Results of a Protocol Standardization Study for the LUMI-CELL® Estrogen Receptor (ER) Transcriptional Activation (TA) Bioassay

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Abstract

In 2003, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) concluded that none of the ER TA assays considered for inclusion in the EPA Endocrine Disruptor Screening Programs Tier 1 battery had been adequately validated. NICEATM subsequently conducted a study to standardize protocols to be used in formal validation studies for the XDS LUMI-CELL® ER TA (LUMI-CELL® ER) bioassay, a proposed Tier 1 in vitro screening assay for the detection of ER agonists and antagonists. The objective of the study was to develop standardized protocols that can be easily transferred to other laboratories and be used to obtain reproducible results for the detection of ER agonists and antagonists. The study included optimization of the use of estrogen reference standards and controls, and compared quantitative and qualitative methods for evaluating cytotoxicity. The intralaboratory reproducibility and accuracy of the standardized protocols was demonstrated using eight coded substances covering a range of ER agonist activities, including negatives, and eight coded substances covering a range of ER antagonist activities, including negatives. The test substances used to evaluate ER agonist activity were atrazine (ATZ), bisphenol A (BPA), bisphenol B (BPB), corticosterone (COR), o,p'-DDT (DDT), diethylstilbestrol (DES), 17α-ethinyl estradiol (EE), and flavone (FLA); the test substances used to evaluate ER antagonist activity were butylbenzyl phthalate (BBP), dibenzo[a,h]anthracene (DBA), FLA, genistein (GEN), nonylphenol (NON), progesterone (PRO), DDT, and tamoxifen (TAM). EE, DES, BPA, BPB, DDT, and FLA are estrogenic agonists, while ATZ and COR are negative for ER agonist activity. TAM, DBA, FLA, and GEN are estrogenic antagonists, while BBP, PRO, NON, and DDT are negative for ER antagonist activity. This study demonstrated the intralaboratory reproducibility and accuracy of this test method and indicated its readiness for a multi-laboratory validation study. Supported by NIEHS Contract N01-ES-85424.

Introduction

In 2000, the U.S. Environmental Protection Agency (EPA) asked ICCVAM to evaluate the validation status of in vitro ER and androgen receptor (AR) test methods, which were proposed as components of the EPA Endocrine Disruptor Screening Program (EDSP). NICEATM subsequently prepared comprehensive background review documents (BRDs) to present validation status of in vitro estrogen receptor (ER) and androgen receptor (AR) binding and transcriptional activation (TA) assays that could be used in the EDSP to screen for potential endocrine disruptors.

An independent expert panel review of the compiled information concluded that there were no adequately validated in vitro ER- or AR-based test methods and recommended that methods that do not require the use of animals as receptor sources should be a priority for further development and validation, and that human recombinant receptors should be evaluated. Based on the expert panel's conclusions and recommendations, along with comments from the public, ICCVAM developed test method recommendations that included minimum procedural standards and a list of reference substances that should be used to standardize and validate in vitro ER and AR test methods and issued an FR notice (FR Vol. 68, No. 106, pp. 33171-33172, June 3, 2003) announcing the availability of a report defining these recommendations entitled, "ICCVAM Evaluation of In Vitro Test Methods for Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays," (ICCVAM 2003). The FR notice also invited the nomination for validation studies of in vitro ER and AR test methods that meet the published recommendations and for which there are standardized test method protocols, prevalidation study data, or proposed validation study designs. It is anticipated that data obtained during the validation of in vitro ER- and AR-based test methods will help characterize the extent to which individual in vitro endocrine disruptor test methods (or test method batteries) might be used to further reduce the expected requirements for animal use in the screening of potential endocrine disruptors.

In response to the FR notice, the LUMI-CELL® ER TA bioassay developed by Xenobiotic Detection Systems, Inc.[1] was nominated to ICCVAM for validation studies. In accordance with the ICCVAM nomination process, NICEATM conducted a pre-screen evaluation of a BRD containing the historical development and rationale for the assay, assay protocols, and supporting materials to determine the extent that the proposed nomination addressed ICCVAM prioritization criteria, submission guidelines, and recommendations for the standardization and validation of in vitro endocrine disruptor test methods. ICCVAM sought public comments and comments from the Scientific Advisory Committee for Alternative Test Methods (SACATM) on the nomination (FR Vol. 69, No. 77, pp. 21564, April, 2004), which supported the proposed validation study as a high priority. Following consideration of SACATM and public comments, ICCVAM recommended that the nomination for validation studies should have a high priority, and that following completion of the validation study, performance standards should be developed that can be used to assess other functionally and structurally similar ED TA methods.

NICEATM will be conducting an interlaboratory validation study on this ER TA test method in conjunction with the ECVAM and the JaCVAM (see 2007 SOT Poster 1166-440).

In preparation for the validation study, NICEATM conducted a protocol standardization study for the LUMI-CELL® ER bioassay. Protocol standardization procedures were based on ICCVAM minimum procedural standards (ICCVAM 2003).

Specific goals of the study were to:

- Standardize procedures for using the LUMI-CELL® ER bioassay to identify ER agonists and antagonists
- Standardize procedures for a quantitative test of cell viability for use with the LUMI-CELL® ER agonist and antagonist bioassays
- Develop two GLP-compliant protocols: one for identifying substances with ER agonist activity, and one for identifying substances with ER antagonist activity
- Develop a historical database for reference standards and controls for LUMI-CELL® ER agonist and antagonist bioassays
- Demonstrate the adequacy of the standardized protocols for detecting agonists or antagonists using eight substances covering a range of ER agonist and antagonist activities, respectively

[1]The development of the assay was supported by a Small Business Innovation Research grant from the U.S. National Institutes of Environmental Health Sciences (Grant # 5R44ES010533-03)

Overview of the LUMI-CELL® ER Bioassay

The LUMI-CELL® ER bioassay measures whether and to what extent a substance induces or blocks TA activity via an ER-mediated pathway in recombinant BG-1Luc4E2 cells (Dennison et al. 1998). The BG-1Luc4E2 cell line was derived from BG-1 immortalized human adenocarcinoma cells that have been stably transfected with the plasmid pGudLuc7.ERE. This plasmid contains four copies of a synthetic oligonucleotide containing the estrogen response element upstream of the mouse mammary tumor viral (MMTV) promoter and the firefly luciferase gene (Figure 1). BG1 adenocarcinoma cells that endogenously express ER were transfected with the reporter gene construct and stable transfectants were selected by growth in minimal essential medium (MEM) containing gentamycin (G418) (Rogers and Denison 2000). The resultant cell line expresses luciferase activity in response to estrogen and estrogen-like substances.
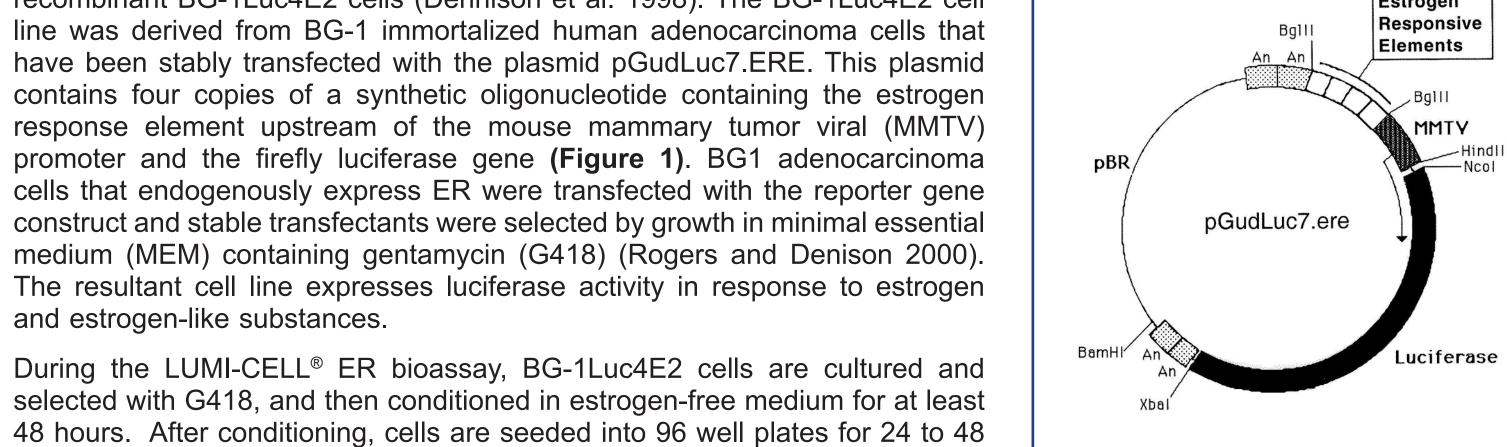
During the LUMI-CELL® ER bioassay, BG-1Luc4E2 cells are cultured and selected with G418, and then conditioned in estrogen-free medium for at least 48 hours. After conditioning, cells are seeded into 96 well plates for 24 to 48 hours and then incubated in estrogen-free medium containing solvent and/or reference standard, control, or test substance for 19 to 24 hr. Cytotoxicity is then evaluated and cells are subsequently treated with luciferase reagent, and luminescence in each well is measured in a luminometer as relative light units (RLU). RLUs are normalized for background and adjusted such that the maximal TA response induced by 17β-estradiol (E2) controls is 10,000 RLUs.

Figure 1 pGudLuc7.ERE Plasmid

Selection and Standardization of Reference Standards and Controls

The agonist reference standard consists of a 10-point double serial dilution of E2, and the antagonist reference standard is a 9-point double serial dilution of raloxifene with a fixed concentration of E2 (2.5 x 10^{-5} μg/mL). Controls include p,p'-methoxychlor (methoxychlor) for the agonist protocol and E2 and 25 μg/mL flavone + 2.5 x 10^{-5} μg/mL E2 (flavone/E2) for the antagonist protocol. The solvent control for both protocols is 1% volume per volume (v/v) dimethyl sulfoxide (DMSO). Reference standards and controls are presented in Table 1.

Table 1   Solvent, Reference Estrogen, Agonist, and Antagonist Controls

Use	Substance Name	CASRN	Supplier	Catalog Number	Purity	ER TA Agonist Activity[1,2]	ER TA Antag Activity[1,3,4]
Solvent	Dimethyl sulfoxide	67-68-5	Sigma-Aldrich Corp	D8418	99.9%	−	−
Agonist Reference Standard	17β-estradiol	50-28-2	Sigma-Aldrich Corp	E8875	98%	+++	−
Agonist Positive Control	p,p'-methoxychlor	72-43-5	Supelco	49004	99.9%	+	−
Antagonist Reference Standard	Raloxifene HCl	82640-04-8	Sigma-Aldrich Corp	R1402	99.5%	−	+++
Antagonist Positive Control	Flavone	525-82-6	Sigma-Aldrich Corp	F2003	99%	+	+
Antagonist E2 Control	17β-estradiol	50-28-2	Sigma-Aldrich Corp	E8875	98%	+++	−

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; Corp = Corporation.

[1]Data on agonist and antagonist activities were derived from ICCVAM 2006

[2]+++ Indicates that the substance is strongly active (half-maximal effective concentration [EC50] value is <0.001 μM); + indicates that the substance is weakly active (EC50 value is >0.1 μM), or a positive response was reported without an EC50 value; − indicates that the substance is negative.

[3]Antag = Antagonist

[4]+++ Indicates that the substance is strongly active (concentration inhibiting reference estrogen response by 50% [IC50] value is <0.001 μM); + indicates that the substance is weakly active (IC50 value is >0.1 μM) − indicates that the substance is negative.

The agonist historical database was established by conducting 10 independent studies using the 10-point E2 reference standard run in duplicate, DMSO control run in quadruplicate, and the methoxychlor control run in triplicate in each 96 well plate.

The antagonist historical database was established by conducting 10 independent studies using the 9-point raloxifene/E2 reference standard run in duplicate, DMSO control run in triplicate, and the E2 control and flavone/E2 control run in triplicate in each 96 well plate. These data were used to establish test acceptance criteria for subsequent studies.

Test Acceptance Criteria

Acceptance criteria for agonist testing:

- Induction: Plate induction, as measured by dividing the averaged highest E2 reference standard RLU value by the averaged DMSO control RLU value, must be greater than four fold
- Reference standard results: Calculated E2 reference standard half-maximal effective concentration (EC50) must be within 2.5 times the standard deviation of the historical database EC50 mean value
- DMSO control results: DMSO control RLU values must be within 2.5 times the standard deviation of the historical DMSO control mean RLU value
- Methoxychlor positive control results: Methoxychlor control RLU values must be within 2.5 times the standard deviation of the historical methoxychlor control mean RLU value

Acceptance criteria for antagonist testing:

- Reduction: Plate reduction, as measured by dividing the averaged highest raloxifene/E2 reference standard RLU value by the averaged lowest raloxifene/E2 reference standard RLU value, must be greater than three fold
- Reference standard results: Calculated concentration of raloxifene that inhibits E2 reference estrogen response by 50% (IC50) must be within 2.5 times the standard deviation of the historical database IC50 mean value
- DMSO control results: DMSO control RLU values must be within 2.5 times the standard deviation of the historical solvent control mean RLU value
- E2 control results: E2 control RLU values must be within 2.5 times the standard deviation of the historical E2 control mean RLU value
- Flavone/E2 control results: Flavone/E2 control RLU values must be within 2.5 times the standard deviation of the historical database flavone/E2 control mean RLU value

Selection and Standardization of Cell Viability Testing

CellTiter-Glo® (Promega, Inc.), a commercially available, quantitative cell viability assay, was incorporated into the LUMI-CELL® ER bioassay. CellTiter-Glo® is a luminescence-based assay for measuring adenosine triphosphate (ATP) levels and provides the use of a separate plate from the one used to evaluate ER TA activity.

CellTiter-Glo® data indicated that a significant decrease in estrogenic activity as measured by the LUMI-CELL® ER bioassay corresponded with a reduction in ATP levels above 20%. Abnormal cell morphology and alterations in cell density were also observed at concentrations that reduced ATP levels more than 20%. Based on these data, test substance concentrations that caused a reduction in cell viability below 80% were classified as cytotoxic and were not included in the assessment of estrogenic activity. A qualitative method of assessing cell viability using visual observation previously developed by XDS was also conducted for all agonist and antagonist experiments during the protocol standardization study and results demonstrated that this method was comparable to the CellTiter-Glo® assay in assessing cell viability (see SOT Poster No. 138-210 for a detailed evaluation of these results).

Testing of Coded Substances in Agonist and Antagonist Protocols

Eight coded substances (ATZ, BPA, BPB, COR, DDT, DES, EE, and FLA) covering a range of ER agonist activities and eight coded substances (BBP, DBA, FLA, GEN, NON, PRO, DDT, and TAM) covering a range of ER antagonist activities were each tested in three independent experiments to evaluate intralaboratory reproducibility and the ability of the test method to correctly distinguish between ER positive and negative substances. Prior to comprehensive testing, range finder experiments were conducted to establish the maximum test substance concentration based on solubility in 1% DMSO/culture media and either the maximum ER TA response (agonist assay) or the minimum ER TA response (antagonist assay) observed.

Test substances were provided to the laboratory in coded vials and all results were reported in μg/mL 1% DMSO/culture media. Following range finder testing, comprehensive testing of coded substances was conducted as an 11-point double serial dilution in triplicate for each of three independent experiments.

Based on the agonist results obtained, EE, DES, BPA, BPB, DDT, and FLA were classified as estrogenic agonists, while ATZ and COR did not induce a significant ER TA response. None of the substances that tested positive for agonist activity decreased cell viability at any of the concentrations used for comprehensive testing.

ICCVAM recommends that an evaluation of cell viability be included in in vitro tests for TA. The need for an assessment of cell viability is especially critical during antagonist testing to insure that a reduction of ER TA activity is in fact ER mediated and not as a result of cytotoxicity. Several substances (DBA, FLA, GEN, and TAM) were classified as ER antagonists without causing a decrease in cell viability. BBP, DDT, NON, and PRO, also reduced ER TA activity, but with a significant decrease in cell viability (ATP below 80% and alterations in cell morphology and cell density) at all concentrations that caused reduction of ER TA. These substances were therefore classified as negative for antagonist activity.

Absolute versus Relative EC50 and IC50 Values

No standardized methods for analyzing data obtained from in vitro ER TA test methods have been developed. Initially, a four parameter Hill equation, a logistic mathematical model relating substance concentration to RLU values in a sigmoidal shape, was used to determine EC50 and IC50 values (relative EC or IC50 value) with the LUMI-CELL® ER bioassay. However, the Hill equation requires the generation of a full concentration–response curve in order to calculate EC50 or IC50 values. Of the four substances that reduced ER TA activity without causing a decrease in cell viability (i.e., DBA, FLA, GEN, and TAM), a full concentration-response curve (and hence, a relative IC50 value) could only be generated for TAM.

Therefore, an approach that does not require a full dose response curve to calculate EC50 and IC50 values used by Eli Lilly (Eli Lilly 2005) was employed as an alternative to the Hill equation. This approach uses a method that measures the response of the test substance relative to experimental controls to calculate EC50 and IC50 values (absolute EC50 or IC50). The absolute EC50 is defined as the concentration of a substance that increases the measured activity in an agonist assay to 50% of the maximum activity induced by the reference estrogen (E2), and defines the absolute IC50 as the concentration of a substance that decreases the measured activity in an antagonist assay to 50% of the maximum activity induced by the reference estrogen (E2). An absolute EC50 value was calculated for all test substances that were positive for agonism (Table 2). An absolute IC50 value was calculated for FLA, GEN, and TAM (Table 3). An absolute IC50 value could not be calculated for DBA, which did not decrease measured activity to 50% of the E2 response.

Table 2   Relative and Absolute EC50 Values Obtained in the LUMI-CELL® ER Bioassay

Substance	Data Presented as μg/mL			Data Presented as μM		
	Relative EC50	Absolute EC50	Activity Range	Relative EC50	Absolute EC50	Activity Range[1]
Atrazine	Negative	Negative	Negative	Negative	Negative	Negative
Bisphenol A	0.09	0.08	0.08 to 10	0.38	0.33	0.34 to 43.8
Bisphenol B	0.05	0.02	0.02 to 1.25	0.21	0.10	0.08 to 5.16
Corticosterone	Negative	Negative	Negative	Negative	Negative	Negative
o,p'-DDT	0.38	0.34	0.16 to 10	1.08	0.97	0.44 to 28.2
Diethylstilbestrol	1.26 x 10^{-6}	8.74 x 10^{-6}	3.13 x 10^{-6} to 1.00 x 10^{-4}	4.69 x 10^{-6}	3.26 x 10^{-5}	1.17 x 10^{-5} to 3.73 x 10^{-4}
17α-ethinyl estradiol	3.87 x 10^{-6}	2.74 x 10^{-6}	7.81 x 10^{-7} to 1.00 x 10^{-4}	1.31 x 10^{-5}	9.24 x 10^{-6}	2.64 x 10^{-6} to 3.37 x 10^{-4}
Flavone	6.88	5.71	3.1 to 5	31.0	25.7	14.1 to 22.5

[1] The activity range is the range of concentrations at which test substance caused an increase in luminescence greater than three times the mean of the DMSO control plus the standard deviation of the mean.

Table 3   Relative and Absolute IC50 Values Obtained in the LUMI-CELL® ER Bioassay

Substance	Data Presented as μg/mL			Data Presented as μM		
	Relative IC50	Absolute IC50	Activity Range	Relative IC50	Absolute IC50	Activity Range[1]
Butylbenzyl phthalate	Negative	Negative	Negative	Negative	Negative	Negative
Dibenzo[a,h]anthracene	NC*	ND[†]	0.31 to 1.25	NC*	ND[†]	1.12 to 4.49
Genistein	NC	47.1	50	NC	174.0	185.0
Flavone	NC	17.4	12.5 to 50.0	NC	78.5	56.3 to 225.0
Nonylphenol	Negative	Negative	Negative	Negative	Negative	Negative
Progesterone	Negative	Negative	Negative	Negative	Negative	Negative
o,p'-DDT	Negative	Negative	Negative	Negative	Negative	Negative
Tamoxifen	0.16	0.20	0.16 to 5.00	0.43	0.53	0.42 to 13.5

NC = Not Calculated; ND = Not Determined

[1] The activity range is the range of concentrations at which test substance caused a decrease in luminescence greater than three times the mean of the DMSO control minus the standard deviation of the mean.

*A relative IC50 value could not be calculated for this substance

[†]An absolute IC50 value could not be calculated for this substance

Concordance of Testing Results with ICCVAM Published Data

Classification of estrogenic activity for substances tested using the standardized agonist protocol was in complete agreement (100% concordance) with ICCVAM published data (data compiled from a comprehensive review of scientific literature and unpublished data submitted to ICCVAM), classifying six substances (BPA, BPB, DDT, DES, EE, and FLA) as ER agonists and two (ATZ and COR) as negative (Table 4).

Table 4   Concordance of LUMI-CELL® ER Agonist Bioassay and ICCVAM Published Data

		ICCVAM Agonist Classification		
		Positive	Negative	Total
LUMI-CELL® ER Classification	Positive	6	0	6
	Negative	0	2	2
	Total	6	2	8

- Concordance = 100% (8/8)
- Sensitivity = 100% (6/6)
- Specificity = 100% (2/2)
- False Negative Rate = 0% (0/6)
- False Positive Rate = 0% (0/2)
- Positive Predictivity = 100 % (6/6)
- Negative Predictivity = 100% (2/2)

To compare the relative activity of ER agonists with ICCVAM published data, EC50 values were converted from concentration per unit volume to molar concentration. The relative activity of the ER agonists, based on their calculated EC50 concentrations, was in agreement with ICCVAM reported median activity (Table 5).

Table 5   Comparison of LUMI-CELL® ER Agonist Bioassay and ICCVAM Published EC50 Values

Substance	Relative EC50	Absolute EC50	ICCVAM EC50*
Atrazine	Negative	Negative	Negative
Bisphenol A	0.38	0.08	0.40
Bisphenol B	0.21	0.02	NR
Corticosterone	Negative	Negative	Negative
o,p'-DDT	1.08	0.34	0.66
Diethylstilbestrol	4.69 x 10^{-6}	3.26 x 10^{-5}	1.9 x 10^{-5}
17α-ethinyl estradiol	3.87 x 10^{-6}	9.24 x 10^{-6}	1.1 x 10^{-5}
Flavone	31.0	25.7	NR

NR = Not Reported

*Values are reported in μM

Classification of estrogenic activity for substances tested using the standardized antagonist protocol was in agreement with ICCVAM published data for DBA, FLA, GEN, and TAM, which were classified as ER antagonists, and BBP and PRO, which were classified as negative. Two substances, DDT and NON, classified as ER antagonists in the ICCVAM published data were classified as negative in the LUMI-CELL® ER bioassay resulting in a concordance of 75% (Table 6). Although NON and DDT caused a significant decrease in ER TA activity in the LUMI-CELL® ER bioassay, they also caused a significant decrease in cell viability over the same concentration range and were therefore classified as cytotoxic rather than as estrogenic antagonists. It is noted that studies from which ICCVAM published data for antagonism was derived did not evaluate cell viability.

Table 6   Concordance of LUMI-CELL® ER Antagonist Bioassay and ICCVAM Published Data

		ICCVAM Antagonist Classification		
		Positive	Negative	Total
LUMI-CELL® ER Classification	Positive	4	0	4
	Negative	2	2	4
	Total	6	2	8

- Concordance = 75% (6/8)
- Sensitivity = 100% (4/4)
- Specificity = 50% (2/4)
- False Negative Rate = 0% (0/4)
- False Positive Rate = 50% (2/4)
- Positive Predictivity = 67% (4/6)
- Negative Predictivity = 100% (2/2)

No antagonist IC50 values were reported in the ICCVAM data (Table 7).

Table 7   Comparison of LUMI-CELL® ER Antagonist Bioassay and ICCVAM Published IC50 Values

Substance	Relative IC50	Absolute IC50	ICCVAM IC50*
Butylbenzyl phthalate	Negative	Negative	NR
Dibenzo[a,h]anthracene	NC[1]	ND[2]	NR
Genistein	NC	47.1	NR
Flavone	NC	17.4	NR
Nonylphenol	Negative	Negative	NR
Progesterone	Negative	Negative	Negative
o,p'-DDT	Negative	Negative	NR
Tamoxifen	0.43	0.53	NR

NC = Not Calculated; NR = Not Reported

*Values are reported in μM

[1] A relative IC50 value could not be calculated for this substance

[2] An absolute IC50 value could not be calculated for this substance

Summary

NICEATM has conducted a protocol standardization study for the in vitro LUMI-CELL® ER bioassay. The reference standards and controls selected for the LUMI-CELL® ER bioassay were:

- 1% v/v DMSO, used as solvent control in both agonist and antagonist protocols
- A 10-point double serial dilution of E2 as agonist reference standard
- p,p'-methoxychlor as agonist control
- A 9-point double serial dilution of raloxifene with a fixed concentration of E2 as antagonist reference standard
- E2 and flavone/E2 as antagonist controls

The 'CellTiter-Glo' assay was incorporated into the LUMI-CELL® ER bioassay to assess cell viability. Concentrations of substance that caused a reduction in cell viability below 80% were classified as cytotoxic and were not included in the assessment of estrogenic activity. Under these conditions:

- None of the agonists tested significantly decreased cell viability at any of the concentrations tested
- BBP, DDT, NON, and PRO decreased cell viability to below 80% at all concentrations that caused reduction of ER TA and were classified as negative

Concordance of estrogenic activity for substances tested using the standardized agonist protocol with ICCVAM published data was:

- 100% for agonists tested
- 75% for antagonists tested
  - NON and DDT were classified as ER antagonists in the ICCVAM published data but classified as negative in the LUMI-CELL® ER protocol standardization study due to significant decreases in cell viability
  - Cell viability was not evaluated in studies from which ICCVAM published data were derived. Further reviews of current literature will be conducted to determine if studies have been conducted with assessment of cell viability.

The LUMI-CELL® ER agonist and antagonist protocols have been standardized and the intralaboratory reproducibility and accuracy of the standardized test method protocol has been demonstrated using a group of representative substances, indicating the test method's readiness for a multi-laboratory validation study.

Acknowledgments

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Current validation study information available at: http://iccvam.niehs.nih.gov/methods/endocrine.htm

NIEHS National Institute of Environmental Health Sciences

U.S. Department of Health and Human Services National Institutes of Health

ICCVAM The Interagency Coordinating Committee on the Validation of Alternative Methods

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

More information on ICCVAM and NICEATM can be accessed at http://iccvam.niehs.nih.gov/