Coumestrol	479-13-0	POS	POS	8.77×10^{-8}	Heterocyclic Compound	Natural Product
4-*tert*-Octylphenol	140-66-9	POS	POS	3.19×10^{-8}	Phenol	Chemical Intermediate, Pharmaceutical Intermediate
17∝-Estradiol	57-91-0	POS	POS	1.54×10^{-9}	Steroid	Pharmaceutical, Veterinary Agent
Norethynodrel	68-23-5	POS	POS	9.39×10^{-10}	Steroid	Pharmaceutical
Estrone	53-16-7	POS	POS	2.57×10^{-10}	Steroid	Pharmaceutical, Veterinary Agent
Diethylstilbestrol	56-53-1	POS	POS	3.34×10^{-11}	Hydrocarbon (Cyclic)	Pharmaceutical, Veterinary Agent
meso-Hexestrol	84-16-2	POS	POS	1.65×10^{-11}	Steroid	Pharmaceutical, Veterinary Agent
17ß-Estradiol	50-28-2	POS	POS	8.37×10^{-12}	Steroid	Pharmaceutical, Veterinary Agent
17∝-Ethinyl estradiol	57-63-6	POS	POS	7.31×10^{-12}	Steroid	Pharmaceutical, Veterinary Agent
Atrazine	1912-24-9	NEG	NEG	-	Heterocyclic Compound	Herbicide
Bicalutamide	90357-06-5	NEG	NEG	-	Amide	Pharmaceutical
Corticosterone	50-22-6	NEG	NEG	-	Steroid	Pharmaceutical
Hydroxyflutamide	52806-53-8	NEG	NEG	-	Amide	Pharmaceutical
Linuron	330-55-2	NEG	NEG	-	Urea	Herbicide
Phenobarbital	50-06-6	NEG	NEG	-	Heterocyclic Compound, Pyrimidine	Pharmaceutical, Veterinary Agent
Spironolactone	52-01-7	NEG	NEG	-	Lactone, Steroid	Pharmaceutical

Abbreviations: BG1Luc ER TA = LUMI-CELL BG1Luc4E2 ER TA test method; CASRN = CAS Registry Number (American Chemical Society); EC_{50} = half-maximal effective concentration of a test substance; ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; M = molar; MeSH = Medical Subject Headings (U.S. National Library of Medicine); NEG = negative; POS = positive.

[a] Substances are listed in order based upon EC_{50} values.

[b] BG1Luc ER TA consensus classification represents the majority classification among the three validation laboratories.

[c] Mean EC_{50} values were calculated with values reported by the laboratories of the BG1Luc ER TA validation study (XDS, ECVAM, and Hiyoshi).

[d] Substances were assigned to one or more chemical or product classes using the U.S. National Library of Medicine's Medical Subject Headings (MeSH), an internationally recognized standardized classification scheme (available at http://www.nlm.nih.gov/mesh).

[e] Substances were assigned to one or more product classes using the U.S. National Library of Medicine's Hazardous Substances Data Bank (available at http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB).

Table 4-2 10 Reference Substances for Evaluation of ER Antagonist Accuracy

Substance[a]	CASRN	ICCVAM Consensus[b]	BG1Luc ER TA Consensus	BG1Luc ER TA Mean IC_{50} (M)[c]	MeSH Chemical Class[d]	Product Class[d]
Tamoxifen	10540-29-1	POS	POS	8.17×10^{-7}	Hydrocarbon (Cyclic)	Pharmaceutical
4-Hydroxytamoxifen	68047-06-3	POS	POS	2.08×10^{-7}	Hydrocarbon (Cyclic)	Pharmaceutical
Raloxifene HCl	82640-04-8	POS	POS	1.19×10^{-9}	Hydrocarbon (Cyclic)	Pharmaceutical
17∝-Ethinyl estradiol	57-63-6	NEG	NEG	-	Steroid	Pharmaceutical, Veterinary Agent
Apigenin	520-36-5	NEG	NEG	-	Heterocyclic Compound	Dye, Natural Product, Pharmaceutical Intermediate
Chrysin	480-40-0	NEG	NEG	-	Flavonoid, Heterocyclic Compound	Natural Product
Coumestrol	479-13-0	NEG	NEG	-	Heterocyclic Compound	Natural Product
Genistein	446-72-0	NEG	NEG	-	Flavonoid, Heterocyclic Compound	Natural Product, Pharmaceutical
Kaempferol	520-18-3	NEG	NEG	-	Flavonoid, Heterocyclic Compound	Natural Product
Resveratrol	501-36-0	NEG	NEG	-	Hydrocarbon (Cyclic)	Natural Product

Abbreviations: BG1Luc ER TA = LUMI-CELL BG1Luc4E2 ER TA test method; CASRN = CAS Registry Number (American Chemical Society); IC_{50} = half-maximal inhibitory concentration; ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; M = molar; MeSH = Medical Subject Headings (U.S. National Library of Medicine); NEG = negative; POS = positive.

[a] Substances are listed in order based upon IC_{50} values.

[b] BG1Luc ER TA consensus classification represents the majority classification among the three validation laboratories.

[c] Mean IC_{50} values were calculated with values reported by the laboratories of the BG1Luc ER TA validation study (XDS, ECVAM, and Hiyoshi).

[d] Substances were assigned to one or more chemical classes using the U.S. National Library of Medicine's Medical Subject Headings (MeSH), an internationally recognized standardized classification scheme (available at http://www.nlm.nih.gov/mesh).

[e] Substances were assigned to one or more product classes using the U.S. National Library of Medicine's Hazardous Substances Data Bank (available at http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB).

4.2.3.2 Characteristics of Selected Reference Substances

The reference substances include a range of chemical and product classes representative of the classes commonly associated with endocrine disruption.

Agonist and antagonist test method intralaboratory reproducibility was evaluated using nine substances and four substances, respectively, that were each tested three times on three separate days at each laboratory. Agonist and antagonist test method interlaboratory reproducibility was

evaluated using 27 and 8 substances, respectively, that were tested at least once in each laboratory during the validation study.

4.2.4 Accuracy and Reliability Performance Values

The final elements of performance standards are the accuracy and reliability values (i.e., test method performance) that should be met or exceeded by the proposed test method when evaluated with the reference substances. *Accuracy* is defined as the closeness of agreement between a test method result and an accepted reference value. *Reliability* is the degree to which a test method can be performed reproducibly within and among laboratories over time (ICCVAM 2003b). For these performance standards, the proposed test method should have accuracy and reliability characteristics that approximate those of the validated ER TA test method, which are detailed below. Although it is not realistic to expect test methods to perform identically, discordant results should be addressed in terms of the test method's ability to accurately classify other substances with similar potencies and from the same chemical/product classes.

4.2.4.1 Test Method Accuracy

The analysis of agonist activity for the 34 substances in **Table 4-1** indicated an overall accuracy of 100% (34/34), sensitivity of 100% (27/27), specificity of 100% (7/7), false positive rate of 0% (0/7), and false negative rate of 0% (0/27).

The analysis of antagonist activity for the 10 substances in **Table 4-2** indicated an overall accuracy of 100% (10/10), sensitivity of 100% (3/3), specificity of 100% (7/7), false positive rate of 0% (0/7), and false negative rate of 0% (0/3).

4.2.4.2 Test Method Reliability

For the BG1Luc ER TA agonist test method, there was 100% agreement within each laboratory for each of the three repeat tests for nine reference substances tested in Phase 2 of the agonist validation study. When results were compared across laboratories for these nine substances, there was 78% (7/9) agreement among the three laboratories for the substances. An additional 17 substances tested once in each laboratory for agonist activity produced a definitive result in at least two laboratories. There was agreement among the laboratories for 82% (14/17) of these substances.

For the BG1Luc ER TA antagonist test method, there was 100% agreement within each laboratory for each of the three repeat tests for four reference substances tested in Phase 2 of the antagonist validation study. When results were compared across laboratories for these four substances, there was 100% agreement among the three laboratories for all four substances. An additional five substances tested once in each laboratory for antagonist activity produced a definitive result in at least two laboratories. There was agreement among the laboratories for 80% (4/5) of these substances.

5.0 ICCVAM Consideration of Public Comments

The ICCVAM evaluation process incorporates a high level of scientific peer review and transparency. The evaluation process on the use of the BG1Luc ER TA test method as a screening method to identify *in vitro* ER agonists and antagonists included one public review meeting by an independent scientific peer review panel, multiple opportunities for public comments, and comments from SACATM. ICCVAM and the EDWG considered the Panel report, SACATM comments, and all public comments before finalizing the ICCVAM test method evaluation report and BRD for the use of the BG1Luc ER TA test method. This section summarizes the ICCVAM consideration of public comments (see **Appendix E**).

5.1 ICCVAM Consideration of Public and SACATM Comments

Six opportunities for public comment were provided during the ICCVAM evaluation of the BG1Luc ER TA test method (**Table 5-1**). A total of nine comments were submitted. *Federal Register* notices published by NICEATM–ICCVAM during evaluation of the BG1Luc ER TA test method are available in **Appendix E** and from the NICEATM–ICCVAM website (http://iccvam.niehs.nih.gov/). Comments received in response to or related to the *Federal Register* notices are available on the NICEATM–ICCVAM website.[4] The following sections, delineated by *Federal Register* notice and public meeting, briefly discuss the public comments received.

Table 5-1 Opportunities for Public Comments

Opportunity for Public Comment	Date	Number of Public Comments Received
69 FR 21564 - *In Vitro* Endocrine Disruptor Test Methods: Request for Comments and Nominations	April 21, 2004	0
71 FR 13597 - Notice of Availability of a Revised List of Recommended Reference Substances for Validation of *In Vitro* Estrogen and Androgen Receptor Binding and Transcriptional Activation Assays: Request for Comments and Submission of *In Vivo* and *In Vitro* Data	March 16, 2006	0
74 FR 62317 - Evaluation of *In Vitro* Estrogen Receptor Transcriptional Activation and *In Vitro* Cell Proliferation Assays for Endocrine Disruptor Chemical Screening: Request for Nominations for an Independent Expert Peer Review Panel and Submission of Relevant *In Vitro* and *In Vivo* Data	November 27, 2009	6
76 FR 4113 - Announcement of an Independent Scientific Peer Review Panel Meeting on an *In Vitro* Estrogen Receptor Transcriptional Activation Test Method for Endocrine Disruptor Chemical Screening; Availability of Draft Background Review Document (BRD); Request for Comments	January 24, 2011	1
76 FR 23323 - Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)	April 26, 2011	2
76 FR 28781 - Independent Scientific Peer Review Panel Report: Evaluation of the Validation Status of an *In Vitro* Estrogen Receptor Transcriptional Activation Test Method for Endocrine Disruptor Chemical Screening: Notice of Availability and Request for Public Comments	May 18, 2011	0

[4] http://ntp-apps.niehs.nih.gov/iccvampb/searchPubCom.cfm

5.1.1 Public Comments in Response to 69 FR 21564 (April 21, 2004)

***In Vitro* Endocrine Disruptor Test Methods: Request for Comments and Nominations**
NICEATM requested nomination of ER and AR binding and TA test methods for validation studies.

No public comments were received in response to this *Federal Register* notice.

5.1.2 Public Comments in Response to 71 FR 13597 (March 16, 2006)

Notice of Availability of a Revised List of Recommended Reference Substances for Validation of *In Vitro* Estrogen and Androgen Receptor Binding and Transcriptional Activation Assays: Request for Comments and Submission of *In Vivo* and *In Vitro* Data
NICEATM announced the availability of an addendum (ICCVAM 2006) to the *ICCVAM Evaluation of In Vitro Test Methods for Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays* (ICCVAM 2003a). The addendum describes the rationale for proposed revisions to the original list of recommended reference substances for validation of *in vitro* ER and AR binding and TA assays. NICEATM requested public comments on the substances proposed as substitutes for six of the 78 substances in the original list. Data were also requested from *in vitro* and *in vivo* studies evaluating the estrogenic and androgenic activity of the 78 substances in the revised list of reference substances.

No public comments were received in response to this *Federal Register* notice.

5.1.3 Public Comments in Response to 74 FR 62317 (November 27, 2009)

Evaluation of *In Vitro* Estrogen Receptor Transcriptional Activation and *In Vitro* Cell Proliferation Assays for Endocrine Disruptor Chemical Screening: Request for Nominations for an Independent Expert Peer Review Panel and Submission of Relevant *In Vitro* and *In Vivo* Data
NICEATM requested:

- Nominations of expert scientists for consideration as potential Panel members
- Submission of existing data from the LUMI–CELL ER and the CertiChem MCF–7 cell proliferation assays
- Submission of data from *in vivo* or other *in vitro* assessments for the 78 reference substances recommended by ICCVAM for the validation of *in vitro* ER and AR binding and TA test methods

NICEATM received six public comments in which nine potential panelists were nominated for consideration. The nominees were included in the database of experts from which the Panel was selected.

5.1.4 Public Comments in Response to 76 FR 4113 (January 24, 2011)

Announcement of an Independent Scientific Peer Review Panel Meeting on an *In Vitro* Estrogen Receptor Transcriptional Activation Test Method for Endocrine Disruptor Chemical Screening; Availability of Draft Background Review Document (BRD); Request for Comments
NICEATM invited public comments on the draft BRD and draft ICCVAM test method recommendations. One public comment was received that included a number of suggestions.

The commenter proposed assigning a level of confidence ranking to the reference data. Substances for which there is a low degree of confidence in the reference data should be deleted

from the reference list and omitted from validation studies. With regard to specific test substances, the commenter stated that the discordant results among laboratories for atrazine, corticosterone, and dicofol were not fully explained.

ICCVAM Response
The independent scientific peer review panel concluded that it is reasonable to use the majority classification criteria among published study results (i.e., >50%) to establish the consensus reference classification for each reference substance. The Panel suggested that this approach could be improved by a ranking method, such as Klimisch criteria (Klimisch et al. 1997), that focuses primarily on the reliability of the data. Such a method would clarify the relative quality of the reference data and strengthen the classification. ICCVAM concurred that additional review and ranking of the published reports would strengthen the utility of literature citations for classifying the reference substances and agreed to take this into consideration in future evaluations.

The commenter questioned the use of flavone as the weak positive control in the antagonist protocol. The commenter further stated that differences among the laboratories in range finder starting concentrations were not fully explained.

ICCVAM Response
During protocol standardization, a number of substances were evaluated for use as the weak antagonist control. Flavone produced a dose response and an $IC_{50} = 4.3 \times 10^{-7}$ M, which was consistent with the single literature reference for this compound (reported $IC_{50} = \sim 15$ μM) and was two times below that of raloxifene. Based on these results, flavone was chosen as the weak antagonist control for the validation study. However, after review of the data from the completed study, it was apparent that the vast majority of test substances classified as "negative" or "presumed negative" produced a "positive" response at concentrations above ~10 μM. Use of flavone as a weak antagonist control was therefore reconsidered.

The commenter suggested including quantitative comparison of test substances (such as EC_{50} values) and indicated that it would be helpful to include data presented as a relative potency index (the EC_{50} of the positive control divided by the EC_{50} of the test substances, multiplied by 100).

ICCVAM Response
Quantitative measures of activity (i.e., EC_{50} and IC_{50} values) were generated and presented in the BRD. The independent scientific peer review panel considered the descriptive approach for evaluating test method reliability acceptable but also suggested additional statistical analyses that could be performed to better characterize and clarify variability. The Panel suggested that a quantitative measure of activity should be included in each future study report, and the uncertainty associated with these estimates should also be reported.

5.1.5 Public Comments in Response to 76 FR 23323 (April 26, 2011)

Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)
NICEATM announced the SACATM meeting and requested written and public oral comments on the agenda topics. Two public comments were received.

One commenter supported the validation of the BG1Luc ER TA test method and recommended modifications of the protocol that would allow for the implementation of a liquid handling system. The commenter felt that the use of a liquid handling system would greatly increase sample throughput.

ICCVAM Response
The use of a liquid handling system represents a potential improvement to the protocol that could

increase throughput. Use of a liquid handling system at the lead laboratory was considered during the initial phases of the validation study. However, because of difficulties experienced with the system that was acquired at the outset of the study, a decision was made to focus on the "benchtop" version of the assay and perhaps reconsider incorporating automated procedures into the assay at a later time.

A second commenter also supported the validation of the BG1Luc ER TA test method and recommended improvements. The commenter recommended a quantitative comparison of the BG1Luc ER TA data to EPA OPPTS 890.1300/OECD TG 455 data and development of a relative potency index for the reference substances.

ICCVAM Response
As stated above, ICCVAM concurred that additional review and ranking of the published reports would strengthen the utility of literature citations for classifying the reference substances. A comparison of median EC_{50} and IC_{50} values from the BG1Luc ER TA test method and literature references is provided in the BRD (**Appendix C**). A relative potency index for the reference substances has not been calculated; however, data provided in the current review permit calculation of such an index.

5.1.6 Public Comments in Response to 76 FR 28781 (May 18, 2011)

Independent Scientific Peer Review Panel Report: Evaluation of the Validation Status of an *In Vitro* Estrogen Receptor Transcriptional Activation Test Method for Endocrine Disruptor Chemical Screening: Notice of Availability and Request for Public Comments
NICEATM requested submission of written public comments on the *Peer Review Panel Report: Evaluation of the LUMI-CELL ER® (BG1Luc ER TA) Test Method* (**Appendix D2**). No comments were received in response to this request.

6.0 References

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