Testing of Coded Substances for a Multi-phased International Validation Study of an Estrogen Receptor Transcriptional Activation Assay

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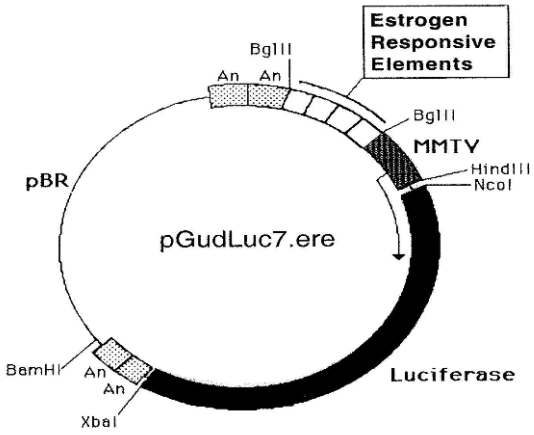
Introduction

- The LUMI-CELL® estrogen receptor (ER) assay is an ER transcriptional activation (TA) test method developed to detect ER agonists and antagonists. NICEATM, ECVAM, and JaCVAM are conducting an international multi-laboratory validation study to evaluate the reproducibility and accuracy of this assay.
- This four-phased study will evaluate all 78 of the reference substances recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) for validation of *in vitro* ER test methods (53 of which are considered as minimum for validation).
- In Phase 1, the repeated testing of reference standards and controls to establish laboratory-specific acceptance criteria was conducted in each of three participating laboratories (XDS, Inc., in the United States, ECVAM in Europe, and Hiyoshi, Corp., in Japan).
- Phase 1 results demonstrated acceptable initial intralaboratory reproducibility and established quality controls for testing of coded reference substances in subsequent phases.
- In Phase 2, repeated testing of coded reference substances covering a range of estrogenic activities was conducted in each of three participating laboratories in two stages (four agonist and four antagonist substances in Phase 2a and eight agonist and eight antagonist substances in Phase 2b) to optimize protocols to be used for the testing of the remaining reference substances in Phases 3 and 4 (currently in progress) and evaluate the accuracy of the assay.

Overview of the LUMI-CELL® ER Assay

- The assay measures whether and to what extent a substance induces or blocks TA activity via an ER-mediated pathway in recombinant BG-1Luc4E2 cells.
- The BG-1Luc4E2 cell line (Rogers et al. 2000) was derived from BG-1 human adenocarcinoma cells that endogenously express human ER and that have been stably transfected with the pGudLuc7.ERE plasmid **(Figure 1)**.
- BG-1Luc4E2 cells express luciferase activity in response to estrogen and estrogen-like substances.
- BG-1Luc4E2 cells are selected with G418 and then conditioned in estrogen-free medium (EFM) for at least 48 hours.
- Cells are then seeded into 96-well plates for 24 to 48 hours and then incubated in EFM containing reference standard, control, or test substance for 19 to 24 hr.
- Cytotoxicity is evaluated visually, after which cells are lysed and treated with luciferase reagent.
- Luminescence is measured and expressed as relative light unit (RLUs).

Figure 1 The pGudLuc7.ERE Plasmid[1]



[1] The pGudLuc7.ERE Plasmid[1] 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

Agonist Reference Standard and Controls

- Vehicle control = dimethyl sulfoxide (DMSO, CASRN 67-68-5): 1% (v/v) DMSO in EFM

- Reference standard = 17β-estradiol (E2, CASRN 50-28-2) using an eleven point serial dilution
- Weak positive control = p,p'-methoxychlor (methoxychlor, CASRN 72-43-5) at 3.13µg/mL

Antagonist Reference Standards and Controls

- Vehicle control = 1% (v/v) DMSO in EFM
- Reference standard = raloxifene HCl (Ral, CASRN 84449-90-1 with E2): A nine-point serial dilution of Ral with a fixed concentration of E2 (a concentration that results in approximately 80% of the maximum response for E2 in the assay [Ral/E2])
- Reference estrogen = E2 used as the base line reference estrogen.
- Positive control = flavone (CASRN 525-82-6), at 25 µg/mL, with E2 at 2.5 x 10^{-5} µg/mL, (flavone/E2)

Phase 1 Testing

- Reference standards and controls were tested 10 times in all 3 laboratories
- Intralaboratory reproducibility evaluated
- Established initial quality controls for testing of coded reference substances

Figure 2 Phase 1 Agonist Results

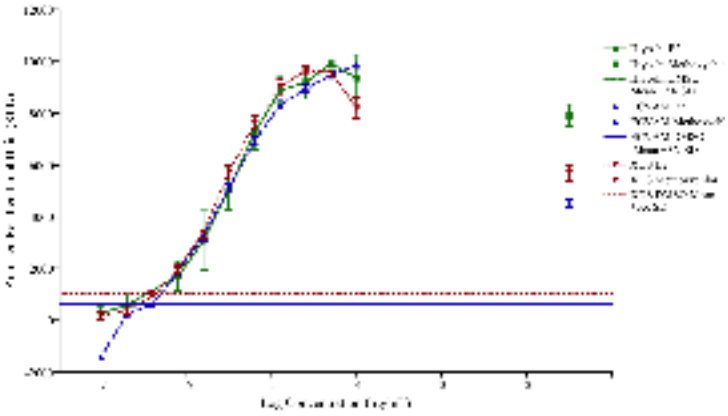
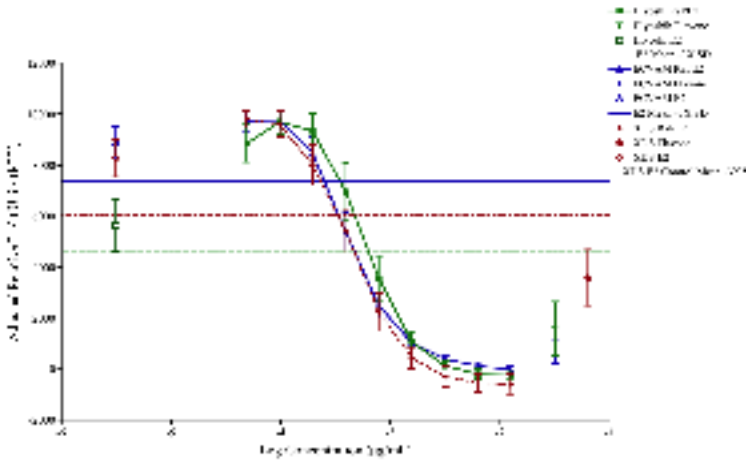


Figure 3 Phase 1 Antagonist Results



Phase 2 Testing

- Coded agonist and antagonist substances tested (**Table 1**)
- Intra- and inter-laboratory reproducibility was evaluated
- Accuracy and reliability were evaluated

Table 1 Substances Tested in Phase 2*

	Agonist Test Substances		Antagonist Test Substances	
	Substance Name	**CASRN**	**Substance Name**	**CASRN**
Phase 2a	Bisphenol A (BPA)	80-05-7	Dibenzo[*a.h*]anthracene (DBA)	53-70-3
	Bisphenol B (BPB)	77-40-7	*p*-n-nonylphenol (NON)	104-40-5
	Corticosterone (CORT)	50-22-6	Progesterone (PROG)	57-83-0
	Diethylstilbestrol (DES)	56-53-1	Tamoxifen (TAM)	10540-29-1
Phase 2b	Atrazine (ATR)	1912-24-9	Apigenin (API)	520-36-5
	Butylbenzyl phthalate (BBP)	85-68-7	Atrazine (ATR)	1912-24-9
	o.p'-DDT (DDT)	789-02-6	Butylbenzyl phthalate (BBP)	85-68-7
	17-α ethinyl estradiol (EE)	57-63-6	Corticosterone (CORT)	50-22-6
	Flavone (FLA)	525-82-6	*o.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
	Vinclozolin (VIN)	50471-44-8	Resveratrol (RES)	501-36-0

Abbreviation: CASRN = Chemical Abstracts Chemical Registry Number

*Substances selected from the ICCVAM list of minimum reference substances (ICCVAM 2003)

Phase 2a Results

Table 2 Results of Phase 2a Testing

Agonist Test Substance	Laboratory	Agonist Test Results*	Antagonist Test Substance	Laboratory	Antagonist Test Results*
BPA	ICCVAM[1] Reference Data	Positive	DBA	ICCVAM Reference Data	Positive
	XDS	Positive		XDS	Positive
	ECVAM	Positive		ECVAM	Positive
	Hiyoshi	Positive		Hiyoshi	Positive
BPB	ICCVAM Reference Data	Positive	NON	ICCVAM Reference Data	Positive
	XDS	Positive		XDS	**Negative**
	ECVAM	Positive		ECVAM	Positive
	Hiyoshi	Positive		Hiyoshi	Positive
CORT	ICCVAM Reference Data	Negative	PROG	ICCVAM Reference Data	Negative
	XDS	Negative		XDS	**Positive**
	ECVAM	**Positive**		ECVAM	**Positive**
	Hiyoshi	Negative		Hiyoshi	**Positive**
DES	ICCVAM Reference Data	Positive	TAM	ICCVAM Reference Data	Positive
	XDS	Positive		XDS	Positive
	ECVAM	Positive		ECVAM	Positive
	Hiyoshi	Positive		Hiyoshi	Positive

*Results in **bold** are discordant from ICCVAM Reference Data

[1]From ICCVAM (2003)

Phase 2a Test Plate Acceptance Criteria

Agonist test plate acceptance criteria used in Phase 2a testing:

- Plate induction > 3-fold (averaged highest E2 reference standard RLU divided by the averaged DMSO RLU)
- E2 $EC_{50} \leq 2.5$ times the standard deviation (SD) of the historical database E2 EC_{50}
- DMSO RLU ≤ 2.5 times the standard deviation of the historical DMSO RLU
- Methoxychlor control RLU ≤ 2.5 times the standard deviation of the historical methoxychlor control RLU

Antagonist test plate acceptance criteria used in Phase 2a testing:

- Plate reduction > 3 fold (averaged highest Ral/E2 reference standard RLU divided by the averaged lowest Ral/E2 reference standard RLU)
- Ral/E2 IC_{50} values $\leq$ 2.5 times the standard deviation of the historical database Ral/E2 IC_{50} value
- DMSO RLU ≤ 2.5 times the standard deviation of the historical DMSO RLU
- E2 control RLU must be $\leq$ 2.5 times the standard deviation of the historical E2 control
- Flavone/E2 control RLU must be ≤ 2.5 times the standard deviation of the historical flavone/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 of antagonist classifications.
    - Only changes to acceptance criteria for agonist E2 reference standard EC_{50} and methoxychlor control, and antagonist Ral/E2 reference standard IC_{50} and flavone/E2 control values were considered for modification.
    - Acceptance criteria based on DMSO control values, agonist E2 reference standard fold induction, antagonist Ral/E2 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 EC_{50} and IC_{50} 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 EC_{50} and Methoxychlor Control Acceptance Criteria[1]

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	+ (3/3)	+ (4/4)	DNF	DNF
	ECVAM	13	+ (3/3)	+ (7/7)	+ (3/3)	DNF
	Hiyoshi	4	+ (3/3)	DNF	+ (1/1)	DNF
BPB	XDS	7	+ (3/3)	+ (4/4)	DNF	DNF
	ECVAM	9	+ (3/3)	+ (4/4)	DNF	+ (2/2)
	Hiyoshi	4	+ (3/3)	DNF	+ (1/1)	DNF
CORT	XDS	7	+ (1/3)	+ (1/4)	DNF	DNF
	ECVAM	13	+ (3/3)	+ (5/7)	+ (3/3)	DNF
	Hiyoshi	4	+ (0/4)	DNF	DNF	DNF
DES	XDS	7	+ (3/3)	+ (4/4)	DNF	DNF
	ECVAM	9	+ (3/3)	+ (4/4)	DNF	+ (2/2)
	Hiyoshi	4	+ (4/4)	DNF	DNF	DNF

Abbreviations: DNF = did not fail acceptance criteria; E2 = 17β-estradiol; EC_{50} = half-maximal effective concentration

[1]Data in parentheses represent the proportion of positive results out of the total number of tests

Table 4 Qualitative Evaluation of Antagonist Ral/E2 Reference Standard IC_{50} and Flavone/E2 Positive Control Acceptance Criteria[1]

Antagonist Test Substances	Laboratory	Number of Tests	Passed All Acceptance Criteria	Failed Ral/E2 IC_{50} Only	Failed Flavone/E2 Only	Failed both Ral/E2 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	+ (0/3)	+ (0/3)	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 acceptance criteria; E2 = 17β-estradiol; IC_{50} = concentration of test substance that inhibits E2 response by 50%; Ral = raloxifene HCL

[1] Data in parentheses represent the proportion of positive results out of the total number of tests

Table 5 Comparison of Test Substance EC_{50} and IC_{50} Values from Plates that Passed or Failed Agonist and Antagonist Reference Standard and Positive Control 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[1]
	N	Mean EC_{50} Value[2]	SD[2]	N	Mean EC_{50} Value[2]	SD[2]	
XDS/BPA	3	8.8×10^{-2}	7.2×10^{-3}	4	9.9×10^{-2}	1.4×10^{-2}	0.40
ECVAM/BPA	3	1.9×10^{-1}	7.6×10^{-3}	10	1.6×10^{-1}	5.6×10^{-2}	0.16
XDS/BPB	3	3.9×10^{-2}	6.0×10^{-3}	4	4.3×10^{-2}	1.1×10^{-2}	0.63
ECVAM/BPB	3	4.2×10^{-2}	1.3×10^{-2}	4	7.5×10^{-2}	1.7×10^{-2}	0.06
XDS/DES	4	1.4×10^{-5}	5.0×10^{-6}	4	2.6×10^{-5}	1.1×10^{-5}	0.20
Laboratory and Substance Evaluated	**Antagonist Plates that Passed All Acceptance Criteria**			**Antagonist Plates that did not Pass Ral/E2 IC_{50} and/or Flavone/E2 Acceptance Criteria**			**P Value[1]**
	N	**Mean IC_{50} Value[2]**	**SD[2]**	**N**	**Mean IC_{50} Value[2]**	**SD[2]**	
XDS/TAM	4	1.5×10^{-1}	5.7×10^{-2}	3	3.1×10^{-1}	8.8×10^{-2}	0.11

Abbreviations: E2 = 17β-estradiol; EC_{50} = half-maximal effective concentration; IC_{50} = concentration of test substance that inhibits E2 response by 50%; Flavone/E2 = antagonist positive control; Methoxychlor = agonist positive control; N = number of plates; Ral = raloxifene HCL; SD = Standard Deviation

[1] $P>0.05$ indicates that EC_{50} or IC_{50} values are not significantly different

[2] EC_{50} and IC_{50} values are expressed in μg/mL

Modifications to the Protocols

Modifications to Acceptance Criteria

Acceptance criteria based on EC_{50}, IC_{50}, methoxychlor control and flavone/E2 control values were removed from the protocols.

Acceptance criteria were modified as follows:

- Agonist 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 methoxychlor RLU > 3x SD of the mean DMSO RLU.
- Antagonist acceptance criteria:
  - Ral/E2 standard curve should be sigmoidal in shape and have at least three values within the linear portion of the curve.
  - Mean flavone/E2 RLU < 3x SD of the mean E2 control RLU
  - E2 control RLU must be ≤ 2.5 times the standard deviation of the historical E2 control

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/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 Hiyoshi 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 Hiyoshi using the modified solubility procedures.

Table 6 Results of Phase 2b Testing

Agonist Test Substance	Laboratory	Agonist Test Results*	Antagonist Test Substance	Laboratory	Antagonist Test Results*
ATZ	ICCVAM Reference Data[1]	Negative	API	ICCVAM Reference Data	Positive
	XDS	Negative		XDS	Positive
	ECVAM	Negative		ECVAM	Positive
	Hiyoshi	Negative		Hiyoshi	Positive
BBP	ICCVAM Reference Data	Positive	ATZ	ICCVAM Reference Data	Negative
	XDS	Positive		XDS	**Positive**
	ECVAM	Positive		ECVAM	**Positive**
	Hiyoshi	Positive		Hiyoshi	**Positive**
DDT	ICCVAM[1] Reference Data	Positive	BBP	ICCVAM Reference Data	Negative
	XDS	Positive		XDS	**Positive**
	ECVAM	Positive		ECVAM	**Positive**
	Hiyoshi	Positive		Hiyoshi	**Positive**
EE	ICCVAM Reference Data	Positive	CORT	ICCVAM Reference Data	Negative
	XDS	Positive		XDS	**Positive**
	ECVAM	Positive		ECVAM	**Positive**
	Hiyoshi	Positive		Hiyoshi	**Positive**
FLA	ICCVAM Reference Data	Positive	DDT	ICCVAM Reference Data	Positive
	XDS	Positive		XDS	Positive

	ECVAM	Positive		ECVAM	Positive
	Hiyoshi	Positive		Hiyoshi	Positive
GEN	ICCVAM Reference Data	Positive	FLA	ICCVAM Reference Data	Positive
	XDS	Positive		XDS	Positive
	ECVAM	Positive		ECVAM	Positive
	Hiyoshi	Positive		Hiyoshi	Positive
NON	ICCVAM Reference Data	Positive	GEN	ICCVAM Reference Data	Positive
	XDS	Positive		XDS	Positive
	ECVAM	Positive		ECVAM	Positive
	Hiyoshi	Positive		Hiyoshi	Positive
VIN	ICCVAM Reference Data	Negative	RES	ICCVAM Reference Data	Positive
	XDS	Negative		XDS	Positive
	ECVAM	**Positive**		ECVAM	Positive
	Hiyoshi	Negative		Hiyoshi	Positive

*Results in **bold** are discordant from ICCVAM reference data

[1]ICCVAM reference data from NIH Pub. No. 03-4503 (ICCVAM 2003)

Summary

Phase I (Reference Standards and Controls)

- Completed in Feb 2008
- Results demonstrated acceptable initial intralaboratory reproducibility and established initial quality controls for testing of coded reference substances in Phase 2

Phase 2a (4 Agonists and 4 Antagonists)

- Completed in Sep 2008
- A large number of tests failed one or more study acceptance criteria
- Acceptance criteria were modified to reduce test plate failure rates before progressing top Phase 2b

Phase 2b (8 Agonists and 8 Antagonists)

- Completed in Mar 2009
- Test plate failure rates were significantly reduced compared to Phase 2a
- Differences in the solubility of test substances in 1% DMSO/EFM resulted in discordance in antagonist testing
- Substances discordant for antagonist activity were retested using modified procedures and discordance was eliminated
- Acceptable intra- and inter-laboratory reproducibility
- These results underscore the importance of a phased study design to allow for protocol refinements
- Modified protocols to be used for Phase 3 testing of 41 agonists and 41 antagonists

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