表7．アンタゴニスト試験の結果一覧

					DMSO Average	3155000		
Cell name	Project/Sample ID	Dosing Fraction	Comments	results	Absolute RLU Value	% Cell Death	% Viable	Score
B2	EA1-3.1	40.00	β-estradiol a	-275000	275000	8.7%	91.3%	1
B3	EA1-3.1	20.00	β-estradiol a	-95000	95000	3.0%	97.0%	1
B4	EA1-3.1	10.00	β-estradiol a	-45000	45000	1.4%	98.6%	1
B5	EA1-3.1	5.00	β-estradiol a	-35000	35000	1.1%	98.9%	1
B6	EA1-3.1	2.50	β-estradiol a	15000	15000	0.5%	100.5%	1
B7	EA1-3.1	1.25	β-estradiol a	5000	5000	0.2%	100.2%	1
B8	EA1-3.1	0.63	β-estradiol a	35000	35000	1.1%	101.1%	1
B9	EA1-3.1	0.31	β-estradiol a	35000	35000	1.1%	101.1%	1
B10	EA1-3.1	0.08	β-estradiol a	-15000	15000	0.5%	99.5%	1
B11	EA1-3.1	0.04	β-estradiol a	-25000	25000	0.8%	99.2%	1
C2	EA1-3.1	40.00	β-estradiol b	-225000	225000	7.1%	92.9%	1
C3	EA1-3.1	20.00	β-estradiol b	-165000	165000	5.2%	94.8%	1
C4	EA1-3.1	10.00	β-estradiol b	-65000	65000	2.1%	97.9%	1
C5	EA1-3.1	5.00	β-estradiol b	-45000	45000	1.4%	98.6%	1
C6	EA1-3.1	2.50	β-estradiol b	-35000	35000	1.1%	98.9%	1
C7	EA1-3.1	1.25	β-estradiol b	-15000	15000	0.5%	99.5%	1
C8	EA1-3.1	0.63	β-estradiol b	15000	15000	0.5%	100.5%	1
C9	EA1-3.1	0.31	β-estradiol b	15000	15000	0.5%	100.5%	1
C10	EA1-3.1	0.08	β-estradiol b	-5000	5000	0.2%	99.8%	1
C11	EA1-3.1	0.04	β-estradiol b	-85000	85000	2.7%	97.3%	1
D2	EB-1.1	1.25E+06	Methoxychlor	-435000	435000	13.8%	86.2%	1
D3	EB-1.1	1.25E+06	Methoxychlor	-265000	265000	8.4%	91.6%	1
D4	EB-1.1	1.25E+06	Methoxychlor	-265000	265000	8.4%	91.6%	1
D5	#105K00451	DMSO	control	-5000	5000	0.2%	99.8%	1
D6	#105K00451	DMSO	control	35000	35000	1.1%	101.1%	1
D7	#105K00451	DMSO	control	-25000	25000	0.8%	99.2%	1
D8	#105K00451	DMSO	control	-5000	5000	0.2%	99.8%	1
D9	EG1-1.1	4.00E+07	Bisphenol A	-2936844	2936844	93.1%	6.9%	4
D10	EG1-1.1	3.60E+07	Bisphenol A	-3012886	3012886	95.5%	4.5%	4
D11	EG1-1.1	2.88E+07	Bisphenol A	-2859835	2859835	90.6%	9.4%	4
E2	EG1-1.1	2.02E+07	Bisphenol A	-2065000	2065000	65.5%	34.5%	4
E3	EG1-1.1	1.21E+07	Bisphenol A	-1615000	1615000	51.2%	48.8%	3
E4	EG1-1.1	6.05E+06	Bisphenol A	-605000	605000	19.2%	80.8%	2

4．1　問題となった点

・　“Compound Tracking Form”におけるデーターの採用[PASS or Fail]の基準があいまいであった。Phase I 結果より QC SCATTER CHARTS を作成し、日吉でのデータ許容基準を作成した。基準は、QC/QA　Memorandam に記載し、今後の Phase でのデータ許容基準とする。

・　Ag3 及び Ag4 のデータの不採用理由は、Induction not > 3fold であった。一部の DMSO の Background が高く出たことが原因である。作業中でのコンタミネーションの可能性が考えられたため、それ暴露工程は、安全キャビネット内で行うこととした。

・　細胞の継代数の限界が未確認であった。Phase I では、10～60 代までを使用可能数と判断し分析を行ったが、今後、限界代数の基準が必要と考える。

4．2　試験プロトコールへの質問・提案

・　転写活性測定用試薬(本 Project では、Promega Lusiferase Assay Kit を使用している。)についても様々な商品が販売されており、発光強度が長時間保持し、細胞を溶解さ

せる成分も発光試薬に含まれているワンステップの試薬を使用が可能です。

・　BG-1Cell は、エッジエフェクトが少ないことがわかっているので、Test Plate Layout を変更が可能です。

・　LUMI-CELL®ER International Validation SOW では、細胞の Plete への播種前に一旦 DMEM 培地への置換が必要となります。原因は、現在の SOW 中の RPMI1640 培地・FBS にエストロゲン活性があるためと聞いていますが、別の培地・FBS を使用することで別の培地への置換のいらない方法の検討が必要です。

・　LUMI-CELL®ER International Validation SOW では、細胞がコンフルエントになった状態からの継代操作で G418 の添加を行っていますが、不要ではないでしょうか?

参考文献

1) Xenobiotic Detection Systems, Inc. LUMI-CELL ®Estrogen Receptor(ER) Transcriptional Activation Assays for the Detection of ER Agonist. GLP-COMPLIANT PROTOCOL FORMAT TEST METHOD PROTOCOL August 1.2006
2) Xenobiotic Detection Systems, Inc. LUMI-CELL ®Estrogen Receptor(ER) Transcriptional Activation Assays for the Detection of ER Antagonist. GLP-COMPLIANT PROTOCOL FORMAT TEST METHOD PROTOCOL August 1.2006
3) QA/QC の手引き(概略版)

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

ICCVAM — *Interagency Coordinating Committee on the Validation of Alternative Methods*

Results of Phase I of the LUMI-CELL® ER Assay International Validation Study

Overview (1)

Overview (2)

List of Acronyms and Abbreviations (1)

ANOVA	Analysis of Variance
BPA	Bisphenol A
CASRN	Chemical Abstracts Service Registry Number
CV	Coefficient of Variation
DMSO	Dimethyl Sulfoxide
E2	17ß-Estradiol
EC_{50}	Half-maximal effective concentration
ER	Estrogen receptor
ECVAM	European Centre for the Validation of Alternative Methods
Hiyoshi	Hiyoshi Corporation
IC50	Concentration of substance that inhibits the reference estrogen response by 50%

List of Acronyms and Abbreviations (2)

ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
JaCVAM	Japanese Center for the Validation of Alternative Methods
NICEATM	National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Methods
Ral	Raloxifene HCl
RLU	Relative Light Units
SD	Standard Deviation
Standardization	LUMI-CELL® ER Protocol Standardization Study conducted at XDS
TA	Trancriptional Activation
XDS	Xenobiotic Detection Systems, Inc

Background Information: History and Overview of the LUMI-CELL® Protocol Standardization Study

Nomination and Submission of the LUMI-CELL® ER Assay for Validation

- April, 2004 - NICEATM issued an *FR* notice inviting the nomination of *in vitro* test methods for the detection of potential endocrine disruptors.
- NICEATM received a submission from Xenobiotic Detection Systems (XDS) nominating the LUMI-CELL® ER assay. The submission was evaluated and found to meet ICCVAM's submission guidelines and prioritization criteria.
- ICCVAM recommended that:
  - the assay be considered as a high priority for validation studies as an *in vitro* test method for the detection of test substances with ER agonist and antagonist activity
  - further protocol standardization of the test method be performed

Overview of the LUMI-CELL® ER Assay

- The LUMI-CELL® ER assay is based on a stable recombinant cell line (BG1Luc4E2)
  - BG1 - human ovarian carcinoma cell that expresses endogenous alpha (95%) and beta (5%) estrogen receptors
  - Plasmid pGudLUC7 ERE used to transfect cell line
    - Contains 4 copies of synthetic oligonucleotide containing estrogen response element (ERE)
    - Mouse mammary tumor promoter
    - Firefly luciferase gene
  - Exposure to estrogenic substances causes activation of ERE, which drives transcription of luciferase
  - Luminometer is used to quantify luciferase expression

Standardization of the LUMI-CELL® ER Test Method

- Between October 2005 and July 2006, a protocol standardization study was conducted at XDS.
- The primary goal of the study was to develop standardized agonist and antagonist protocols for use in a multi-laboratory validation study. This included:
  - Selection and standardization of reference standards, vehicle and weak positive controls, and a method for assessing cell viability.
  - Establishing a historical control database to be used for deciding acceptability criteria for tests conducted using a limited number of ICCVAM recommended substances for validation of ER TA binding and transcriptional activation (TA) assays.
  - Testing the adequacy of the protocols with coded ER agonists and antagonists (8 each) selected from among the ICCVAM recommended list of substances to cover the range of responses (negative to strongly positive)

Solvent, Reference Estrogen, Agonist, and Antagonist Controls

- During the protocol standardization study, the following solvent, reference estrogen/anti-estrogen, and controls were selected:

Use	Substance Name	CASRN	Concentration
Solvent	Dimethyl sulfoxide	67-68-5	1%
Agonist Reference Standard	17β-estradiol	50-28-2	10 point serial dilution (1.00×10^{-4} – 9.78×10^{-8} µg/mL)
Agonist Weak Positive Control	*p,p'*-methoxychlor	72-43-5	3.13 µg/mL
Antagonist Reference Standard	Raloxifene HCl	82640-04-8	9 point serial dilution (1.25×10^{-2} – 4.88×10^{-5} µg/mL)
Antagonist Weak Positive Control	Flavone	525-82-6	25 µg/mL
Agonist Reference Standard Used in the Antagonist Assay	17β-estradiol	50-28-2	2.5×10^{-5} µg/mL

Evaluation of Cell Viability

- ICCVAM recommended the use of quantitative tests for the measurement of cell viability in ER TA assays.
- During the protocol standardization study, CellTiter-Glo®, a luminescence-based assay that measures ATP, was compared to an assessment of cell viability based on visual observations of cellular morphology and cell density already in use at XDS.
- The visual observation method can be conducted in the same plate in which luminescence is being evaluated, as opposed to CellTiter-Glo ®, which must be tested in parallel plates. Thus, the visual observation method requires fewer test plates, less test substance and cell culture supplies, and is less costly to use.

Coded Substances for Agonist Testing

Code	Substance Name	CASRN	Supplier	Purity	ER TA Agonist Activity	Additional Basis for Selection
N0001	Atrazine	1912-24-9	ChemService, Inc	98%	-	Cytotoxic
N0002	Bisphenol A	80-5-7	Sigma-Aldrich Corp	100%	+	
N0003	Bisphenol B	77-40-7	City Chemical, LLC	97%	++	
N0004	Corticosterone	50-22-6	Sigma-Aldrich Corp	99%	-	
N0005	*o,p'*-DDT	789-2-6	ChemService, Inc	98%	+	Cytotoxic
N0006	Diethylstilbestrol	56-53-1	Sigma-Aldrich Corp	99%	+++	
N0007	17α-ethinyl estradiol	57-63-6	Sigma-Aldrich Corp	99%	+++	
N0008	Flavone	525-82-6	Sigma-Aldrich Corp	99%	+	

Footnotes for this table are available on slide number 13

Footnotes for Slide 12

- Abbreviations: CASRN = Chemical Abstracts Service Registry Number; Co = Company; Corp = Corporation; Inc = Incorporated; LLC = Limited Liability Corporation
- [1]Data on agonist and antagonist activities were derived from the March, 2006 Addendum to ICCVAM Evaluation of In Vitro Test Methods For Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays, NIH Publication No. 03-4503. May 2003 (Addendum)
- [2]+++ Indicates that the substance was strongly active (EC_{50} value was <0.001 μM); ++ indicates that the substance was moderately active (EC_{50} value was between 0.001 and 0.1 μM); + indicates that the substance was weakly active (EC_{50} value was >0.1 μM), or a positive response was reported without an EC_{50} value. The EC_{50} is the effective concentration that causes half-maximal activation of the receptor.
- [3]Information on solubility and cytotoxicity were derived from the addendum and from the scientific literature.

Coded Substances for Antagonist Testing

Code	Substance Name	CASRN	Supplier	Purity	ER TA Antagonist Activity[1,2]	Additional Basis for Selection[3]
N0009	Butylbenzyl phthalate	85-68-7	Sigma-Aldrich Corp	98%	-	
N0010	Dibenzo [a,h] anthracene	53-70-3	Sigma-Aldrich Corp	99%	##	
N0011	Genistein	446-72-0	Sigma-Aldrich Corp	99%	#	Insoluble
N0012	Flavone	525-82-6	Sigma-Aldrich Corp	99%	###	
N0013	*p*-n-nonylphenol	104-40-5	Alfa Aesar, Co	100%	#	
N0014	Progesterone	57-83-0	Sigma-Aldrich Corp	100%	-	
N0015	*o,p'*-DDT	789-2-6	ChemService, Inc	98%	#	Cytotoxic
N0016	Tamoxifen	10540-29-1	Sigma-Aldrich Corp	99%	###	Cytotoxic

Footnotes for this table are available on slide number 15

Footnotes for Slide 14

- Abbreviations: CASRN = Chemical Abstracts Service Registry Number; Co = Company; Corp = Corporation; Inc = Incorporated; LLC = Limited Liability Corporation
- [1]Data on agonist and antagonist activities were derived from the March, 2006 Addendum to ICCVAM Evaluation of In Vitro Test Methods For Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays, NIH Publication No. 03-4503. May 2003 (Addendum)
- [2]### Indicates that the substance was uniformly positive in multiple assays; ## indicates that the substance was positive in the majority of assays in which it was tested; # indicates that the substance was positive in the single assay in which it was tested; #- indicates the substance was positive in one assay but was also negative in one or more assays; - indicates that the substance was uniformly negative in multiple assays
- [3]Information on solubility and cytotoxicity were derived from the addendum and from the scientific literature.

Results of Coded Substance Testing

- Eight coded substances (one set for agonism, one set for antagonism) covering a range of ER agonist and antagonist activities were each tested in three independent experiments for either agonism or antagonism.
- 17α-ethinyl estradiol, diethylstilbestrol, bisphenol A, bisphenol B, o,p'-DDT, and flavone were positive for agonism, while atrazine and corticosterone were negative.
- Tamoxifen, dibenzo[a,h]anthracene, and genistein, were positive for antagonism, while butylbenzyl phthalate, progesterone, nonylphenol, and o,p'-DDT was negative for antagonism when tested up to concentrations that did not induce cytotoxicity.

Summary of Protocol Standardization Study (1)

- Reference standards and controls were selected and standardized for both agonist and antagonist protocols
- Quantitative and qualitative evaluations of cell viability were conducted; there was a high degree of correlation between the visual observation and CellTiterGlo® methods of assessing cell viability.
- Eight coded substances covering a range of ER agonist and antagonist activities tested in three independent experiments gave consistent results, and the results had a high degree of correlation with ICCVAM published data.
- Measuring cytotoxicity was critical in the antagonist assay to avoid false positives.

Summary of Protocol Standardization Study (2)

- Protocol standardization results were reviewed by ICCVAM, who concluded that:
  - The LUMI-CELL® ER agonist and antagonist protocols were standardized.
  - The intralaboratory reproducibility of the standardized protocols was demonstrated.
  - The assay was ready for a multi-laboratory validation study.

The LUMI-CELL® ER Assay International Validation Study

The International Validation Study Design

- A four phase international validation study to evaluate the reproducibility and accuracy of the LUMI-CELL® ER bioassay was organized by NICEATM, ECVAM, and JaCVAM.
- The study uses three laboratories, one each in the United States, Europe, and Japan.
- The study includes:
  - An evaluation of the ability of the standardized LUMI-CELL® ER assay (agonist and antagonist) protocols developed at XDS to be transferred to other laboratories.
  - An opportunity between phases of the validation study for protocol refinement.
  - Testing of 78 coded ICCVAM-recommended test substances
  - Evaluation of assay performance (comparison of results against the published literature and intra- and inter-laboratory reproducibility)

LUMICELL® ER Assay International Validation Study: Study Management Team and Participating Labs

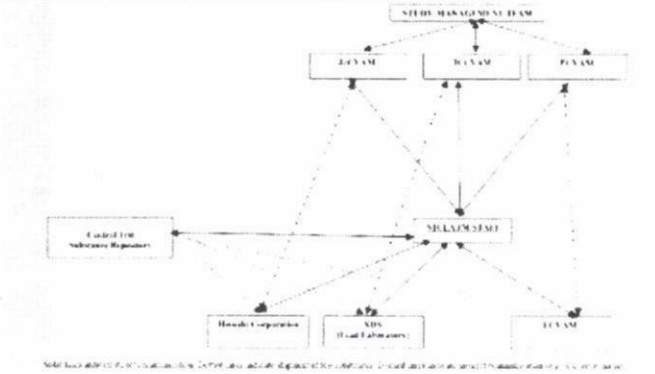
Study Management Team:

- **NICEATM**
  - William S. Stokes, D.V.M., D.A.C.L.A.M. (NIEHS/NTP) Chair
  - Raymond Tice, Ph.D. (NIEHS/NTP) Co-Chair
  - Frank Deal (ILS, Inc.) Project Coordinator
  - Patricia Ceger (ILS, Inc.) Asst. Project Coordinator
  - David Allen, Ph.D. (ILS, Inc.) Principal Investigator, NICEATM Support Contract
- **ECVAM**
  - Thomas Hartung, Ph.D.
  - Susanne Bremer, Ph.D.
- **JaCVAM**
  - Hajime Kojima, Ph.D.
  - Atsushi Ono, Ph.D.

Participating Laboratories:

- Xenobiotic Detection Systems, Inc. (Lead Laboratory), Durham, North Carolina, U.S.
  - John Gordon, Ph.D. (Study Director)
- ECVAM Internal Laboratory, Ispra, Italy
  - Miriam Jacobs, Ph.D. (Study Director)
  - Jan de Lange
- Hiyoshi Corporation, Omi Hachiman, Japan
  - Masafumi Nakamura (Study Director)
  - Hiyoshi Handa

Information Flow for the LUMI-CELL® ER Assay International Validation Effort



International Validation Study Testing Phases and Timelines

STUDY PHASE	ACTIVITY	ORIGINAL TIMELINE	CURRENT TIMELINE
Phase I	Each laboratory conducts multiple testing of reference standards and controls (n = 10) to demonstrate proficiency with agonist and antagonist protocols, establish historical databases to be used to develop acceptability criteria for tests conducted in Phase 2A, and to provide measured or calculated reference standard and control data for an evaluation of intra- and inter-laboratory reproducibility	Jan 07 – May 07	Mar 07 – Jan 08
Phase IIa	Four substances each from the ICCVAM recommended ER minimum list tested independently by each laboratory three times for agonist and antagonist activity	Jun 07 – Jul 07	Mar 08 – Apr 08
Phase IIb	Eight substances each from the ICCVAM recommended ER minimum list tested independently by each laboratory three times for agonist and antagonist activity	Aug 07 – Oct 07	May 08 – Jun 08
Phase III	Remaining 41 substances from ICCVAM recommended ER minimum list tested once by each laboratory for agonist and antagonist activity	Nov 07 – Dec 07	Jul 08 – Aug 08
Phase IV	Remaining 25 substances from ICCVAM recommended ER list tested once each by the lead laboratory only for agonist and antagonist activity	Jan 07 – Feb 07	Sep 08 – Oct 08

Phase I of the LUMI-CELL® ER Assay Validation Study

Overview of Phase I Activities

- XDS evaluated new range finder and comprehensive test plate designs that were modified to improve testing efficiency.
- Conduct multiple testing of agonist and antagonist protocol reference standards and controls using standardized protocols to:
  - Demonstrate proficiency with agonist and antagonist protocols
  - Demonstrate intra- and inter-laboratory reproducibility
  - Develop quality control criteria for Phase IIa testing from a historical database established from the Phase I testing of reference standards and controls

Phase I Validation Study Timeline

Apr 07	Phase I of validation study initiated
	– Two laboratories (XDS, Inc. and Hiyoshi Corp.) began in April 2007
	– ECVAM made decision to conduct validation study using ECVAM "in-house" laboratory
Jun 07	ECVAM laboratory technical lead received training at XDS
Aug 07	NICEATM conducted site visit of Hiyoshi Corp.
	– Laboratories were well organized and maintained, and much of the equipment was state of the art
	– Management and technical personnel were knowledgeable and well trained
Sep 07	ECVAM laboratory began Phase I
Nov 07	NICEATM conducts site visit of ECVAM lab
Feb 08	All laboratories completed Phase I testing

Phase I Protocol Amendment #1

- Before Phase I testing of agonist and antagonist reference standards and controls was initiated, the requirement for a quantitative assessment of cell viability using the CellTiter-Glo® method was removed from the agonist and antagonist protocols. The qualitative visual observation method will be used only.
- This amendment was approved at the 15 May 2007 SMT teleconference .

Phase I Testing and Evaluation of Modified Test Plate Designs at the Lead Laboratory (XDS)

Range Finder Protocol

- The purpose of range finder testing is to select starting concentrations for comprehensive testing.
- For agonist range finder testing, the starting concentration that is selected for comprehensive testing (and subsequently diluted in 11- point double serial dilutions) is one log dilution higher than the concentration giving the highest RLU value.
- For antagonist range finder testing, the concentration that is selected for comprehensive testing (and subsequently diluted in 11- point double serial dilutions) is the concentration giving the lowest RLU value.

Rationale for Modifying Range Finder Plate Designs (1)

- Range finder testing in the protocols developed during the protocol standardization study limited testing to logarithmic (log) serial dilutions for five substances, with each concentration tested in a single well only.
- This approach sometimes resulted in studies where the selection of the starting concentration to be used for comprehensive testing was problematic.
- To minimize this problem in future studies, it was proposed that the study design for range finder testing be made more robust by testing duplicates of each test substance concentration.

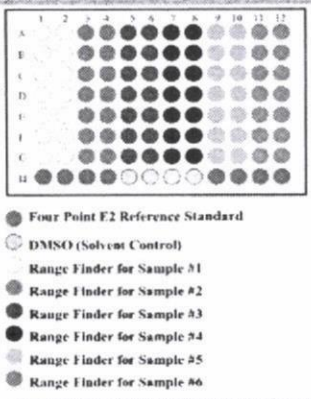
Rationale for Modifying Range Finder Plate Designs (2)

- However, this change would result in a reduction in the number of substances that could be tested on a single plate when using the plate layout used in the protocol standardization study, which excluded using outer wells.
- In order to increase testing efficiency, plate designs were modified to use all 96 wells to run reference standards, controls, and test substances.

Modified Agonist Range Finder Plate Design (1)

- In order to maximize the number of substances that can be tested in duplicate on each agonist range finder plate:
  - The number of concentrations of the E2 reference standard run in duplicate per plate was reduced to four (5.00×10^{-5}, 1.25×10^{-5}, 3.13×10^{-6} and 7.83×10^{-7} µg/mL)
  - The methoxychlor weak positive control was eliminated

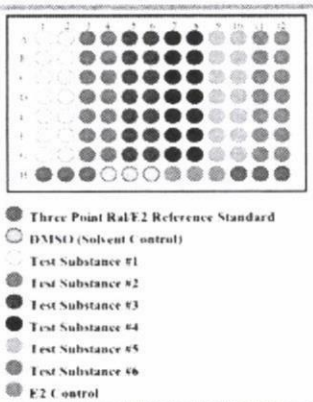
Modified Agonist Range Finder Plate Design (2)



Modified Antagonist Range Finder Plate Design (1)

- In order to maximize the number of substances that can be tested in duplicate on each antagonist range finder plate:
  - The number of concentrations of the Ral/E2 reference standard run in duplicate per plate was reduced to three (1.56×10^{-3}, 3.91×10^{-4}, and 9.77×10^{-5} µg/mL of Ral, with a fixed concentration of E2 (2.5×10^{-5} µg/mL)
  - The flavone/E2 weak positive control was eliminated

Modified Antagonist Range Finder Plate Design (2)



Testing of Modified Range Finder Plate Designs

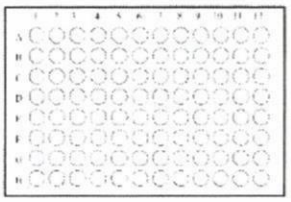
- The outermost wells on 96-well plates are often not used due to possible edging effects resulting from differences in vapor pressure or temperature between outer and inner wells.
- The evaluation of edging effects for the modified range finder plate design using all 96 test plate wells addressed the following questions:
  - Are there significant differences in observed responses (as measured in relative light units [RLUs] recorded from a luminometer) between outside and inside wells (edging effects) using the revised plate design?
  - If edging effects are observed in range finder testing, do they have a significant impact on the selection of concentrations for comprehensive testing?

Testing of "Edging Effects" (1)

- To evaluate the modified plate designs for possible edging effects, observed responses (i.e., RLUs) between outside and inside wells were compared for:
  - ten plates using the agonist protocol to test seven point logarithmic (log) serial dilutions of bisphenol A (BPA, 100 µg/mL to 1 x10^{-4} µg/mL) in the revised plate layout
  - seven plates using the antagonist protocol to test seven point log serial dilutions of tamoxifen (50 µg/mL to 5 x10^{-5} µg/mL) in the revised plate layout

Testing of "Edging Effects" (2)

- Serial dilutions were run in plate columns 1-12 at descending concentrations in rows A-G.



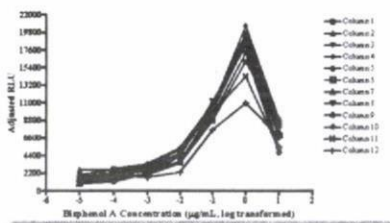
A comparison of RLUs was made between column 1 (left outside wells) and column 2 (adjacent left inside wells), and column 12 (right outside wells) and column 11 (adjacent right insides wells), and using rows B-G (row A was excluded because it is the top outside row on the plate)

Testing of "Edging Effects" (3)

- A total of 204 pairs were evaluated with a Z statistic ($Z \geq 1.96$, 95% confidence interval) using a Sign Test to determine significant differences between outer and inner wells. Analysis of paired values in columns 1 and 2, and 11 and 12 resulted in Z statistics of 5.67 and 2.87 respectively, indicating statistically significant differences in observed RLUs between outer and inner wells.

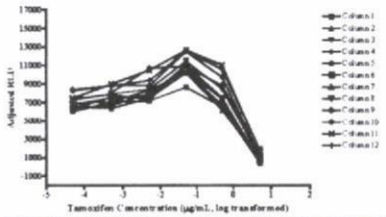
Example of Edging Effects in an Agonist Range Finder Test

- An example of edging effects from the testing of a 7-point log serial dilution of BPA is presented in the graph below (RLUs from plate column 12 [outside right column] are significantly different from those in columns 1 through 11).
- This difference results in a concentration-response curve that is clearly lower in magnitude for the BPA serial dilutions run in plate column 12. However, the shape of all concentration-response curves are similar and importantly, the concentration giving the highest RLU value is identical in all columns.



Example of Edging Effects in an Antagonist Range Finder Test

- An example of edging effects from the testing of a 7-point log serial dilution of TAM is presented in the graph below (RLUs from plate column 1 [outside left column] are significantly different from those in columns 2 to 12)
- This difference results in a concentration-response curve that is clearly lower in magnitude than the remaining curves. However, the shape of all concentration-response curves are similar and importantly, the concentrations giving the highest and lowest RLU values are identical in all columns

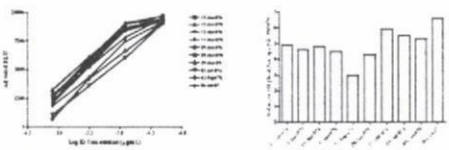


Impact of Edging Effects on Concentration Selection for Comprehensive Testing

- Results of this testing demonstrated that although there are statistical differences between the level of RLUs in the outer and inner wells, these differences do not impact selection of the appropriate starting concentration for comprehensive testing.

Evaluation of Modified Agonist Range Finder E2 Reference Standard

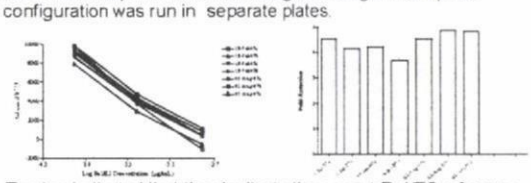
- Acceptance of test plates for agonist range finding is based on induction of E2 (i.e., the highest averaged E2 RLU value divided by the average DMSO control RLU value must be greater than three fold). The modified range finder plate design was run in 10 separate plates.



- Testing indicated that the duplicate four point E2 reference standard produced a repeatable concentration response curve that consistently exceeded the three-fold plate induction requirement, thus demonstrating the efficacy of the modified range finder E2 reference standard.

Evaluation of Modified Antagonist Range Finder Ral/E2 Reference Standard

- Acceptance of test plates for antagonist range finding is based on reduction of Ral\E2 (i.e., the highest averaged Ral/E2 RLU value divided by the lowest averaged Ral/E2 RLU value must be greater than three fold). The modified antagonist range finder plate configuration was run in separate plates.

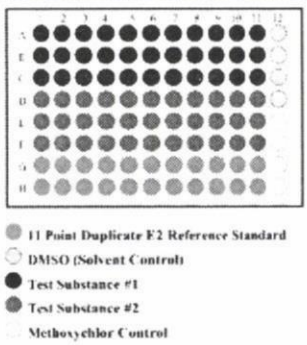


- Testing indicated that the duplicate three point Ral/E2 reference standard produced a repeatable concentration response curve that consistently exceeded the three-fold plate reduction requirement, thus demonstrating the efficacy of the modified range finder Ral/E2 reference standard.

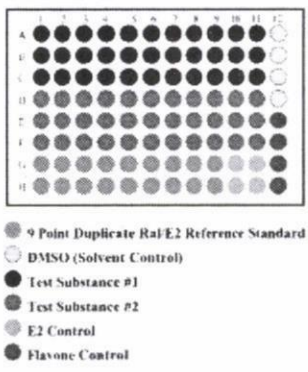
Modification of Plate Designs for Comprehensive Testing

- To increase testing throughput, the plate designs for agonist and antagonist comprehensive testing were also modified to use all 96 wells.
- This modification allows for the testing of 11-point double serial dilutions of two substances in triplicate instead of only one substance, as would occur when using the original plate design developed during the protocol standardization study.

Modified Agonist Comprehensive Testing Plate Design



Modified Antagonist Comprehensive Testing Plate Design



Rationale for Modifying Comprehensive Testing Plate Designs (1)

- The modified plate designs for comprehensive testing uses row A, an outside set of wells, for one of the three replicates of the 11-point double serial dilution of one of the substances to be tested per plate.
- To evaluate the effect of using this outer well on comprehensive testing, EC_{50} values were calculated for the seven point log serial dilutions of BPA (100 µg/mL to 1 x10^{-4} µg/mL) tested in the revised agonist range finder plates.
  - EC_{50} values derived from replicates using outside wells were compared to EC_{50} values derived from replicates using inside wells.

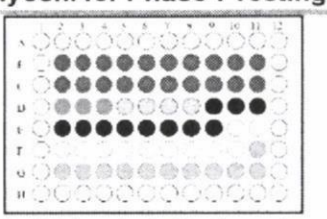
Rationale for Modifying Comprehensive Testing Plate Designs (2)

- The comparison of EC_{50} values was conducted using the Friedman Test, a nonparametric test that compares matched groups by ranking group values and conducting a two-way analysis of variance.
- Based on this analysis, no significant difference was observed ($p > 0.05$) between EC_{50} values derived from replicates using outside wells and those derived from using inside wells.

Phase I Protocol Amendment #2

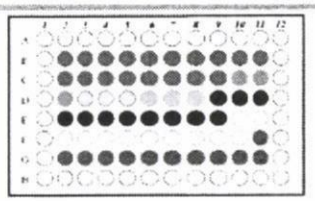
- Based on the results from the testing of the modified agonist and antagonist plate designs, the range finder and comprehensive test plate layouts were amended to include all 96 wells of test plates.
- This modification was approved at a 25 July 2007 SMT teleconference
  - Hiyoshi began Phase I testing of reference standards and controls before completion of testing, evaluation, and amendment of comprehensive test plate designs and therefore conducted testing using the plate designs developed during the protocol standardization study that did not use outside wells.
  - ECVAM and XDS began Phase I testing of reference standards and controls after approval of modified plate designs and therefore conducted testing with plate designs that used all 96 wells.

Agonist Plate Design Used by Hiyoshi for Phase I Testing



- E2 Reference Standard Dose Response Curve
- - Methoxychlor Control (3.13 µg/mL)
- - DMSO Control (1% v/v)
- - Test Substance Replicate #1
- - Test Substance Replicate #2
- - Test Substance Replicate #3
- - Media only wells, not used for assay

Antagonist Plate Design Used by Hiyoshi for Phase I Testing



- 9 Point Duplicate Ral/E2 Reference Standard
- DMSO (Solvent Control)
- Test Substance Replicate #1
- Test Substance Replicate #2
- Test Substance Replicate #3
- F2 Control
- Flavone Control
- Media Only Wells

The LUMI-CELL® ER Assay International Validation Study - Phase I ER Agonist Testing

Phase I Agonist Reference Standards and Controls

- Multiple testing of agonist reference standards and controls was conducted to:
  - Demonstrate proficiency with the agonist protocol
  - Provide reference standard and control data for an evaluation of intra- and inter-laboratory reproducibility
  - Establish historical databases to be used to develop acceptance criteria for tests to be conducted in Phase IIa

Historical Database for Phase IIa Agonist Testing

- Acceptance or rejection of agonist tests to be conducted in Phase IIa will be based on evaluation of test plate reference standard and control results. Results will compared to acceptance criteria derived from the historical databases established from Phase I testing at each laboratory. Agonist test plate acceptance criteria to be used in Phase IIa are summarized as follows:
  - Plate induction, as measured by dividing the averaged highest E2 reference standard RLU value by the averaged DMSO control value, must be greater than three-fold
  - E2 EC_{50} values must be within 2.5 times the standard deviation of the historical database E2 EC_{50} value
  - DMSO control RLU values must be within 2.5 times the standard deviation of the historical DMSO control value
  - Methoxychlor (the weak positive control) RLU values must be within 2.5 times the standard deviation of the historical E2 control value

Adjustment and Normalization of Agonist Assay Luminescence Measurements

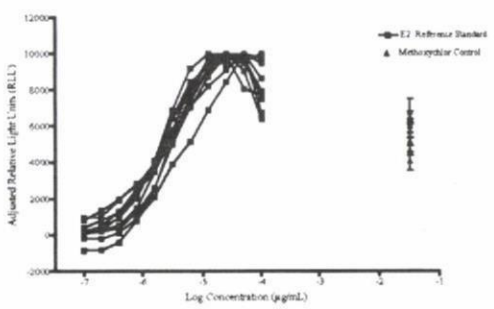
- Luminescence measurements are adjusted and normalized by:
  - Subtracting the averaged RLU values for the DMSO control wells from RLU values from wells containing E2 reference standard, methoxychlor control, or test substance
  - Luminescence measurements are further adjusted (normalized) by scaling RLU values to the highest mean RLU value from E2 reference standard, which is assigned an RLU value of 10,000

Testing of Agonist Reference Standards and Controls at XDS, ECVAM, and Hiyoshi

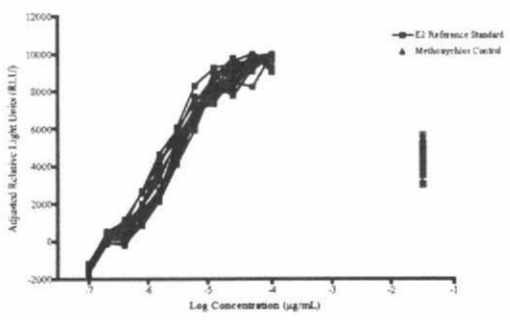
- At XDS, reference standard and controls were tested in 10 separate plates on 3 separate days (2 plates each on 2 separate days and 6 plates on another day)
- At ECVAM, reference standard and controls were tested in 18 separate plates on 9 separate days (2 plates each on 9 separate days)
- At Hiyoshi, reference standard and controls were tested in 12 separate plates on 12 separate days (**note:** induction in two plates was less than three-fold and were not included in data analysis)

The LUMI-CELL® ER Assay International Validation Study - Phase I ER Agonist Intralaboratory Analyses

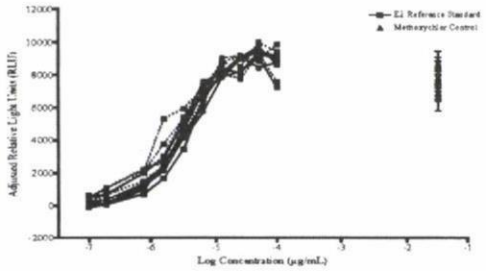
Test Plate Results from Agonist Testing at XDS



Test Plate Results from Agonist Testing at ECVAM



Test Plate Results from Agonist Testing at Hiyoshi

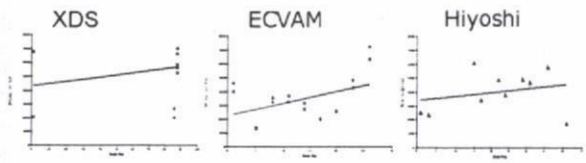


Experiments on 6/04/07 and 6/06/07 did not meet acceptance criteria

Intralaboratory Reproducibility of Agonist Reference Standards and Controls

- Intralaboratory reproducibility of the RLU values associated with the DMSO control wells, the fold-induction of E2 at its maximum response, the calculated E2 EC_{50} values, and the adjusted and normalized RLU values associated with the methoxychlor weak positive control wells were statistically analyzed.
  - A linear regression analysis was conducted to assess intralaboratory reproducibility over time for each laboratory
  - At XDS and ECVAM, reference standards and controls were tested in multiple plates on three or more separate days so within-day and across-day variability was analyzed using an ANOVA

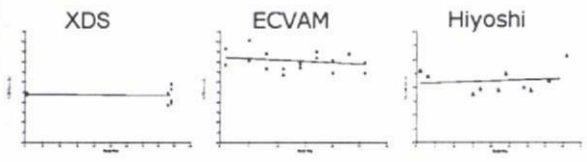
Agonist DMSO Linear Regression Analysis



	N[1]	Intercept[2]	Slope	p-value (Slope)
XDS	10	4308	15.5	0.540
ECVAM	18	2286	86.4	0.064
Hiyoshi	10	3400	29.0	0.483

[1]Number of plates tested.
[2]Intercept values are in relative light units.

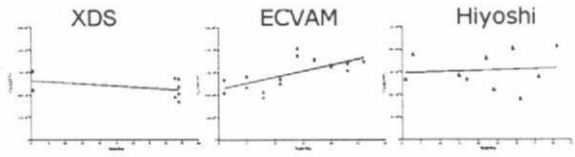
E2 Fold-Induction Linear Regression Analysis



	N[1]	Intercept[2]	Slope	p-value (Slope)
XDS	10	4.6	-0.002	0.800
ECVAM	18	8.4	-0.030	0.351
Hiyoshi	10	4.3	0.010	0.686

[1]Number of plates tested.
[2]Intercept values are fold-induction.

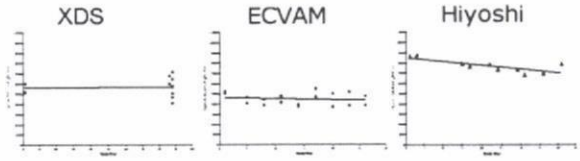
E2 EC_{50} Linear Regression Analysis



	N[1]	Intercept[2]	Slope	p-value (Slope)[3,4]
XDS	9*	2.6×10^{-6}	-5.0×10^{-9}	0.262
ECVAM	*18*	*2.3×10^{-6}*	*5.4×10^{-8}*	*0.002*
Hiyoshi	10	3.0×10^{-6}	5.7×10^{-8}	0.793

*A single EC_{50} value was excluded from analysis after failing the Q-test for outliers.
[1]Number of plates tested.
[2]Intercept units are in µg/mL.
[3]Statistically significant from zero at $p<0.05$.
[4]Values in italics have p values that are less than 0.05.

Methoxychlor Linear Regression Analysis



	N[1]	Intercept[2]	Slope	p-value (Slope)[3,4]
XDS	10	5592	1.7	0.865
ECVAM	18	4641	-10.74	0.564
Hiyoshi	10	*8506*	*-37.0*	*0.009*

[1]Number of plates tested.
[2]Intercept units are in adjusted relative light units.
[3]Statistically significant from zero at $p<0.05$.
[4]Values in italics have p values that are less than 0.05.

Agonist ANOVA Results for Intralaboratory Comparison of Reference Standard and Controls

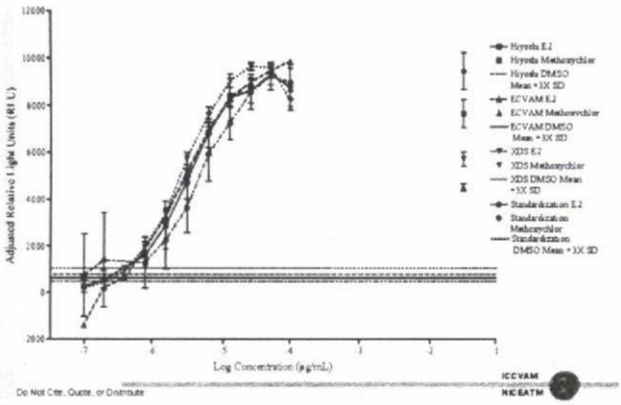
	XDS		ECVAM	
	p-Value[1,2]	F Value[3]	p-Value[1,2]	F Value[3]
DMSO	0.068	4.0	*<0.001*	*49.0*
E2 Maximum Fold-Induction	0.749	0.3	0.256	1.6
E2 EC_{50}	0.529	0.7	<0.001	6.0
Methoxychlor	0.596	0.6	0.485	1.0

[1]Variability is statistically significant at $p<0.05$.
[2]Values in italics have p values that are less than 0.05.
[3]F = ratio of between-day variability to within-day variability – a ratio of 1.0 indicates that the within-day variability to between-day variability is equal and a ratio of zero indicates that all means are equal.

At XDS and ECVAM, reference standards and controls were tested in multiple plates on three or more separate days. The within-day and across-day variability of the RLU values associated with the DMSO wells, the fold-induction of E2 at its maximum response, the E2 EC_{50} values, and the adjusted RLU values associated with the methoxychlor weak positive control were analyzed using an ANOVA.
Results from the analysis indicate that with-in day variability is not statistically different from between-day variability for reference standard and control values at XDS but was statistically different for DMSO control and E2 EC_{50} values at ECVAM.

The LUMI-CELL® ER Assay International Validation Study - Phase I ER Agonist Interlaboratory Analyses

Comparison of Agonist Historical Databases



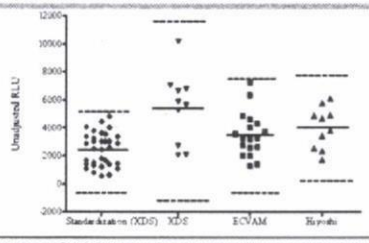
Interlaboratory Reproducibility of Reference Standard and Controls (1)

- Interlaboratory reproducibility of the RLU values associated with the DMSO control wells, the fold-induction of E2 at its maximum response, the calculated E2 EC_{50} values, and the adjusted and normalized RLU values associated with the methoxychlor weak positive control wells was evaluated:
  - Means, standard deviations and coefficients of variation (CV) of reference standard and control values were compared
  - Variability of reference standard and control values across laboratories was evaluated by conducting an analysis of variance (ANOVA)

Interlaboratory Reproducibility of Reference Standard and Controls (2)

- If a significant p-value was obtained for the ANOVA, a Newman-Keuls post-test was used to test for significant differences in reference standard and control values between pairs of laboratories.
- To test for significant differences between the reference standard and control values obtained in each laboratory versus the corresponding endpoint values obtained in the protocol standardization study, a Dunnett's analysis was conducted.

Interlaboratory Comparison of Agonist DMSO Control



Data points represent DMSO RLU values from plates tested in the protocol standardization and Phase I studies. Solid horizontal lines represent the mean DMSO RLU value for each data set. Dashed lines indicate the mean agonist DMSO value plus and minus 2.5 times the standard deviation from the mean.

	Agonist DMSO			
	N[1]	Mean[2]	SD[2]	CV
XDS	10	5394	2558	47%
ECVAM	18	3486	1582	45%
Hyoshi	10	4006	1500	37%
Standardization	33	2429	1188	49%

[1]Number of plates tested
[2]Units are expressed in relative light units