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

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Abstract[1]

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 EDSP 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.

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

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.

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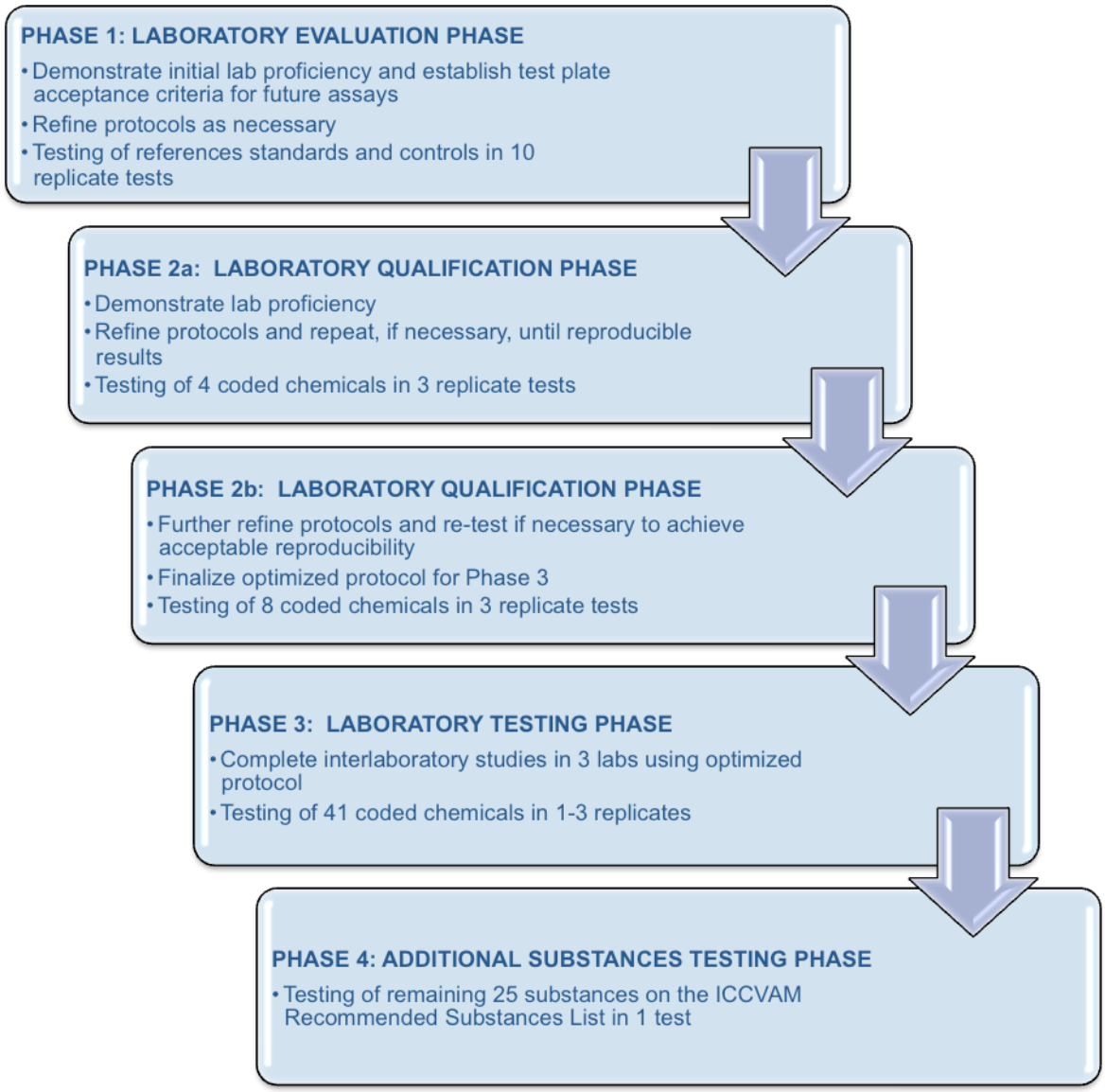
Introduction

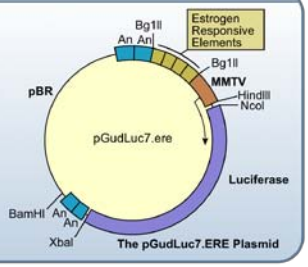
- The LUMI-CELL® ER assay is a TA test method developed to detect ER agonists and antagonists.
  - There are currently no adequately validated *in vitro* test methods for identifying estrogen antagonists
    - The LUMI-CELL(R) ER assay will provide the first validated screening method for estrogen antagonists
- 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

- The assay measures how much a substance induces or blocks transcriptional activation activity via an estrogen receptor-mediated pathway in recombinant BG-1Luc4E2 cells (Rogers et al. 2000).
- The cell line was derived from human cells that endogenously express human estrogen receptor. It has been stably transfected with the pGudLuc7.ERE plasmid (pictured at right).
- These cells express luciferase activity in response to estrogen and estrogen-like substances.



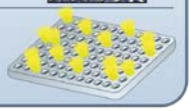
Assay Protocol

1. Select BG-1Luc4E2 cells with G418 and condition in estrogen-free medium for at least 48 hours.

2. Seed cells into 96-well plates for 24 to 48 hours
   - Incubate in estrogen-free medium containing reference standard, control, or test substance for 19 to 24 hr.

3. Evaluate cells visually for cytotoxicity, then lyse and treat with luciferase reagent.

4. Measure luminescence (expressed as relative light units or RLUs).



Reference Substances

	Vehicle Control	Reference Standard	Reference Estrogen	Positive Control
Agonist Protocol	1% DMSO	17β-estradiol	(none used)	p,p'-methoxychlor (weak 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**)
- Intra-laboratory reproducibility evaluated
- Established initial quality controls for testing of coded reference substances

Figure 2 Phase 1 Agonist Results

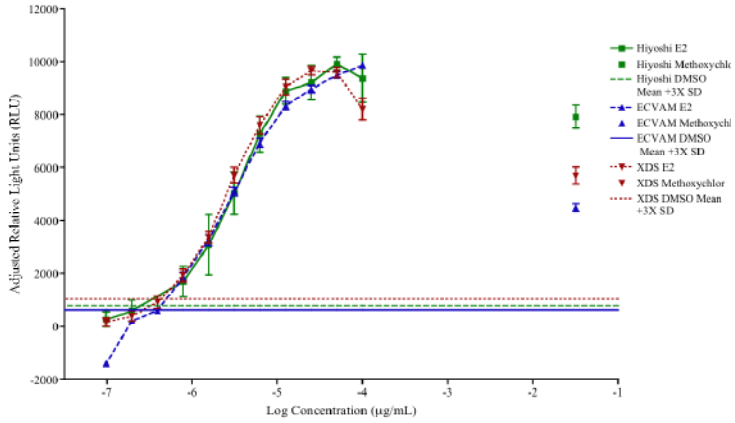
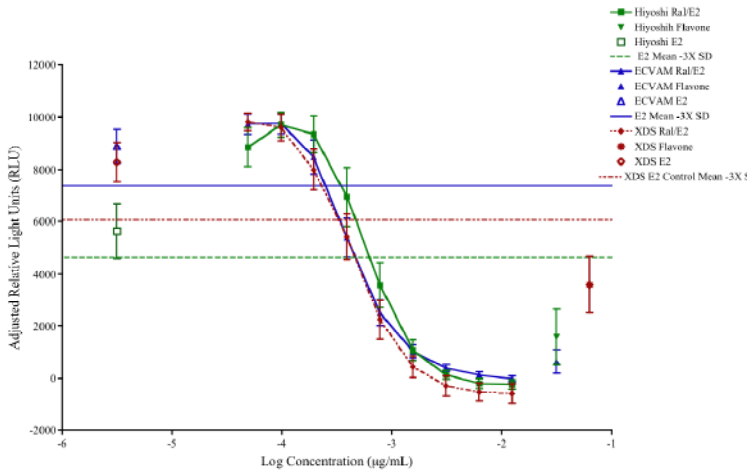


Figure 3 Phase 1 Antagonist Results



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*

Study Phase	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 2006)

Table 2 Results of Phase 2a Testing

Agonist Test Substance	Laboratory	Agonist Test Results*	Antagonist Test Substance	Laboratory	Antagonist Test Results*
BPA	IRD[1]	Positive	**DBA**	IRD[1]	Positive
	XDS	Positive		XDS	Positive
	ECVAM	Positive		ECVAM	Positive
	Hiyoshi	Positive		Hiyoshi	Positive
BPB	IRD[1]	Positive	**NON**	IRD[1]	Positive
	XDS	Positive		XDS	**Negative[2]**
	ECVAM	Positive		ECVAM	Positive
	Hiyoshi	Positive		Hiyoshi	Positive
CORT	IRD[1]	Negative	**PROG**	IRD[1]	Negative
	XDS	Negative		XDS	**Positive**
	ECVAM	**Positive[3]**		ECVAM	**Positive**
	Hiyoshi	Negative		Hiyoshi	**Positive**
DES	IRD[1]	Positive	**TAM**	IRD[1]	Positive
	XDS	Positive		XDS	Positive
	ECVAM	Positive		ECVAM	Positive
	Hiyoshi	Positive		Hiyoshi	Positive

Abbreviations: IRD = ICCVAM Reference Data
*Results in **bold** are discordant from ICCVAM Reference Data
[1]IRD=ICCVAM reference data; from ICCVAM (2006)
[2]Positive during standardization studies at one non-cytotoxic concentration. Negative results in Phase 2a due to cytotoxicity at all doses that could be considered positive.
[3]Borderline positive 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 EC_{50} ≤ 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 (MET) RLU ≤ 2.5 times the standard deviation of the historical MET RLU

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

- Plate reduction > 3 fold (averaged highest Raloxifene/E2 [Ral/E2] reference standard RLU divided by the averaged lowest Ral/E2 reference standard RLU)

- Ral/E2 IC_{50} values ≤ 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 ≤ 2.5 times the standard deviation of the historical E2 control
- Flavone/E2 control (FLA/E2) RLU must be ≤ 2.5 times the standard deviation of the historical FLA/E2 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 EC_{50} and MET, and antagonist Ral/E2 reference standard IC_{50} and FLA/E2 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 Test Plate Acceptance Criteria[1]

Agonist Test Substances	Laboratory	Number of Tests	Passed All Test Plate Acceptance Criteria	Failed E2 EC_{50} Only	Failed Methoxy-chlor 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 acceptance criteria; E2 = 17β-estradiol; EC_{50} = half-maximal effective concentration

[1]Data 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 produces an adjusted RLU value greater than the RLU value of the mean DMSO control plus three times the standard deviation of the DMSO mean. Otherwise, it is considered negative (-).

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 Test Plate 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 (+)	DNF	DNF
	ECVAM	3	3 (+)	DNF	DNF	DNF
	Hiyoshi	3	3 (+)	DNF	DNF	DNF
NON	XDS	6	3 (-)	3 (-)	DNF	DNF
	ECVAM	3	3 (+)	DNF	DNF	DNF
	Hiyoshi	3	3 (+)	DNF	DNF	DNF
PROG	XDS	6	3 (+)	3 (-)	DNF	DNF
	ECVAM	3	3 (+)	DNF	DNF	DNF
	Hiyoshi	3	3 (+)	DNF	DNF	DNF
TAM	XDS	6	3 (+)	3 (+)	DNF	DNF
	ECVAM	5	3 (+)	DNF	2 (+)	DNF
	Hiyoshi	3	3 (+)	DNF	DNF	DNF

Abbreviations: DNF = did not fail test plate acceptance criteria; E2 = 17β-estradiol; IC_{50} = concentration of test substance that inhibits E2 response by 50%; Ral = raloxifene HCL

[1]Data in parentheses indicate the qualitative response (positive or negative) of the test substance. A test substance is considered to be positive (+) for ER antagonism if the test substance has an adjusted RLU value less than the RLU value of the mean E2 control minus three times the standard deviation of the E2 control mean. Otherwise, it is considered negative (-).

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 Test Plate Acceptance Criteria

Laboratory and Substance Evaluated	Agonist Plates that Passed All Test Plate Acceptance Criteria			Agonist Plates that did not Pass E2 EC_{50} and/or Methoxychlor Test Plate 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 Test Plate Acceptance Criteria**			**Antagonist Plates that did not Pass Ral/E2 IC_{50} and/or Flavone/E2 Test Plate 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
[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 Test Plate Acceptance Criteria

During Phase 2a, test plates that failed test plate acceptance due to failure of the E2 EC_{50}, Ral\E2 IC_{50}, MET or flavone\E2 controls did not alter the expected test substance results (**Table 5**). Test plate acceptance criteria based on EC_{50}, IC_{50}, 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 methoxychlor RLU > 3x SD of the mean DMSO RLU
- Antagonist Test Plate 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 methoxychlor RLU < 3x SD of the mean DMSO RLU
  - Mean flavone/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 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[1]	Laboratory	Antagonist Test Results*
ATZ	IRD[2]	Negative	**API**	IRD[2]	Positive
	XDS	Negative		XDS	Positive
	ECVAM	Negative		ECVAM	Positive
	Hiyoshi	Negative		Hiyoshi	Positive
BBP	IRD[2]	Positive	**ATZ**	IRD[2]	Negative
	XDS	Positive		XDS	**Positive**
	ECVAM	Positive		ECVAM	**Positive**
	Hiyoshi	Positive		Hiyoshi	**Positive**
DDT	IRD[2]	Positive	**BBP**	IRD[2]	Negative
	XDS	Positive		XDS	**Positive**
	ECVAM	Positive		ECVAM	**Positive**
	Hiyoshi	Positive		Hiyoshi	**Positive**
EE	IRD[2]	Positive	**CORT**	IRD[2]	Negative
	XDS	Positive		XDS	**Positive**
	ECVAM	Positive		ECVAM	**Positive**
	Hiyoshi	Positive		Hiyoshi	**Positive**
FLA	IRD[2]	Positive	**DDT**	IRD[2]	Positive
	XDS	Positive		XDS	Positive
	ECVAM	Positive		ECVAM	Positive
	Hiyoshi	Positive		Hiyoshi	Positive
GEN	IRD[2]	Positive	**FLA**	IRD[2]	Positive
	XDS	Positive		XDS	Positive
	ECVAM	Positive		ECVAM	Positive
	Hiyoshi	Positive		Hiyoshi	Positive
NON	IRD[2]	Positive	**GEN**	IRD[2]	Positive
	XDS	Positive		XDS	Positive
	ECVAM	Positive		ECVAM	Positive
	Hiyoshi	Positive		Hiyoshi	Positive
VIN	IRD[2]	Negative	**RES**	IRD[2]	Positive
	XDS	Negative		XDS	Positive
	ECVAM	**Positive**		ECVAM	Positive
	Hiyoshi	Negative		Hiyoshi	Positive

Abbreviations: IRD = ICCVAM Reference Data

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

[1]Results shown for Flavone and Genistein are results obtained during retesting.

[2]ICCVAM reference data from ICCVAM 2006

Phase 3 Testing

- Coded agonist and antagonist substances were tested (**Table 7**)
- Each substance was tested at least once in each laboratory
- Inter-laboratory reproducibility are being evaluated
- Accuracy and Reliability are being evaluated

Table 7 Substances Tested in Phase 3*

Agonist Test Substances		Antagonist Test Substances	
Substance Name	**CASRN**	**Substance Name**	**CASRN**
Actinomycin D	50-76-0	Actinomycin D	57-76-0
4-androstenedione	63-05-8	4-androstenedione	63-05-8
Apigenin	520-36-5	Bisphenol A	80-05-7
Clomiphene citrate	50-41-9	Bisphenol B	77-40-7
Coumestrol	479-13-0	Clomiphene citrate	50-41-9
4-cumylphenol	599-64-4	Coumestrol	479-13-0
Daidzein	486-66-8	4-cumylphenol	599-64-4
Dibenzo[*a.h*]anthracene	53-70-3	Daidzein	486-66-8
Di-*n*-butyl phthalate	84-74-2	Di - *n* -butyl phthalate	84-74-2
p.p'-DDE	72-55-9	*p.p'*-DDE	72-55-9
Diethylhexyl phthalate	117-81-7	Diethylhexyl phthalate	117-81-7
Dexamethasone	50-02-2	Diethylstilbestrol	56-53-1
5α-dihydrotestosterone	521-18-6	Dexamethasone	50-02-2
Dicofol	115-32-2	5α̃dihydrotestosterone	521-18-6
17-α estradiol	57-91-0	Dicofol	115-32-2
17-β estradiol	50-28-2	17a-Estradiol	57-91-0
Ethyl paraben	120-47-8	17ß-estradiol	50-28-2
Estrone	53-16-7	17a- ethinyl estradiol	57-63-6
Fluoranthene	206-44-0	Ethyl paraben	120-47-8
meso-hexestrol	84-16-2	Estrone	53-16-7
Hydroxyflutamide	52806-53-8	Fluoranthene	206-44-0
Kepone	143-50-0	*meso*-hexestrol	84-16-2
Kaempferol	520-18-3	Hydroxyflutamide	52806-53-8
p.p'-methoxychlor	72-43-5	Kepone	143-50-0
Morin	480-16-0	Kaempferol	520-18-3
Methyl testosterone	58-18-4	*p,p'*-methoxychlor	72-43-5
Norethynodrel	68-23-5	Morin	480-16-0
4-*tert*-octylphenol	140-66-9	Methyl testosterone	58-18-4
4-hydroxytamoxifen	68047-06-3	Norethynodrel	68-23-5
Phenobarbital[1]	50-06-6	4-*tert*-octylphenol	140-66-9
Phenolphthalin	81-90-3	4-hydroxytamoxifen	68047-06-3
Progesterone	57-83-0	Phenobarbital[1]	50-06-6
Propylthiouracil	51-52-5	Phenolphthalin	81-90-3
Raloxifene HCl	82640-04-8	Propylthiouracil	51-52-5
Resveratrol	501-36-0	Raloxifene HCl	82640-04-8

Agonist Test Substances		Antagonist Test Substances	
Substance Name	**CASRN**	**Substance Name**	**CASRN**
Sodium azide	26628-22-8	Sodium azide	26628-22-8
2-*sec*-butylphenol	89-72-5	2-*sec*-butylphenol	89-72-5
Tamoxifen	10540-29-1	2,4,5-Trichlorophenoxyacetic acid	93-76-5
2,4,5-trichlorophenoxyacetic acid	93-76-5	Testosterone	58-22-0
Testosterone	58-22-0	12 - *O* - Tetradecanoylphorbol-13-acetate	16561-29-8
12 – *O* - Tetradecanoylphorbol-13-acetate	16561-29-8	Vinclozolin	50471-44-8

Abbreviation: CASRN = Chemical Abstracts Chemical Registry Number
*Substances selected from the ICCVAM list of minimum reference substances (ICCVAM 2006)
[1]Hiyoshi Corp. did not posses necessary licensing to test phenobarbital, a Schedule 4 controlled substance. This substance was not tested at Hiyoshi Corp.

Phase 4 Testing

- Additional testing Phase. Testing was conducted at XDS, Inc.
- Coded agonist and antagonist substances were tested (**Table 8**)
- Each substance was tested at least once
- Results of Phase 4 testing are being evaluated

Table 8 Substances Tested in Phase 4*,†

Agonist and Antagonist Test Substances	
Substance Name	**CASRN**
Ammonium perchlorate	7790-98-9
Apomorphine	58-00-4
Bicalutamide	90357-06-5
Cycloheximide	66-81-9
Chrysin	480-40-0
Cyproterone acetate	427-51-0
Finasteride	98319-26-7
Fenarimol	60168-88-9
Flutamide[3]	13311-84-7
Fluoxymestrone	76-43-7
Haloperidol	52-86-8
Ketoconazole	65277-42-1
Linuron	330-55-2
L-thyroxine	51-48-9
Mifepristone	84371-65-3

Agonist and Antagonist Test Substances	
Substance Name	**CASRN**
Medroxyprogesterone acetate	71-58-9
Nilutamide	63612-50-0
19-nortestosterone	434-22-0
4-hydroxy androstenedione	566-48-3
Oxazepam	604-75-1
Procymidone	32809-16-8
Pimozide	2062-78-4
Reserpine	50-55-5
Spironolactone	52-01-7
17β-trenbolone	10161-33-8

*Substances selected from the ICCVAM list of reference substances (ICCVAM 2006)

†The same set of substances were tested for both agonism and antagonism.

SUMMARY

Phase I

- Completed in February 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, 4 antagonists)

- Completed in September 2008
- A large number of tests failed one or more test plate acceptance criteria
- Test plate acceptance criteria were revised prior to Phase 2b to consist of:
  - DMSO control values should be ≤ 2.5 times the standard deviation of the historical DMSO control
  - Reference standard curves should be sigmoidal in shape
  - Methoxychlor (agonist) and flavone/E2 (antagoinst) controls should be positive
  - In the antagonist assay, the E2 control RLU must be ≤ 2.5 times the standard deviation of the historical E2 control

Phase 2b (8 agonists, 8 antagonists)

- Completed in March 2009
- Test plate failure rates were significantly reduced compared to Phase 2a
- Differences in the solubility of test substances in 1% DMSO/estrogen-free medium between the laboratories resulted in discordance in antagonist testing
  - This discordance appeared to be due to differences in the maximum concentrations that were being reported as soluble in 1% DMSO/EFM
  - To eliminate these differences, the laboratory that most often reported the lowest soluble concentrations would retest the using the solubility in 100% DMSO.
- Retested substances using the solubility in 100% DMSO eliminated the discordance.

- Protocols were updated prior to Phase 3 and 4 testing to require solubility determinations in 100% DMSO prior to testing

Phase 3 (41 agonists, 41 antagonists)

- Completed in December 2009
- Results and inter-laboratory reproducibility are being evaluated

Phase 4 (25 agonists, 25 antagonists)

- Completed in January 2010
- Results are being evaluated

Conclusions

- These results underscore the importance of a phased study design to allow for protocol refinements necessary to ensure a high level of interlaboratory reproducibility
- Final study results will be used to update the available *in vitro* data for the list of 78 ICCVAM recommended reference substances and to develop a final standardized protocol that will form the basis for:
  - Test method performance standards to validate mechanistically and functionally similar ER TA test methods and
  - A new OECD Test Guideline

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