Establishing a Historical Database for a Multi-phased International Validation Study of a Stably Transfected Estrogen Receptor (ER) Transcriptional Activation (TA) Test Method

R Tice[1], F Deal[2], P Ceger[2], D Allen[2], J Gordon[3], J de Lange[4], S Bremer[4], M Nakamura[5], H Kojima[6], A Ono[6], W Stokes[1]

[1]National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)/NIEHS/NIH/DHHS, RTP, NC; [2]ILS, Inc., RTP, NC; [3] Xenobiotic Detection Systems, Inc. (XDS, Inc.), Durham, NC;

[4]European Centre for the Validation of Alternative Methods (ECVAM), Ispra, Italy; [5]Hiyoshi Corp., Omihachiman, Japan; [6]Japanese Center for the Validation of Alternative Methods (JaCVAM), Tokyo, Japan

Abstract

The LUMI-CELL® ER assay is a stably transfected ER TA method developed for the detection of ER agonists and antagonists. Based on an Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommendation, NICEATM, ECVAM, and JaCVAM initiated a validation study, using three laboratories (one each in the United States, Japan, and Europe), to evaluate the reproducibility and accuracy of the LUMI-CELL® ER assay. This four-phased study will ultimately evaluate all of the 78 reference substances recommended by ICCVAM for validation of *in vitro* ER test methods (http://iccvam.niehs.nih.gov/methods/endocrine/endocrine.htm). During Phase I, multiple testing of reference standards and controls was conducted using standardized LUMI-CELL® agonist and antagonist protocols to demonstrate proficiency, establish historical databases to be used as quality controls for subsequent testing, and provide measured or calculated reference standard and control data for an evaluation of intra- and inter-laboratory reproducibility. Phase I also included an evaluation at the U.S. laboratory for "edge" effects on the 96-well plate used for testing, which resulted in a redesign of the plate layout to include all 96 wells. Additional testing was also conducted at the European and Japanese laboratories to demonstrate proficiency with a visual observation method of assessing cell viability. Results of Phase I testing demonstrated the ability of the three laboratories to conduct the assay in a reproducible manner and supported modifications made to the protocols to increase testing efficiency. The three laboratories also established a historical database to use as quality controls when testing coded reference substances in the subsequent phases of the validation study.
Supported by NIEHS Contract N01-ES-35504.

Introduction

The LUMI-CELL® ER assay, developed by XDS, Inc., is a stably transfected ER TA method for the detection of ER agonists and antagonists. This test method incorporates the ICCVAM recommended ER TA essential test method components (ICCVAM 2003) and was recommended by ICCVAM as a high priority activity for validation. In response, NICEATM conducted a protocol standardization study and the test method protocols, which were revised based on study results, were considered to be sufficiently reproducible to warrant initiating a multi-laboratory international validation study. Subsequently, NICEATM, ECVAM, and JaCVAM initiated the recommended validation study, using three laboratories (one each in the United States, Japan, and Europe), to evaluate the reliability and accuracy of the LUMI-CELL® ER assay.

The validation study is ongoing and will ultimately evaluate all 78 ICCVAM-recommended reference substances for the validation of *in vitro* ER test methods (ICCVAM 2006). The study is being conducted in a multi-phased process in order to maximize the likelihood of developing highly reproducible agonist and antagonist assay protocols that can be used for international regulatory use (see flowchart below).

The first phase of the validation study has been completed. Phase I focused on the transferability of the protocols developed in the standardization study by establishing and comparing a historical control database in each of the three participating laboratories (XDS, Inc., in the United States, ECVAM in Europe, and Hiyoshi Corp., in Japan). Revised test plate layouts that used all 96 wells in order to maximize the number of substances that could be tested per plate were also evaluated during this phase.

Study Phases and Activities

Phase I: Laboratory Qualification Phase
(Development of Historical Database for Each Laboratory)

- Qualify the participating laboratories based on results from testing reference standards and controls
- Evaluate agonist and antagonist assay intra- and inter-laboratory reproducibility
- Modify assay protocols as necessary to reduce intra- and inter-laboratory variation
- Establish individual laboratory historical database for reference standards and controls by conducting independent experiments (at least 10 each for the agonist and antagonist protocols)
- Establish initial test acceptance criteria for each laboratory based on historical database for use in Phase II
- Evaluate redesigned test plate layouts for differences in responses between cells in the outermost wells of the plate and all other wells (so-called "edge" effects)

Phase II: Protocol Optimization Phase
(Repeated Testing of Coded Substances/Evaluation of Protocol Modifications)

- Test 12 coded substances from ICCVAM-recommended minimum list independently three times for agonism and antagonism at each laboratory
- Evaluate reproducibility and accuracy
- Modify assay protocols as necessary to reduce intra- and inter-laboratory variation
- Repeat testing if major changes are made
- Finalize optimized test method protocol for use in Phases III and IV

Phase III: Laboratory Validation Testing Phase

- Test remaining 41 coded test substances from ICCVAM-recommended minimum list of 53 substances once for agonism and antagonism at each laboratory using final optimized protocols
- Evaluate inter-laboratory reproducibility and accuracy

Phase IV: Expansion of Validation Database Using Additional Reference Substances

- Test 25 remaining coded test substances from ICCVAM recommended list of 78 for agonism and antagonism once at the lead laboratory (XDS, Inc.) using final protocols
- Evaluate test method accuracy to further characterize the usefulness and limitations of the LUMI-CELL® ER assay

Overview of the LUMI-CELL® ER Assay

The LUMI-CELL® ER assay measures whether and to what extent a substance induces or blocks TA activity via an ER-mediated pathway in recombinant BG-1Luc4E2 cells (Rogers et al. 2000). The BG-1Luc4E2 cell line was derived from BG-1 human adenocarcinoma cells that endogenously express ER and that have been stably transfected with the plasmid pGudLuc7.ERE. This plasmid contains four copies of a synthetic oligonucleotide containing the estrogen response element upstream of the mouse mammary tumor viral (MMTV) promoter and the firefly luciferase gene (**Figure 1**). The resulting cell line expresses luciferase activity in response to estrogen and estrogen-like substances.

Figure 1: pGudLuc7.ERE Plasmid

During the LUMI-CELL® ER bioassay, BG-1Luc4E2 cells are selected with G418, and then conditioned in estrogen-free medium for at least 48 hours. After conditioning, cells are seeded into 96-well plates for 24 to 48 hours and then incubated in estrogen-free medium containing solvent and/or reference standard, control, or test substance for 19 to 24 hr. Cytotoxicity is evaluated visually, after which cells are lysed and treated with luciferase reagent. Luminescence is measured in each well with a luminometer and expressed as relative light units (RLUs). RLU values are first normalized for background by subtraction of the solvent control (dimethyl sulfoxide; DMSO) and then adjusted such that the maximal TA response induced by 17ß-estradiol (E2) (the reference standard for agonist testing) or by raloxifene/E2 (the reference standard for antagonist testing) is 10,000 RLUs.

Evaluation of "Edge" Effects

- Agonist and antagonist testing procedures developed during the protocol standardization study did not use the outer wells of 96-well test plates due to possible "edge" effects that may occur because of differences in evaporation or temperature between outer and inner wells. In order to maximize the number of substances that could be tested per plate, plate layouts using all 96 wells were considered.
- Revised test plate layouts using all 96-wells were evaluated by addressing the following questions:
  - Are there significant differences in observed responses between outer and inner wells (edge effects) using revised plate layouts?
  - If edge effects are observed, do they have a significant impact on the selection of concentrations for comprehensive testing?
  - If edge effects are observed, do they have a significant impact on the measurement of estrogenic activity of substances run in triplicate on comprehensive test plates
- To assess test plates for edge effects, log serial dilutions of bisphenol A (BPA) and tamoxifen were tested in plates using all 96 wells.

Question 1: Are there significant differences in observed responses between outer and inner wells (edge effects)?

- Serial dilutions of BPA or tamoxifen were run in plate columns 1-12 at descending concentrations in rows A-G (see **Figure 2**).
- A comparison of RLUs was made between columns 1 and 2 and between columns 12 and 11 (values from row A were not used for the comparison).
- A total of 204 pairs were evaluated with a Z statistic (Z = 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 RLU values between outer and inner wells.

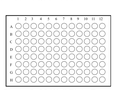


Figure 2: 96-Well Plate Rows and Columns

Question 2: If edge effects are observed in range finder test plates, do they have a significant impact on the selection of concentrations for comprehensive testing?

- In the LUMI-CELL® ER assay, if results from range finder testing suggest that a substance is positive for agonist or antagonist activity, the starting concentrations for comprehensive testing are based on the maximum response observed from the testing of seven-point log serial dilutions. In general:
  - For agonist testing, the starting concentration is one log concentration higher than the concentration that gives the highest RLU value in the range finder
  - For antagonist testing, the starting concentration is one log concentration higher than the concentration that gives the lowest non–cytotoxic RLU value in the range finder
- **Figure 3** presents an example of edge effects from the agonist testing of seven-point log serial dilutions of BPA where the concentration response curve of BPA tested in plate column 12 (outside right column) is clearly lower in magnitude than those tested in columns 1-11. **Figure 4** presents an example of edge effects from the antagonist testing of seven-point serial dilutions of tamoxifen where the concentration response curve of the tamoxifen tested in plate column 1 (outside left column) is clearly lower in magnitude than those tested in columns 2-12. However, the shape of the concentration response curves are similar and importantly, the concentration giving the highest RLU value in the agonist plate and the lowest RLU value in the antagonist plate is identical in all columns.

Figure 3: Example of Edge Effects in Range Finder Test of BPA

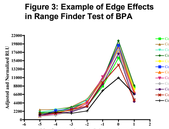
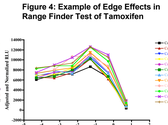


Figure 4: Example of Edge Effects in Range Finder Test of Tamoxifen



Although there are significant differences between RLU values in inner and outer test plate wells, these differences did not impact selection of the appropriate starting concentration for comprehensive testing. Therefore, the Validation Study Management Team approved revising protocols to include all 96 wells during range finder testing (see **Figures 5** and **6**)

Evaluation of "Edge" Effects (cont'd)

Figure 5: Revised Agonist Range Finder Test Plate Layout

Figure 6: Revised Antagonist Range Finder Test Plate Layout

Reference Standard and Control for Agonist Range Finder Testing

- **Solvent Control** = dimethyl sulfoxide (DMSO, CASRN 67-68-5): 1% (v/v) DMSO in tissue culture media
- **Reference Standard** = 17β-estradiol (E2, CASRN 50-28-2): at four concentrations

Reference Standard and Control for Antagonist Range Finder Testing

- **Solvent Control**: = dimethyl sulfoxide (DMSO) 1% (v/v) DMSO in tissue culture media
- **Reference Standard** = raloxifene HCl (Ral, CASRN 84449-90-1): at three concentrations of Ral with a fixed concentration of E2* (Ral/E2)
- **Reference Estrogen** = E2* at one concentration used as the base line reference estrogen

*2.5 x 10^{-3} µg/mL, which results in approximately 80% of the maximum response for E2 in the assay

Question 3: If edge effects are observed do they have a significant impact on the measurement of estrogenic activity of substances run in triplicate on comprehensive test plates?

- Comprehensive test plate layouts used in the protocol standardization study did not use outer wells, which restricted assay throughput to one substance per plate.
- To increase testing throughput, agonist and antagonist comprehensive plate layouts were developed that would increase throughput to two substances per plate (see **Figures 7** and **8**).

Figure 7: Agonist Comprehensive Test Plate Layout

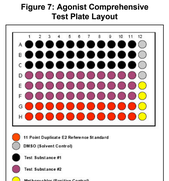
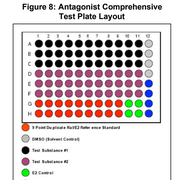


Figure 8: Antagonist Comprehensive Test Plate Layout



Reference Standard and Control for Agonist Comprehensive Testing

- **Solvent Control** = dimethyl sulfoxide (DMSO, CASRN 67-68-5): 1% (v/v) DMSO in tissue culture media
- **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): One concentration of methoxychlor used as a weak positive control for comprehensive testing

Reference Standard and Control for Antagonist Comprehensive Testing

- **Solvent Control** = dimethyl sulfoxide (DMSO): 1% (v/v) DMSO in tissue culture media
- **Reference Standard**: raloxifen HCL (Ral, CASRN 84449-90-1): A nine-point serial dilution of Ral with a fixed concentration of E2* (Ral/E2)
- **Reference Estrogen** = E2: One concentration of E2* used as the base line reference estrogen
- **Positive Control =** flavone (CASRN 525-82-6), with E2* (flavone/E2) used as a weak positive control for comprehensive testing

*2.5 x 10^{-3} µg/mL, which results in approximately 80% of the maximum response for E2 in the assay

- The revised plate layouts for comprehensive testing use row A, an outside set of wells, for one of the three replicates of one of the substances to be tested per plate.
- To evaluate the effect of using the outer wells on comprehensive testing, EC_{50} values derived from replicates using outside wells were compared to EC_{50} values derived from replicates using inside wells from the testing of BPA that demonstrated edge effects using the plate layout presented in **Figure 2**.
- The comparison of EC_{50} values was conducted using the Friedman Test, which compares matched groups by ranking group values and conducting a two-way analysis of variance.

Based on the Friedman analysis, no significant differences were observed ($p>0.05$) between EC_{50} values derived from replicates using outside wells and those derived from using inside wells. Based on these results, significant differences in IC_{50} values for substances to be tested for antagonist activity are not expected. Therefore, the Validation Study Management Team approved revising the protocols to include all 96 wells during comprehensive testing.

Demonstration of Proficiency with the Visual Observation Method of Assessing Cell Viability

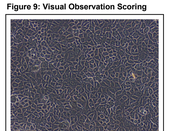
To demonstrate proficiency with the visual observation method of assessing cell viability that was developed in the LUMI-CELL® ER assay protocol standardization study by the lead laboratory (XDS, Inc.), the ECVAM and Hiyoshi, Corp. laboratories tested concentrations of BPA that had previously been shown to induce a complete range of cytotoxicity (i.e., Categories 1, 2, 3, and 4 as defined in the LUMI-CELL® ER Assay Visual Observation Cell Viability Manual, see **Table 1** and **Figure 9**).

- Test plate wells treated with the cytotoxic and non-cytotoxic concentrations of BPA were assessed by technical staff from each laboratory.
  - Technical staff from the ECVAM laboratory demonstrated proficiency as part of formal training in LUMI-CELL® ER assay procedures at XDS, Inc.
  - Technical staff from the Hiyoshi Corp. laboratory demonstrated proficiency by providing photomicrographs for review of treated test plate wells and corresponding cell viability scores.

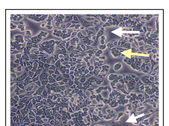
Table 1: Visual Observation Scoring

Viability Score	Brief Description
1	Normal cell morphology and cell density
2	Altered cell morphology and/or small gaps between cells
3	Altered cell morphology and/or large gaps between cells
4	Few or no visible cells
P	Denotes wells containing precipitation

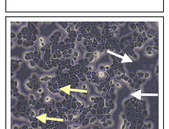
Figure 9: Visual Observation Scoring



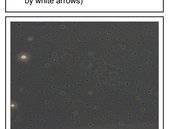
Viability score 1:
- Cells grow in a monolayer and exhibit normal morphology
- No gaps between cells



Viability score 2:
- Some cells are abnormally rounded (indicated by yellow arrow)
- Small gaps between cells (indicated by white arrows)



Viability score 3:
- Most cells are abnormally rounded or exhibit other abnormal morphology (indicated by yellow arrows)
- Large gaps between cells (indicated by white arrows)



Viability score 4:
- No visible cells

Testing of LUMI-CELL® ER Assay Reference Standards and Controls

- Ten independent experiments of each of the of LUMI-CELL® ER agonist and antagonist assays using reference standards and controls were conducted to:
  - Demonstrate proficiency with the agonist and antagonist protocols
  - Establish initial historical databases to be used to develop acceptance criteria for the testing of coded test substances in Phases II-IV
  - Produce data to be used for an evaluation of intra- and inter-laboratory reproducibility

LUMI-CELL® ER Test Plate Acceptance Criteria

Acceptance or rejection of plates used for the testing of coded substances in Phase II is based on evaluation of test plate reference standard and control results. Results are compared to acceptance criteria derived from the historical databases established from Phase I testing at each laboratory.

Summary of agonist test plate acceptance criteria used in initial Phase II testing:

- 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.

Testing of LUMI-CELL® ER Assay Reference Standards and Controls (cont'd)

Summary of antagonist test plate acceptance criteria used in initial Phase II testing:

- Plate reduction, as measured by dividing the averaged highest RalE2 reference standard RLU value by the averaged lowest RalE2 reference standard value, must be greater than three-fold.
- RalE2 IC_{50} values must be within 2.5 times the standard deviation of the historical database RalE2 IC_{50} value.
- DMSO control RLU values must be within 2.5 times the standard deviation of the historical DMSO control value.
- E2 control RLU values must be within 2.5 times the standard deviation of the historical E2 control value.
- Flavone/E2 control RLU values must be within 2.5 times the standard deviation of the historical flavone/E2 control value.

Reproducibility of LUMI-CELL® ER Assay Reference Standards and Controls

- In the agonist assay, reproducibility was evaluated based on the variability of 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.
- In the antagonist assay, reproducibility was evaluated based on the variability of RLU values associated with the DMSO control wells, the fold-induction of Ral/E2 at its maximum response, the calculated Ral/E2 IC_{50} values, and the adjusted and normalized RLU values associated with the E2 control and flavone/E2 weak positive control wells.
- A linear regression analysis was conducted to assess interlaboratory reproducibility of each endpoint over time for each laboratory.
  - Reference standard and control values were reproducible over time ($p>0.05$) with the exception of:
    - E2 and flavone E2 control values at XDS, Inc.
    - E2 EC_{50}, Ral/E2 IC_{50}, and E2 control values at the ECVAM laboratory
    - E2 EC_{50} and methoxychlor values at Hiyoshi Corp.
- An analysis of variance (ANOVA) was conducted to assess interlaboratory reproducibility of reference standard and control values across laboratories.
  - Although reference standard and control values were similar (see **Figures 10** and **11**), there were statistically significant differences across laboratories ($p<0.05$).

Figure 10: Comparison of LUMI-CELL® ER Assay Reference Standard and Controls

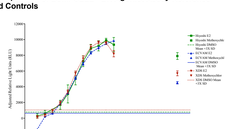
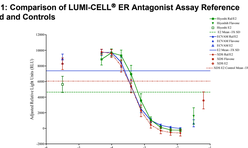


Figure 11: Comparison of LUMI-CELL® ER Antagonist Assay Reference Standard and Controls



Historical Database Values Established for Initial Phase II Testing of Coded Substances

Table 2: Agonist Assay Values

Laboratory: XDS, Inc. (USA)

	Units	Mean	Standard Deviation (SD)	Mean + 2.5 times SD	Mean - 2.5 times SD
DMSO	RLU	5394	2558	11789	0*
E2 EC_{50}	µg/ml	2.3×10^{-6}	4.5×10^{-7}	3.4×10^{-6}	1.2×10^{-6}
Methoxychlor	Adjusted RLU	5709	974	8144	3274

Laboratory: ECVAM (Europe)

	Units	Mean	Standard Deviation (SD)	Mean + 2.5 times SD	Mean - 2.5 times SD
DMSO	RLU	3486	1582	7441	0*
E2 EC_{50}	µg/ml	2.7×10^{-6}	8.5×10^{-7}	4.8×10^{-6}	1.9×10^{-6}
Methoxychlor	Adjusted RLU	4494	590	5969	3019

Laboratory: Hiyoshi Corp. (Japan)

	Units	Mean	Standard Deviation (SD)	Mean + 2.5 times SD	Mean - 2.5 times SD
DMSO	RLU	4006	1500	7756	256
E2 EC_{50}	µg/ml	3.1×10^{-6}	7.9×10^{-7}	5.1×10^{-6}	1.1×10^{-6}
Methoxychlor	Adjusted RLU	7692	633	9275	6110

*Unadjusted DMSO values can not be below zero.

Testing of LUMI-CELL® ER Assay Reference Standards and Controls (cont'd)

Table 3: Antagonist Assay Values

Laboratory: XDS, Inc. (USA)

	Units	Mean	Standard Deviation (SD)	Mean + 2.5 times SD	Mean - 2.5 times SD
DMSO	RLU	1986	1748	6355	0*
Ral/E2 IC_{50}	µg/ml	4.3×10^{-4}	9.0×10^{-5}	6.5×10^{-4}	2.0×10^{-4}
E2	Adjusted RLU	8284	744	10143	6424
Flavone	Adjusted RLU	3583	1089	6305	860

Laboratory: ECVAM (Europe)

	Units	Mean	Standard Deviation (SD)	Mean + 2.5 times SD	Mean - 2.5 times SD
DMSO	RLU	3783	1587	7752	0*
Ral/E2 IC_{50}	µg/ml	4.3×10^{-4}	7.9×10^{-5}	6.3×10^{-4}	2.3×10^{-4}
E2	Adjusted RLU	8881	640	10480	7282
Flavone	Adjusted RLU	644	458	1789	-501

Laboratory: Hiyoshi Corp. (Japan)

	Units	Mean	Standard Deviation (SD)	Mean + 2.5 times SD	Mean - 2.5 times SD
DMSO	RLU	4048	1386	7513	583
Ral/E2 IC_{50}	µg/ml	6.3×10^{-4}	1.3×10^{-4}	9.5×10^{-4}	3.1×10^{-4}
E2	Adjusted RLU	5728	1221	8781	2676
Flavone	Adjusted RLU	1226	724	3036	-584

*Unadjusted DMSO values can not be below zero.

Summary

- LUMI-CELL® ER agonist and antagonist assay reference standards and controls were successfully tested multiple times at the three participating laboratories, and testing results were used to:
  - Demonstrate proficiency with the agonist and antagonist protocols
  - Establish initial historical databases to be used to develop acceptance criteria for the testing of coded test substances
  - Provide reference standard and control data for an evaluation of intra- and inter-laboratory reproducibility
- Statistically significant differences were observed in intra- and inter-laboratory reference standard and control values.
  - It was not possible to identify the causes for these differences, but some of the contributing factors may be:
    - Lot-to-lot differences in cell culture media and tissue culture supplies (for intra- and inter-lab differences)
    - Differences in luminometers (for inter-lab differences)
  - This underscores the importance of developing a historical control database for each individual laboratory.
- Factors supporting reliability of the assay:
  - Assay responds robustly to E2 reference estrogen and raloxifene reference anti-estrogen.
  - Assay consistently responds to weak-acting positive controls at concentrations several orders of magnitude higher than the reference estrogen or anti-estrogen.

Acknowledgments

This poster was supported by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences. ILS staff are supported by NIEHS contract N01-ES 35504. The views expressed on this poster do not necessarily represent the official position of any Federal agency.

References

ICCVAM. 2003. ICCVAM Evaluation of *In Vitro* Test Methods for Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays. NIH Pub. No. 03-4503. Research Triangle Park, NC: National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/methods/endocrine.htm

ICCVAM. 2006. Report Addendum: ICCVAM Evaluation of In Vitro Test Methods for Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays. NIH Pub. No. 03-4503. Research Triangle Park, NC: National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/docs/endo_docs/EDAddendFinal.pdf

Rogers JM, Denison MS. 2000. Recombinant cell bioassays for endocrine disruptors: development of a stably transfected human ovarian cell line for the detection of estrogenic and anti-estrogenic chemicals. In Vitr Mol Toxicol 13(1):67-82.

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

ICCVAM Member Agencies

- *Agency for Toxic Substances and Disease Registry*
- *Consumer Product Safety Commission*
- *Department of Agriculture*
- *Department of Defense*
- *Department of Energy*
- *Food and Drug Administration*
- *National Cancer Institute*
- *Department of Transportation*
- *National Institute for Occupational Safety and Health*
- *National Institute of Environmental Health Sciences*
- *National Institutes of Health*
- *National Library of Medicine*
- *Department of the Interior*
- *Occupational Safety and Health Administration*
- *Environmental Protection Agency*

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
National Institutes of Health

NIEHS National Institute of Environmental Health Sciences

NTP