Standardization of Protocols for the Validation of an *In Vitro* Estrogen Receptor Transcriptional Activation Assay

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Introduction

In 2000, the U.S. Environmental Protection Agency (EPA) asked the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) to evaluate the validation status of *in vitro* estrogen receptor (ER) and androgen receptor (AR) test methods, which were proposed as components of the EPA Endocrine Disruptor Screening Program (EDSP). The NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) subsequently prepared comprehensive background review documents (BRDs) to assess the validation status of *in vitro* ER and 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 tissues or cells from animals should be a priority for further development and validation. Based on the expert panel's conclusions and recommendations, along with comments from the public, ICCVAM developed test method recommendations that included essential test method components (previously called minimum procedural standards) and a list of reference substances that should be used to standardize and validate *in vitro* ER and AR test methods. A *Federal Register (FR)* notice (*FR* Vol. 68, No. 106, pp. 33171-33172, June 3, 2003) was subsequently published announcing the availability of a report defining these recommendations (ICCVAM 2003) and inviting the nomination of *in vitro* ER and AR test methods for validation studies.

In response to the *FR* notice, Xenobiotic Detection Systems, Inc.[1] nominated their LUMI-CELL® ER agonist and antagonist detection assay to ICCVAM for validation. In accordance with the ICCVAM nomination process, NICEATM conducted a pre-screen evaluation of the materials submitted with the nomination to determine the extent that they addressed the ICCVAM prioritization criteria and submission guidelines (ICCVAM 2003). Based on this pre-screen evaluation, the ICCVAM Endocrine Disruptor Working Group (EDWG) and ICCVAM proposed that the LUMI-CELL® ER assay undergo a validation study, with high priority, and published an FR notice (*FR* Vol. 69, No. 77, pp. 21564, April, 2004) seeking public comment. In response to the comments received from the public and from the Scientific Advisory Committee for Alternative Test Methods (SACATM) on the nomination and the proposed priority, the EDWG and ICCVAM recommended the validation of the LUMI-CELL® ER agonist and antagonist assay.

In preparation for the validation study, NICEATM conducted a protocol standardization study for the LUMI-CELL® ER assay. Protocol standardization procedures were based on ICCVAM essential test method components. 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 assays
- 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 assays
- 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

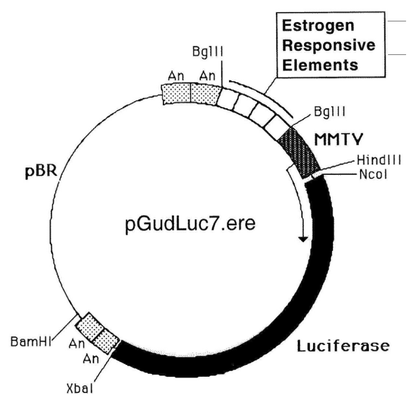
[1] The development of the assay was supported by a Small Business Innovation Research grant from the U.S. National Institute of Environmental Health Sciences (Grant # 5R44ES010533-03)

Overview of the LUMI-CELL® ER Assay

The LUMI-CELL® ER assay 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 immortalized BG-1 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 et al. 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 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, after which cells are lysed and treated with luciferase reagent. Luminescence is then measured in each well with a luminometer and expressed as relative light units (RLU). RLUs are first normalized for background by subtraction of the solvent control dimethyl sulfoxide (DMSO) RLU values from those obtained by every well on the 96-well plate. RLU values are then adjusted such that the maximal TA response induced by 17ß-estradiol (E2) (the reference standard for agonist testing) or by Raloxifene/E2 (the reference standard for antagonist testing) 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 against a fixed concentration of E2 (2.5 x 10^{-5} µg/mL). Controls include 3.13 µg/mL *p,p'*-methoxychlor (methoxychlor) for the agonist protocol 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) 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]
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	49054	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: Antag = Antagonist; 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 [EC_{50}] value is <0.001 µM); + indicates that the substance is weakly active (EC_{50} value is >0.1 µM), or a positive response was reported without an EC_{50} value; - indicates that the substance is negative.

[3]+++ Indicates that the substance is strongly active (concentration inhibiting reference estrogen response by 50% [IC_{50}] value was <0.001 µM); + indicates that the substance is weakly active (IC_{50} value was >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 acceptance criteria for subsequent studies.

Selection and Standardization of Cell Viability Testing

CellTiter-Glo®, a commercially available, quantitative cell viability assay, was incorporated into the LUMI-CELL® ER assay. CellTiter-Glo® is a luminescence-based assay for measuring adenosine triphosphate (ATP) levels; it requires 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 assay 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 by 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.

Testing of Coded Substances in Agonist and Antagonist Protocols

Eight coded substances (atrazine [ATZ], bisphenol A [BPA], bisphenol B [BPB], corticosterone [COR], *o,p'*-DDT[2] [DDT], diethylstilbestrol [DES], 17α-ethinyl estradiol [EE], and flavone [FLA]), covering a range of ER agonist activities and eight coded substances (butylbenzyl phthalate [BBP], dibenzo[*a,h*]anthracene [DBA], flavone [FLA], gensitein [GEN], p-*n*-nonylphenol [NON], progesterone [PRO], *o,p'*-DDT [DDT], and tamoxifen [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 starting concentrations for comprehensive testing.

Test substances were provided to the laboratory in coded vials and all results were reported in µg/mL. Following range finding, 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 (ICCVAM 2003). 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 reduced ER TA activity. These substances were therefore classified as negative for antagonist activity.

[2]1,1,1-Trichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl)ethane

NIEHS National Institute of Environmental Health Sciences

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/

Test Method Reproducibility

Agonist and antagonist results for reference standards, controls, induction or reduction for range finder and comprehensive testing were analyzed for reproducibility and the results are summarized in **Table 2**.

Table 2 Results for Agonist and Antagonist Reference Standards and Controls during Protocol Standardization

	Control	Mean	Standard Deviation	Acceptance Limits	CV (%)[1]	N[2]
Agonist Controls	DMSO Control[3]	2386	1213	-646.5 to 5418.5	50.8	33
	E2 EC_{50} Value[4]	6.38 x 10^{-5}	2.80 x 10^{-5}	6.2 x 10-6 to 1.34 x 10^{-4}	43.9	33
	Fold-Induction[5]	4.2	1.3	0.95 to 7.45	31.0	33
	Methoxychlor Control[6]	6218	2306	453 to 11983	37.1	33
Antagonist Controls	DMSO Control[3]	2252	1304	-1008 to 5512	57.9	28
	E2 Control[6]	4664	2751	-2213.5 to 11541.5	59.0	28
	Flavone/E2 Control[6]	1149	808	-871 to 3169	70.3	28
	Ral/E2 IC_{50} Value[4]	2.22 x 10^{-3}	3.82 x 10^{-4}	1.27 x 10-3 to 3.18 x 10^{-3}	17.2	28
	Fold-Reduction[7]	6.1	1.4	2.66 to 9.46	22.4	28

Abbreviations: DMSO = dimethyl sulfoxide; E2 = 17β–estradiol; EC50 = half-maximal effect concentration; IC_{50} = concentration of test substance that inhibits the reference estrogen response by 50%.

[1]The Coefficient of Variation (CV) is calculated as the standard deviation divided by the mean, expressed as a percentage.

[2]N = Number of experiments. Fewer experiments were conducted for antagonist testing than for agonist testing.

[3]Values are expressed as unadjusted relative light units.

[4]Values are expressed as µM.

[5]Induction is expressed as the ratio of the averaged highest unadjusted RLU value for the E2 reference standard in each experiment over the averaged DMSO control value.

[6]Values are expressed as adjusted relative light units.

[7]Reduction is expressed as the ratio of the averaged highest unadjusted RLU value for the Ral/E2 reference standard in each experiment over the averaged lowest unadjusted RLU value for the Ral/E2 reference standard.

To obtain an assessment of the variation of reference standard and control data over time, a linear regression analysis was conducted, where each test was assigned to a specific "study day" (i.e., the first experiment conducted was assigned as Study Day 1, and an experiment conducted seven days later was assigned as Study Day 8). Agonist and antagonist linear regression results for reference standards, controls, induction or reduction for range finder and comprehensive testing are summarized in **Table 3.**

Table 3 Linear Regression Analysis of Agonist and Antagonist Reference Standards and Controls[1]

	Control	Slope	P-value (Slope)	r^2	y-intercept	N[2]
Agonist Controls	DMSO Control	-11.09	0.58	0.03	2420	33
	E2 EC_{50} Value	-2.63 x 10^{-2}	0.03*	0.35	2.2 x 10^{-5}	33
	Fold-Induction	-0.03	0.29	0.09	4.97	33
	Methoxychlor Control	-45.70	0.19	0.14	6539	33
Antagonist Controls	DMSO Control	-56.02	0.11	0.32	3147	28
	E2 Control	-77.90	0.29	0.16	5837	28
	Flavone/E2 Control	-64.89	0.03*	0.50	2625	28
	Ral/E2 IC_{50} Value	1.18 x 10^{-6}	0.82	0.01	1.14 x 10^{-3}	28
	Fold-Reduction	0.03	0.56	0.05	5.86	28

Abbreviations: DMSO = dimethyl sulfoxide; E2 = 17β–estradiol; EC_{50} = half-maximal effect concentration; IC_{50} = concentration of test substance that inhibits the reference estrogen response by 50%.

[1]Each experiment was assigned a number based on the order in which testing occurred, without respect to the time lapsing between tests.

[2]N = Number of experiments. Fewer experiments were conducted for antagonist testing than for agonist testing.

*The slope of the linear regression across experiments is statistically different from zero.

Table 3 shows that there was statistically significant variation in E2 EC_{50} and Flavone/E2 control values over time. However, in both cases, these values stabilized mid-way through the study.

Results for coded test substances were analyzed for reproducibility and the results are summarized in **Table 4**.

Table 4 EC_{50} and IC_{50} Value Results (µM) for Agonist and Antagonist Testing of Coded Test Substances during Protocol Standardization

	Test Substance	Mean	Standard Deviation	CV (%)[1]	N[2]
Agonist Testing	Atrazine	Negative	-	-	3
	Bisphenol A	0.38	0.087	22.9	3
	Bisphenol B	0.21	0.019	9.1	3
	Corticosterone	Negative	-	-	3
	o,p'-DDT	1.08	0.37	34.0	3
	Diethylstilbestrol	4.69 x 10^{-5}	2.65 x 10^{-5}	56.5	3
	17α-ethinyl estradiol	1.34 x 10^{-5}	4.40 x 10^{-6}	32.8	4
	Flavone	30.96	16.70	53.9	3
Antagonist Testing	BBP	Negative	-	-	3
	DBA	Not Calculated[3]	-	-	3
	Genistein	174.05[4]	-	-	3
	Flavone	78.50	2.93	3.7	3
	Nonylphenol	Negative	-	-	3
	Progesterone	Negative	-	-	3
	o,p'-DDT	Negative	-	-	3
	Tamoxifen	0.43	0.13	30.2	3

Abbreviations: DMSO = dimethyl sulfoxide; E2 = 17β–estradiol; EC^{50} = half-maximal effect concentration; IC_{50} = concentration of test substance that inhibits the reference estrogen response by 50%.

[1]The Coefficient of Variation (CV) is calculated as the standard deviation divided by the mean, expressed as a percentage.

[2]N = Number of experiments. Fewer experiments were conducted for antagonist testing than for agonist testing.

[3]An IC_{50} value could not be calculated for this substance.

[4]An IC_{50} value could only be calculated for a single experiment for this substance. An SD and CV cannot be calculated.

Accuracy of Testing Results with ICCVAM Published Data

For each test substance, there was agreement among the replicate experiments in their classification as either positive or negative for ER agonism or antagonism.

Classification of estrogenic activity for substances tested using the standardized agonist protocol was in complete agreement (100% accuracy) 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 5**).

Table 5 Accuracy of LUMI-CELL® ER Agonist Assay 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

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

To compare the relative activity of ER agonists with ICCVAM published data, EC_{50} values were converted from concentration per unit volume to molar concentration. The relative activity of the ER agonists, based on their calculated EC_{50} concentrations, was in agreement with ICCVAM reported median activity (**Table 6**).

Table 6 Comparison of LUMI-CELL® ER Agonist Assay and ICCVAM Published EC_{50} Values

Substance	Study EC_{50}*	ICCVAM EC_{50}*
Atrazine	Negative	Negative
Bisphenol A	0.38	0.40
Bisphenol B	0.21	NR
Corticosterone	Negative	Negative
o,p'-DDT	1.08	0.66
Diethylstilbestrol	4.69 x 10^{-5}	1.9 x 10^{-5}
17α-ethinyl estradiol	3.87 x 10^{-6}	1.1 x 10^{-5}
Flavone	31.0	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 assay (**Table 7**). Although NON and DDT caused a significant decrease in ER TA activity in the LUMI-CELL® ER assay, 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 7 Accuracy of LUMI-CELL® ER Antagonist Assay and ICCVAM Published Data

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

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

A comparison of the relative ER antagonist activities of the substances tested could not be conducted because no calculated IC_{50} values were provided in the ICCVAM published data.

Summary

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

- 1% v/v DMSO, used as the solvent control in both agonist and antagonist protocols
- A 10-point double serial dilution of E2 as the agonist reference standard covering a concentration range from 1.00 x 10^{-4} µg/mL to 7.83 x 10^{-7} µg/mL.
- 3.13 µg/mL *p,p'*-methoxychlor as the agonist control
- A 9-point double serial dilution of raloxifene covering a concentration range from 1.25 x 10^{-2} µg/mL to 4.88 x 10^{-5} µg/mL, with a fixed concentration of E2 (2.5 x 10^{-5} µg/mL) as the antagonist reference standard
- 25 µg/mL flavone + 2.5 x 10^{-5} µg/mL E2 as antagonist control

The CellTiter-Glo® assay was incorporated into the LUMI-CELL® ER assay 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 used in comprehensive testing
- 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

Accuracy 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 assay protocol standardization study due to significant decreases in cell viability
  - Cell viability was not evaluated in studies from which ICCVAM published data was 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 assay agonist and antagonist protocols have been standardized and the intralaboratory reproducibility and accuracy of the standardized test method protocol was demonstrated using a group of representative substances. NICEATM is currently conducting an interlaboratory validation study on this ER TA test method in conjunction with the European Centre for the Validation of Alternative Methods and the Japanese Center for the Validation of Alternative Methods.

Acknowledgements

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