

Testing of Coded Substances in the NICEATM/ECVAM/JaCVAM LUMI-CELL® STTA Multiphase International Validation Study

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Abstract¹

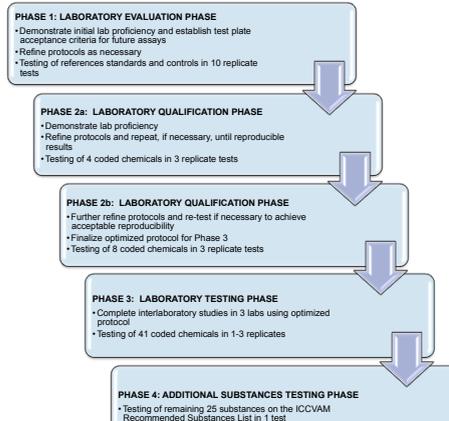
Based on an ICCVAM recommendation, NICEATM, ECVAM, and JaCVAM initiated a four-phase validation study to evaluate the LUMI-CELL® ER assay. The LUMI-CELL® ER transcriptional activation (TA) assay uses the BG-1 cell line, a human ovarian carcinoma cell line with an endogenously expressed estrogen receptor (ER) and a stably transfected luciferase reporter gene to screen for substances that may induce or inhibit ER-mediated transcription. The validated assay will be used to support the EPA EDP5 Tier 1 screening program, and to develop an OECD test guideline with performance standards that can be used to validate mechanistically and functionally similar ER TA test methods. Three laboratories (one each in the United States, Europe, and Japan) tested 53 reference substances recommended by ICCVAM for validation of *in vitro* ER test methods. Phase 1 was the laboratory evaluation phase where each laboratory tested reference standards and controls 10 times to demonstrate initial proficiency, and to establish laboratory-specific acceptance criteria for subsequent phases. In Phase 2, 12 agonist and antagonist substances from the ICCVAM minimum list covering the range of activities (i.e., strong, moderate, weak, negative, agonists and/or antagonists) were tested in two stages (4 in Phase 2a, 8 in Phase 2b). Protocol refinements made during Phase 2 were incorporated into the final optimized protocols used for all subsequent testing. Phase 3 provided the data necessary to evaluate inter-laboratory reproducibility and accuracy of the optimized protocols by testing the remaining 41 substances from the minimum list at least once at each laboratory. In Phase 4 the lead laboratory tested 25 additional substances from the ICCVAM list of 78 recommended substances. Results from Phases 2a and 2b underscore the importance of a phased study design to allow for necessary protocol refinements. Results from Phases 3 and 4 are being evaluated and will form the basis for a standardized protocol to be included in a new OECD test guideline. Supported by ECVAM, JaCVAM, and NIEHS Contract N01-ES-35504.

¹This abstract has been modified from the version presented in the SOT 2010 booklet.

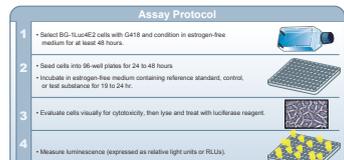
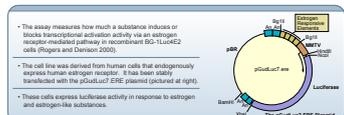
Introduction

- The LUMI-CELL® ER assay is a TA test method developed to detect ER agonists and antagonists.
 - There are currently no adequately validated *in vitro* test methods for identifying estrogen antagonists.
- The LUMI-CELL® ER assay will provide the first validated screening method for estrogen antagonists.
- The test method is based upon an immortalized ovarian cell line, the BG-1 cell, rather than the more commonly used MCF-7 breast cell line.
 - Both cell lines have similar affinities for estradiol. However, BG-1 cells have approximately twice the number of ERs as MCF-7 cells, thereby providing an excellent model for evaluating estrogen responsiveness (Baldwin et al., 2007).
 - BG-1 cells are also more tolerant of dimethyl sulfoxide (DMSO) than MCF-7 cells, tolerating DMSO concentrations of 1%, which allows for the testing of higher concentrations of test substance.
- The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), the European Centre for the Validation of Alternative Methods (ECVAM), and the Japanese Center for the Evaluation of Alternative Methods (JaCVAM) have conducted an international multi-laboratory validation study to evaluate accuracy and reliability in three laboratories:
 - Xenobiotic Detection Systems, Inc. (XDS) (Durham, USA)
 - ECVAM (Ispra, Italy)
 - Hiyoshi Corp. (Omihachiman, Japan)
- The study was conducted in four phases (Figure 1).
- The study evaluated the 78 reference substances recommended by the Interagency Coordination Committee on the Validation of Alternative Methods (ICCVAM) for validation of *in vitro* ER test methods (ICCVAM 2006).

Figure 1: Phases of the LUMI-CELL® ER TA Validation



Overview of the LUMI-CELL® ER Assay

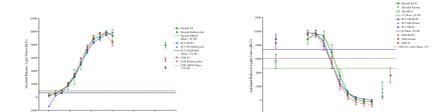


Reference Substances	Vehicle Control	Reference Standard	Reference Estrogen	Positive Control
Agonist Protocol	1% DMSO	17β-estradiol	(none used)	p,p'-methoxychlor (lethal positive)
Antagonist Protocol	1% DMSO	17β-estradiol + raloxifene HCl	17β-estradiol	17β-estradiol + flavone

Phase 1 Testing

- Reference standards and controls were tested at least 10 times in all 3 laboratories (Figures 2 and 3)
- Inter-laboratory reproducibility evaluated
- Established initial quality controls for testing of coded reference substances

Figure 2: Phase 1 Agonist Results Figure 3: Phase 1 Antagonist Results



Phase 2 Testing

- Coded agonist and antagonist substances were tested (Table 1)
- Accuracy and intra- and inter-laboratory reproducibility were evaluated against the ICCVAM reference data published in 2006 (ICCVAM 2006)
- In Phase 2a, three of four test substances were positive for agonism, and four of four test substances were positive for antagonism (Table 2).

Table 1: Substances Tested in Phase 2^a

	Agonist Test Substances		Antagonist Test Substances	
	Substance Name	CASRN	Substance Name	CASRN
Phase 2a	Bisphenol A (BPA)	80-057	Diethylstilbestrol (DES)	53-70-3
	Bisphenol B (BPB)	77-407	Tamoxifen (TAM)	10640-29-1
	Corticosterone (CORT)	50-22-8	Alprazolam (API)	520-36-8
	Diethylstilbestrol (DES)	56-53-1	Alprazolam (API)	520-36-8
Phase 2b	Alprazolam (ATR)	1912-24-9	Alprazolam (ATR)	520-36-8
	Butylnyl phthalate (BBP)	85-68-7	Alprazolam (ATR)	520-36-8
	p,p'-DDT (DDT)	789-02-6	Butylnyl phthalate (BBP)	85-68-7
	17- <i>n</i> -ethyl estradiol (EE)	57-63-6	Corticosterone (CORT)	50-22-8
Phase 3	Flavone (FLA)	525-82-6	p,p'-DDT (DDT)	789-02-6
	Genistein (GEN)	446-72-0	Flavone (FLA)	525-82-6
	p,n-nonylphenol (NON)	104-40-5	Genistein (GEN)	446-72-0
	Ynolindolol (VIN)	50471-44-8	Resveratrol (RES)	501-36-0

^aSubstances selected from the ICCVAM list of reference substances (ICCVAM 2006)

Phase 2 Testing (Continued)

Table 2: Results of Phase 2a Testing

Agonist Test Substance	Laboratory	Agonist Test Results ^a	Antagonist Test Substance	Laboratory	Antagonist Test Results ^a
BPA	IRD ^b	Positive	DBA	IRD ^b	Positive
	XDS	Positive		ECVAM	Positive
	ECVAM	Positive		IRD ^b	Positive
BPB	IRD ^b	Positive	NON	IRD ^b	Positive
	XDS	Positive		ECVAM	Positive
	ECVAM	Positive		Hiyoshi	Positive
CORT	IRD ^b	Positive	PROG	IRD ^b	Positive
	XDS	Negative		ECVAM	Positive
	ECVAM	Positive		Hiyoshi	Positive
DES	IRD ^b	Positive	TAM	IRD ^b	Positive
	XDS	Positive		ECVAM	Positive
	ECVAM	Positive		Hiyoshi	Positive

Abbreviations: IRD = ICCVAM Reference Data. ^aResults in bold are discordant from ICCVAM Reference Data. ^bICCVAM Reference Data. ^cICCVAM Reference Data. ^dPositive using identification studies at low non-cytotoxic concentration. Negative results in Phase 2a due to cytotoxicity at all doses that could be considered positive. ^eNon-cytotoxic results that may have resulted from contamination.

Phase 2a Test Plate Acceptance Criteria

- Agonist test plate acceptance criteria used in Phase 2a testing:
 - Plate induction > 3-fold (averaged highest 17β-estradiol [E2] reference standard RLU divided by the averaged DMSO RLU)
 - E2 EC₅₀ ≤ 2.5 times the standard deviation (SD) of the historical database E2 EC₅₀
 - DMSO RLU ≤ 2.5 times the SD of the historical DMSO RLU
 - Methoxychlor control (MET) RLU ≤ 2.5 times the SD of the historical MET RLU

- Antagonist test plate acceptance criteria used in Phase 2a testing:
 - Plate reduction > 3-fold (averaged highest RalE2 reference standard RLU divided by the averaged lowest RalE2 reference standard RLU)
 - Raloxifene/RalE2 IC₅₀ values ≤ 2.5 times the SD of the historical database RalE2 IC₅₀
 - DMSO RLU ≤ 2.5 times the SD of the historical DMSO RLU
 - E2 control RLU must be ≤ 2.5 times the SD of the historical E2 control
 - Flavone/E2 control (FLA/E2) RLU must be ≤ 2.5 times the SD of the historical FLA/E2 control value

Phase 2a Agonist and Antagonist Test Plate Failure Rates

- Overall failure rates were 61% (33/54) and 38% (13/34) for the agonist and antagonist substances, respectively.
- The relationship between test plate failures and the different test plate acceptance criteria was evaluated to determine if changes to these criteria could reduce the failure rate without affecting agonist or antagonist classifications.
 - Only changes to acceptance criteria for agonist E2 reference standard EC₅₀ and MET, and antagonist RalE2 reference standard IC₅₀ and FLA/E2 values were considered for modification.
 - Acceptance criteria based on DMSO control values, agonist E2 reference standard fold induction, antagonist RalE2 reference standard fold reduction, and the antagonist E2 control were not considered in this evaluation because they are essential for monitoring background activity, assay performance, or determining test substance anti-estrogenic activity.
- Results indicate that test plate failures associated with reference standard EC₅₀ and IC₅₀ and/or positive control RLU values did not affect agonist or antagonist classifications (see Tables 3, 4, and 5).

Table 3: Qualitative Evaluation of Agonist E2 Reference Standard EC₅₀ and Methoxychlor Control Test Plate Acceptance Criteria^a

Agonist Test Substances	Laboratory	Number of Tests	Passed All Acceptance Criteria	Failed E2 EC ₅₀ Only	Failed Methoxychlor Only	Failed Both E2 EC ₅₀ and Methoxychlor
BPA	XDS	7	3 (+)	4 (+)	DNF	DNF
	ECVAM	13	3 (+)	7 (+)	3 (+)	DNF
	Hiyoshi	4	3 (+)	DNF	1 (+)	DNF
BPB	XDS	7	3 (+)	4 (+)	DNF	DNF
	ECVAM	9	3 (+)	4 (+)	DNF	2 (+)
	Hiyoshi	4	3 (+)	DNF	1 (+)	DNF
CORT	XDS	7	3 (+)	4 (+)	DNF	DNF
	ECVAM	13	3 (+)	5 (+)	3 (+)	DNF
	Hiyoshi	4	4 (+)	DNF	DNF	DNF
DES	XDS	7	3 (+)	4 (+)	DNF	DNF
	ECVAM	9	3 (+)	4 (+)	DNF	2 (+)
	Hiyoshi	4	3 (+)	DNF	DNF	DNF

Abbreviations: DNF = did not fail test plate acceptance criteria; E2 = 17β-estradiol; EC₅₀ = half-maximal effective concentration. ^aData in parentheses indicate the qualitative response (positive or negative) of the test substance. A test substance is considered to be positive (+) if the test substance produced an effect that was greater than the RLU value of the mean DMSO control plus three times the standard deviation of the E2 control mean. Otherwise, it is considered negative (-).

Table 4: Qualitative Evaluation of Antagonist RalE2 Reference Standard IC₅₀ and Flavone/E2 Positive Control Acceptance Criteria^a

Antagonist Test Substances	Laboratory	Number of Tests	Passed All Acceptance Criteria	Failed RalE2 IC ₅₀ Only	Failed Flavone/E2 Only	Failed Both RalE2 IC ₅₀ and Flavone/E2
DBA	XDS	6	+ (3/3)	+ (1/3)	DNF	DNF
	ECVAM	3	+ (3/3)	DNF	DNF	DNF
	Hiyoshi	3	+ (3/3)	DNF	DNF	DNF
NON	XDS	6	+ (6/6)	+ (6/6)	DNF	DNF
	ECVAM	3	+ (3/3)	DNF	DNF	DNF
	Hiyoshi	3	+ (3/3)	DNF	DNF	DNF
PROG	XDS	6	+ (3/3)	+ (0/3)	DNF	DNF
	ECVAM	3	+ (3/3)	DNF	DNF	DNF
	Hiyoshi	3	+ (3/3)	DNF	DNF	DNF
TAM	XDS	6	+ (3/3)	+ (3/3)	DNF	DNF
	ECVAM	5	+ (3/3)	DNF	+ (1/2)	DNF
	Hiyoshi	3	+ (3/3)	DNF	DNF	DNF

Abbreviations: DNF = did not fail test plate acceptance criteria; E2 = 17β-estradiol; IC₅₀ = concentration of test substance that inhibits E2 response by 50%. ^aRal = raloxifene HCl. ^bData in parentheses indicate the qualitative response (positive or negative) of the test substance. A test substance is considered to be positive (+) if the test substance produced an effect that was greater than the RLU value of the mean DMSO control plus three times the standard deviation of the E2 control mean. Otherwise, it is considered negative (-).

Phase 2 Testing (Continued)

Table 5: Comparison of Test Substance EC₅₀ and IC₅₀ Values from Plates that Passed or Failed Agonist and Antagonist Reference Standard and Positive Control Test Plate Acceptance Criteria

Laboratory and Substance Evaluated	Agonist Plates that Passed All Acceptance Criteria			Agonist Plates that did not Pass E2 EC ₅₀ and/or Methoxychlor Acceptance Criteria			P Value ^a	
	N	Mean EC ₅₀ Value ^b	SD ^c	N	Mean EC ₅₀ Value ^b	SD ^c		
XDS/BPA	3	8.8 × 10 ²	7.2 × 10 ²	4	9.9 × 10 ²	1.4 × 10 ²	0.40	
	ECVAM/BPA	3	1.9 × 10 ³	9.0 × 10 ²	10	1.8 × 10 ³	5.6 × 10 ²	0.16
	Hiyoshi/BPA	3	3.9 × 10 ²	9.0 × 10 ²	4	4.3 × 10 ²	1.1 × 10 ²	0.83
ECVAM/BPB	3	4.2 × 10 ²	1.3 × 10 ²	4	7.5 × 10 ²	1.7 × 10 ²	0.06	
	ECVAM/DES	4	1.4 × 10 ³	5.0 × 10 ²	4	2.6 × 10 ³	1.1 × 10 ³	0.20
	ECVAM/PROG	3	1.4 × 10 ³	5.0 × 10 ²	4	2.6 × 10 ³	1.1 × 10 ³	0.20
Laboratory and Substance Evaluated	Antagonist Plates that Passed All Acceptance Criteria			Antagonist Plates that did not Pass RalE2 IC ₅₀ and/or Flavone/E2 Acceptance Criteria			P Value ^a	
	N	Mean IC ₅₀ Value ^b	SD ^c	N	Mean IC ₅₀ Value ^b	SD ^c		
	XDS/TAM	4	1.5 × 10 ³	5.7 × 10 ²	3	3.1 × 10 ³	8.8 × 10 ²	0.11

Abbreviations: E2 = 17β-estradiol; EC₅₀ = half-maximal effective concentration; IC₅₀ = concentration of test substance that inhibits E2 response by 50%. ^aFlavone/E2 = antagonist positive control; Methoxychlor = agonist positive control; N = number of plates; Mean = average; SD = Standard Deviation. ^bEC₅₀ and IC₅₀ values are expressed in ng/ml. ^cEC₅₀ and IC₅₀ values are expressed in ng/ml.

Modifications to the Protocols

Modifications to Test Plate Acceptance Criteria

During Phase 2a, test plates that failed test plate acceptance due to failure of the E2 EC₅₀, RalE2 IC₅₀, MET or FLA/E2 controls did not alter the expected test substance results (Table 5). Test plate acceptance criteria based on EC₅₀, IC₅₀, MET and FLA/E2 values were removed from the protocols.

Test plate acceptance criteria were modified as follows:

- Agonist Test Plate Acceptance Criteria:
 - Agonist E2 reference standard curve should be sigmoidal in shape and have at least three values within the linear portion of the curve
 - Mean MET RLU > 3x SD of the mean DMSO RLU
- Antagonist Test Plate Acceptance Criteria:
 - RalE2 standard curve should be sigmoidal in shape and have at least three values within the linear portion of the curve
 - Mean methoxychlor RLU > 3x SD of the mean DMSO RLU
 - Mean FLA/E2 RLU < 3x SD of the mean DMSO RLU
 - E2 control RLU must be ≤ 2.5 times the standard deviation of the historical E2 control

Phase 2b Agonist and Antagonist Results

- In Phase 2b, six of eight test substances were positive for agonism, and eight of eight test substances were positive for antagonism (Table 6).
- Phase 2b Agonist and Antagonist Test Plate Failure Rates

- Overall failure rates were 16% (7/45) and 14% (6/44) for the agonist and antagonist substances, respectively.
- Modifications to Protocol Test Substance Solubility Procedures

- Initial protocols used for Phase 2b specified that substances were to be tested up to the limit concentration of 1 mg/ml or to the limit of solubility in 1% DMSO/estrogen-free medium (EFM) during range finder testing using seven point 1:10 serial dilutions.
- Differences in the solubility of test substances in 1% DMSO/EFM were observed across laboratories, resulting in differences in the maximum concentrations used for comprehensive testing.
 - For example:
 - Flavone and genistein were negative for antagonism when tested at Hiyoishi at 10 μg/ml.
 - Flavone and genistein were positive for antagonism when tested at ECVAM and XDS at 100 μg/ml.

- Protocol procedures for determining maximum solubility were modified to minimize differences across laboratories
 - Maximum concentrations for range finder testing were determined by solubility in 100% DMSO (limit concentration of 100 mg/ml).
- Flavone and genistein were positive when retested at Hiyoishi using the modified solubility procedures.

Table 6: Results of Phase 2b Testing

Agonist Test Substance	Laboratory	Agonist Test Results ^a	Antagonist Test Substance ^b	Laboratory	Antagonist Test Results ^a
ATZ	IRD ^b	Negative	API	IRD ^b	Positive
	XDS	Negative		XDS	Positive
	ECVAM	Negative		ECVAM	Positive
BBP	IRD ^b	Positive	ATZ	IRD ^b	Negative
	XDS	Positive		XDS	Positive
	ECVAM	Positive		ECVAM	Positive
DDT	IRD ^b	Positive	BBP	IRD ^b	Negative
	XDS	Positive		XDS	Positive
	ECVAM	Positive		ECVAM	Positive
EE	IRD ^b	Positive	CORT	IRD ^b	Positive
	XDS	Positive		ECVAM	Positive
	ECVAM	Positive		Hiyoshi	Positive
FLA	IRD ^b	Positive	DDT	IRD ^b	Positive
	XDS	Positive		XDS	Positive
	ECVAM	Positive		ECVAM	Positive
GEN	IRD ^b	Positive			