

Final Results of an International Validation Study of an *In Vitro* ER TA Test Method in BG-1 Cells

W Casey¹, P Ceger², F Deal², D Allen², G Clark³, P Pazos⁴, E Grignard⁴, J de Lange⁴, S Bremer⁴, M Nakamura⁵, H Kojima⁶, A Ono⁶, W Stokes¹.

¹NICEATM/NTP/NIEHS/NIH/DHHS, RTP, NC, ²ILS, Inc., RTP, NC; ³XDS, Inc., Durham, NC, ⁴ECVAM, Ispra, Italy; ⁵Hiyoshi Corp., Omihachiman, Japan, ⁶JaCVAM, Tokyo, Japan.

The LUMI-CELL[®] ER (BG1Luc4E2) stably transfected estrogen receptor (ER) transcriptional activation (TA) assay uses the human ovarian cancer cell line, BG-1, that expresses both human hER-alpha and hER-beta to screen for substances that may induce or inhibit estrogenic activity *in vitro*. NICEATM, in collaboration with ECVAM and JaCVAM, coordinated an international validation study to evaluate the accuracy and reliability of the test method. Three laboratories (one each in the U.S., Europe and Japan) tested ICCVAM recommended reference substances with well-characterized *in vitro* ER TA data. Subsets of this list were used to evaluate test method accuracy and reliability. Phases 1 and 2 were used to demonstrate proficiency, establish historical databases in each laboratory, evaluate intra- and interlaboratory reproducibility, and identify protocol refinements prior to initiating Phases 3 and 4, where the remaining reference substances were tested. Overall accuracy for identifying *in vitro* ER agonists was 97% (34/35), with false positive and false negative rates of 0% (0/7) and 4% (1/28), respectively. For *in vitro* ER antagonists, overall accuracy was 100% (25/25), with false positive and false negative rates of 0% (0/22) and 0% (0/3), respectively. These results will be used to provide the basis for draft ICCVAM recommendations on the usefulness and limitations of the BG1Luc4E2 test method for review by an expert peer panel in March 2011, as well as to develop performance standards for the expedited validation of functionally and mechanistically similar test methods. Results from this study will also be used to support the development of an OECD performance based test guideline for ER TA test methods.

Background

- Endocrine disruptors (EDs) are defined as substances that interfere with the normal function of hormones in the endocrine system, which can lead to abnormal growth, development or reproduction
- In light of the growing concern regarding EDs , the accurate and timely identification of substances with endocrine disrupting potential is an important aspect of protecting public health
- Xenobiotic Detection Systems Inc. (XDS) nominated to NICEATM-ICCVAM the *in vitro* LUMI-CELL[®] BG1Luc4E2 ER TA Test Method (hereafter, BG1Luc ER TA Assay) proposed for screening potential estrogen agonists and antagonists
- NICEATM coordinated an international validation study with its counterparts in Europe (ECVAM) and Japan (JaCVAM) using laboratories sponsored by each validation organization:
 - XDS, Durham, North Carolina, USA
 - European Centre for the Validation of Alternative Methods, Ispra, Italy
 - Hiyoshi Corporation, Omihachiman, Japan

Overview of BG1Luc ER TA

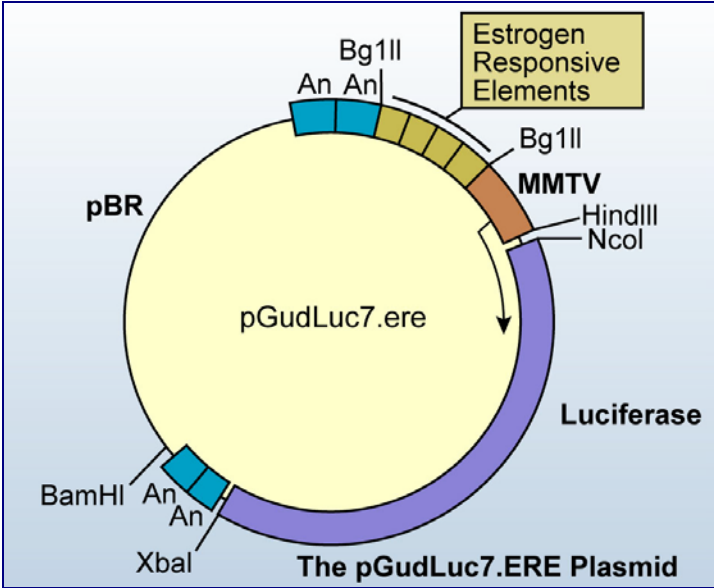
Basis of Assay

- BG1Luc4E2 cells - human ovarian carcinoma cell line that endogenously expresses estrogen receptors (ER α and ER β) and is stably transfected with an estrogen-responsive luciferase reporter gene
- Measures whether and to what extent a substance induces or inhibits TA activity via ER dependent pathways

Key Aspects:

- ER-mediated transcription of the *luc* gene (see **Figure 1**) results in the production of luciferase - enzyme capable of catalyzing a bioluminescent reaction - quantified using a luminometer
- Uses 96-well plates
- Separate protocols for ER agonist and antagonist activity
- 1% DMSO as vehicle.
- Limit concentration of 1 mM (agonist) or 10 μ M (antagonist)
- Cells treated with test substance for 24 hours in estrogen free media
- Cell viability assessed by visual inspection
- Range finder testing (7 concentrations at log serial dilutions)
- Comprehensive testing (11 concentrations at 1:2 or 1:5 serial dilutions)

Figure 1 Luc Reporter Gene Construct



pGudLuc7.ere 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.

Study Phases

The validation study was conducted in four phases, during which all 78 reference substances recommended by ICCVAM for validation of *in vitro* ER test methods (ICCVAM 2006) were tested

Phase 1 – Laboratory qualification and evaluation of intralaboratory reproducibility by repeat testing of reference standards and controls



Phase 2 – Evaluation of accuracy and intra- and inter-laboratory reproducibility by testing 12 coded agonist and antagonist substances from the ICCVAM reference substances in at least 3 independent experiments at each laboratory



Phase 3 – Evaluation of accuracy and interlaboratory reproducibility by testing 41 ICCVAM reference substances at least once at each laboratory



Phase 4 – Remaining 25 ICCVAM reference substances tested once in one laboratory (XDS) to further characterize test method accuracy

Reference Substances used to Evaluate Accuracy

- The ICCVAM list of 78 recommended reference substances was developed to assess the performance *in vitro* ER and androgen receptor binding and TA assays
- Only those substances that could be definitively classified as positive or negative for ER TA agonist and/or antagonist activity based on a preponderance of published data were used to assess accuracy of the BG1Luc ER TA Assay
 - ER agonist activity - 35 substances (28 Positive, 7 Negative) (**Table 1**)
 - ER antagonist activity- 25 substances (3 Positive, 22 Negative) (**Table 2**).

Table 1 Substances used to Assess BG1Luc ER TA Agonist Accuracy

| Substance | CASRN | ICCVAM Classification | BG1Luc ER TA Results |
|--------------------------------|------------|-----------------------|----------------------|
| 17 α -Estradiol | 57-91-0 | POS | POS |
| 17 α -Ethinyl estradiol | 57-63-6 | POS | POS |
| 17 β -Estradiol | 50-28-2 | POS | POS |
| 19-Nortestosterone | 434-22-0 | POS | POS |
| 4-cumylphenol | 599-64-4 | POS | POS |
| 4- <i>tert</i> -octylphenol | 140-66-9 | POS | POS |
| Apigenin | 520-36-5 | POS | POS |
| Bisphenol A | 80-05-7 | POS | POS |
| Bisphenol B | 77-40-7 | POS | POS |
| Butylbenzyl phthalate | 85-68-7 | POS | POS |
| Chrysin | 480-40-0 | POS | POS |
| Coumestrol | 479-13-0 | POS | POS |
| Daidzein | 486-66-8 | POS | POS |
| Dicofol | 115-32-2 | POS | POS |
| Diethylstilbestrol | 56-53-1 | POS | POS |
| Estrone | 53-16-7 | POS | POS |
| Ethyl paraben | 120-47-8 | POS | POS |
| Fenarimol | 60168-88-9 | POS | POS |
| Genistein | 446-72-0 | POS | POS |
| Kaempferol | 520-18-3 | POS | POS |
| Kepone | 143-50-0 | POS | POS |
| L-Thyroxine | 51-48-9 | POS | NEG |
| <i>meso</i> -Hexestrol | 84-16-2 | POS | POS |
| Methyl testosterone | 58-18-4 | POS | POS |
| Norethynodrel | 68-23-5 | POS | POS |
| <i>o,p'</i> -DDT | 789-02-6 | POS | POS |

| Substance | CASRN | ICCVAM Classification | BG1Luc ER TA Results |
|----------------------------|------------|-----------------------|----------------------|
| <i>p</i> -n-nonylphenol | 104-40-5 | POS | POS |
| <i>p,p'</i> - methoxychlor | 72-43-5 | POS | POS |
| Atrazine | 1912-24-9 | NEG | NEG |
| Bicalutamide | 90357-06-5 | NEG | NEG |
| Corticosterone | 50-22-6 | NEG | NEG |
| Hydroxyflutamide | 52806-53-8 | NEG | NEG |
| Linuron | 330-55-2 | NEG | NEG |
| Phenobarbital | 50-06-6 | NEG | NEG |
| Spirolactone | 52-01-7 | NEG | NEG |

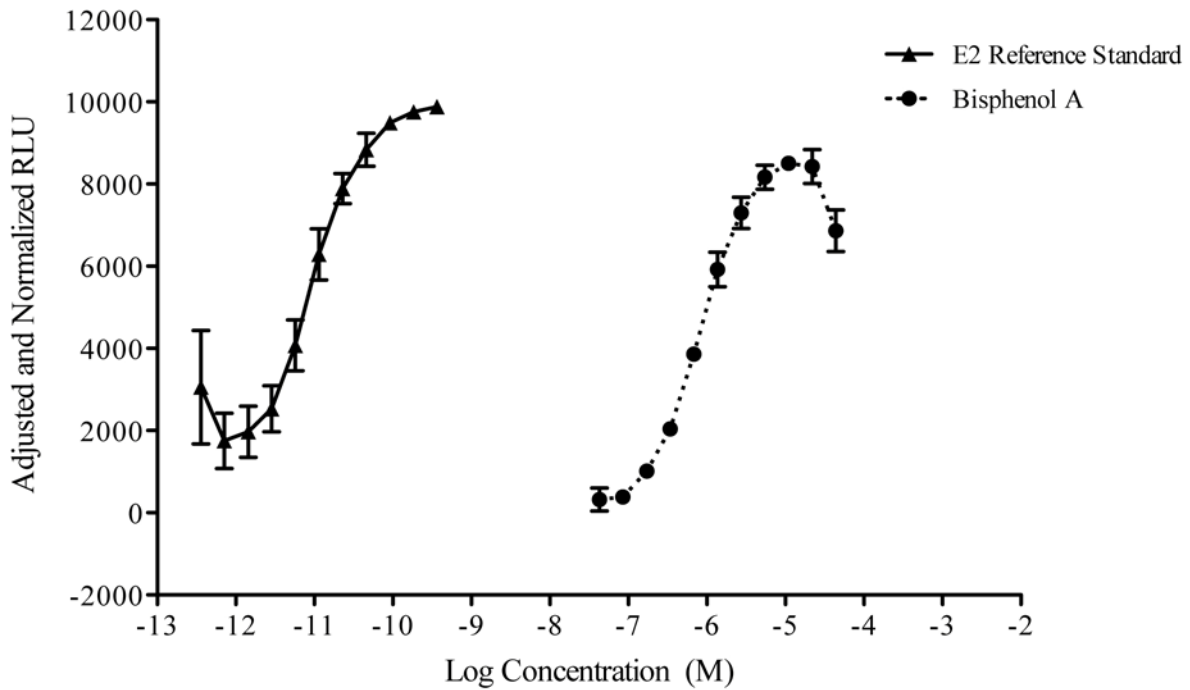
Table 2 Substances Used to Assess ER TA Antagonist Assay Accuracy

| Substance | CASRN | ICCVAM Classification | BG1Luc ER TA Results |
|---------------------------------|------------|-----------------------|----------------------|
| 4-Hydroxytamoxifen | 68047-06-3 | POS | POS |
| Raloxifene HCl | 82640-04-8 | POS | POS |
| Tamoxifen | 10540-29-1 | POS | POS |
| 17 α ethinyl estradiol | 57-63-6 | NEG | NEG |
| 5 α -Dihydrotestosterone | 521-18-6 | NEG | NEG |
| Apigenin | 520-36-5 | NEG | NEG |
| Bisphenol A | 80-05-7 | NEG | NEG |
| Butylbenzyl phthalate | 85-68-7 | NEG | NEG |
| Chrysin | 480-40-0 | NEG | NEG |
| Coumestrol | 479-13-0 | NEG | NEG |
| Daidzein | 486-66-8 | NEG | NEG |
| Di- <i>n</i> -butyl phthalate | 84-74-2 | NEG | NEG |
| Dicofol | 115-32-2 | NEG | NEG |
| Diethylhexyl phthalate | 117-81-7 | NEG | NEG |
| Diethylstilbestrol | 56-53-1 | NEG | NEG |
| Genistein | 446-72-0 | NEG | NEG |
| Kaempferol | 520-18-3 | NEG | NEG |
| Kepone | 143-50-0 | NEG | NEG |
| Mifepristone | 84371-65-3 | NEG | NEG |
| Norethynodrel | 68-23-5 | NEG | NEG |
| <i>o,p'</i> -DDT | 789-02-6 | NEG | NEG |
| <i>p</i> -n-nonylphenol | 104-40-5 | NEG | NEG |
| <i>p,p'</i> -DDE | 72-55-9 | NEG | NEG |
| Progesterone | 57-83-0 | NEG | NEG |
| Resveratrol | 501-36-0 | NEG | NEG |

Abbreviations: NEG = negative; POS = positive

Examples of BG1Luc ER TA Concentration Response Curves

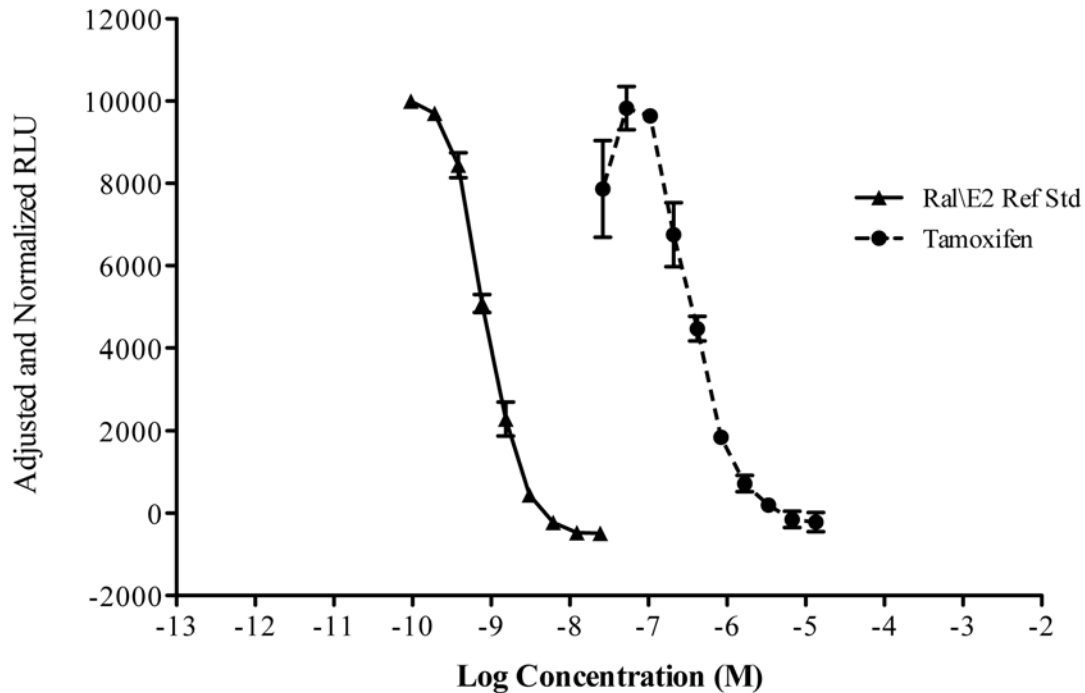
ER TA Agonist Activity



Interpretation of results for AGONIST testing

- "POSITIVE" if concentration response curve is observed:
 - The line defining the positive slope must contain at least three points with non-overlapping error bars.
- The amplitude, the difference between baseline and peak, must be at least 20% of the maximal value of the reference estrogen
- "NEGATIVE" if all data points are below 20% of the maximal value for the reference estrogen

ER TA Antagonist Activity



Interpretation of results for ANTAGONIST testing

- **"POSITIVE"** if concentration response curve is observed:
 - The line defining the positive slope must contain at least three points with non-overlapping error bars.
 - The amplitude, the difference between baseline and bottom, must be at least 20% of the maximal value of the reference estrogen
- **"NEGATIVE"** if all data points are above 80% of the maximal value for the reference estrogen

** Please note: The graphs originally presented on this panel illustrated generalized curves of agonist and antagonist responses, but were not actual examples of curves generated during the validation study. These figures have been replaced with graphs of data produced during the BG1Luc validation study.*

Evaluation of BG1Luc ER TA Accuracy

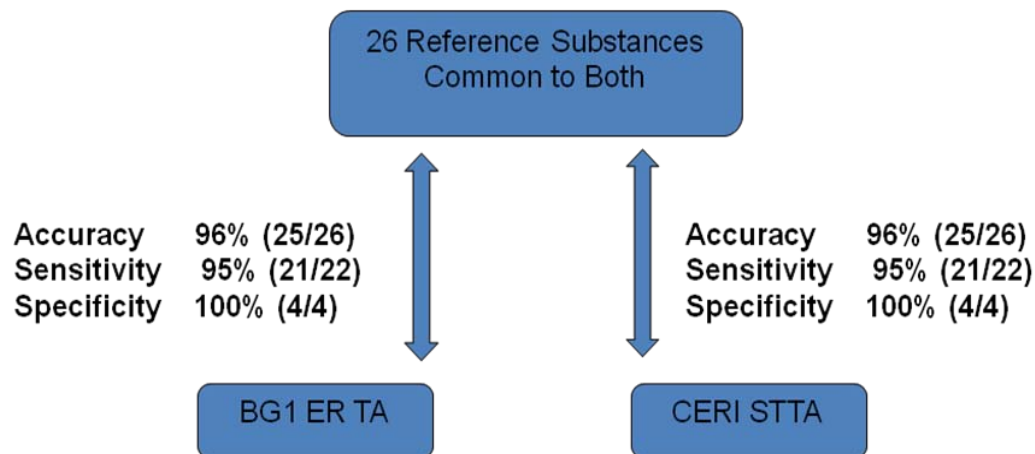
- BG1Luc ER TA results were compared to the reference substance ICCVAM Classification
- The 35 substances listed in **Table 1** and the 25 substances listed in **Table 2** were used to evaluate agonist and antagonist accuracy respectively

| N | Accuracy | Sensitivity | Specificity | False Positive Rate | False Negative Rate |
|-------------------|------------------------|-----------------------|------------------------|---------------------|---------------------|
| Agonist | | | | | |
| 35 | 97% (34/35) | 96% (27/28) | 100% (7/7) | 0% (0/7) | 4% (1/28) |
| Antagonist | | | | | |
| 25 | 100% (25/25) | 100% (3/3) | 100% (22/22) | 0% (0/22) | 0% (0/3) |

Abbreviations: N = number

Comparison of BG1Luc ER TA Results with US EPA OPPTS 890.1300 (CERI STTA)

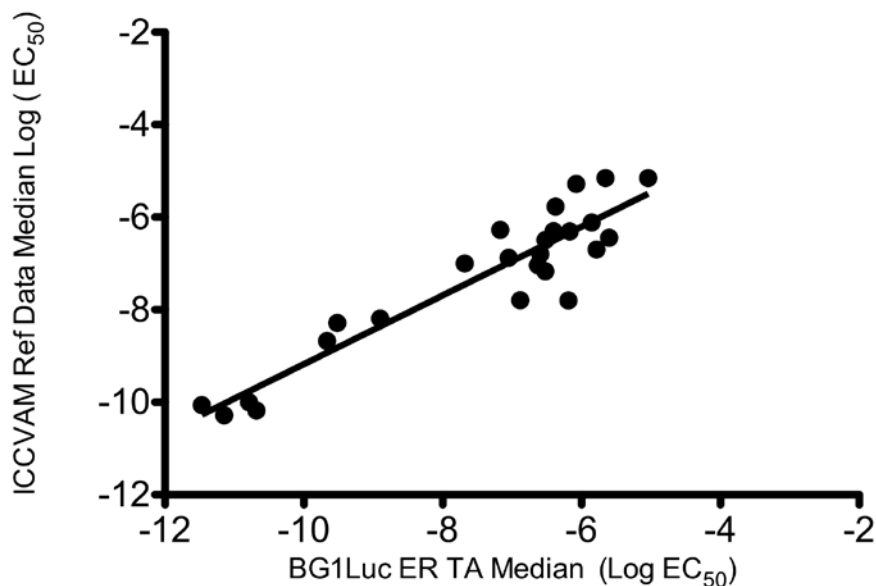
- The CERI STTA uses hER α -HeLa-9903 cells, a human cervical cancer cell line with a stably transfected human ER (OECD 2009; Takeyoshi 2006)
 - Tests for ER TA agonist activity only
- Currently CERI STTA is the only *in vitro* ER TA test method accepted by regulatory agencies
- Therefore, results from BG1Luc ER TA were compared to CERI STTA based on a list of agonist reference substances for which definitive classifications have been produced in both test methods.
- Accuracy statistics were identical for both test methods



Comparison of BG1Luc ER TA and ICCVAM Reference Data EC₅₀ Values

- EC₅₀ values obtained from BG1Luc ER TA substances used to evaluate accuracy were compared to median values from ER TA test methods reported in the literature
- The correlation between the EC₅₀ values was relatively high with an R² value of 0.84

Correlation of EC₅₀ Values



Each point represents a median EC₅₀ value obtained in the BG1Luc ER TA compared with the median ICCVAM reference data EC₅₀ value (EC₅₀ values were obtainable for 26 of the 33 ER TA positive substances used to evaluate accuracy)

Concordance of BG1Luc ER TA Results with ER Binding Data

- Results for substances that tested positive for agonist and/or antagonist activity in BG1Luc ER TA were compared to a list of substances for which definitive classifications have been produced in both ER TA and ER binding assays (34 substances)
- Comparison of BG1Luc ER TA results and ER binding data demonstrated **97% (33/34) concordance** between the two test methods
- The single discordant test substance was medroxy-progesterone acetate (positive in a single BG1Luc ER TA antagonist test but reported negative for ER binding in two published studies)

| | | BG1Luc ER TA Classification | | |
|---------------------------|-------|-----------------------------|-----|-------|
| | | POS | NEG | Total |
| ER Binding Classification | POS | 31 | 0 | 31 |
| | NEG | 1 | 2 | 3 |
| | Total | 32 | 2 | 34 |

Concordance of BG1Luc ER TA Results with Uterotrophic Data

- Results for substances that tested positive for agonist activity in BG1Luc ER TA were compared to a list of substances for which definitive classifications have been produced in both ER TA and the uterotrophic assay (13 substances)
- Comparison of BG1Luc ER TA results and ER binding data demonstrated **92% (12/13) concordance** between the two test methods
- The single discordant test substance was butylbenzyl phthalate (positive BG1Luc ER TA antagonist – negative in uterotrophic assay)

| | | BG1Luc ER TA Classification | | |
|-----------------------------------|-------|-----------------------------|-----|-------|
| | | POS | NEG | Total |
| Uterotrophic Assay Classification | POS | 11 | 0 | 11 |
| | NEG | 1 | 1 | 2 |
| | Total | 12 | 1 | 13 |

Intralaboratory Reproducibility

- Evaluated based on 12 agonist and 12 antagonist substances that were tested at least 3 times for agonist and antagonist activity during Phase 2 at each of the three laboratories
- Although the classifications for some of the test substances differed among the laboratories, there was 100% agreement within each laboratory for each of the three repeat tests

| Activity per Test | XDS | ECVAM | Hiyoshi |
|-----------------------------|---------------------|---------------------|---------------------|
| Agonist Activity | | | |
| Agreement Within Laboratory | 12/12 (100%) | 12/12 (100%) | 12/12 (100%) |
| +++ | 8/12 | 12/12 | 9/12 |
| --- | 4/12 | 0/12 | 3/12 |
| Antagonist Activity | | | |
| Agreement Within Laboratory | 12/12 (100%) | 12/12 (100%) | 12/12 (100%) |
| +++ | 2/12 | 2/12 | 2/12 |
| --- | 10/12 | 10/12 | 10/12 |

Abbreviations: + = positive test result; - = negative test result

Interlaboratory Reproducibility

- The classifications of each of the 41 substances that were tested once for agonist and antagonist activity at all three laboratories during Phase 3 were also used to evaluate the extent of interlaboratory agreement
- Of the 41 substances tested for agonist activity, 36 produced a definitive result in at least two laboratories
 - The three laboratories agreed on 83% (30/36) of these substances
- Definitive results were produced for all 41 substances tested for antagonist activity
 - The three laboratories agreed on 93% (38/41) of the substances.

| Results Among Laboratories^a | Agonist Testing | Antagonist Testing |
|---|------------------------|---------------------------|
| Agreement Among Laboratories | 30/36 (83%) | 38/41 (93%) |
| +++ | 18/36 | 2/41 |
| --- | 4/36 | 33/41 |
| ++i | 2/36 | 1/41 |
| --i | 6/36 | 2/41 |
| Discordance Among Laboratories | 6/36 (17%) | 3/41 (7%) |
| +- | 3/36 | 0/41 |
| +- | 0/36 | 1/41 |
| +-i | 3/36 | 2/41 |

Abbreviations: + = positive test result; - = negative test result; i = inadequate data

^aOnly those substances that produced a definitive result in at least two of the three laboratories were used in this evaluation.

Test Method Transferability

- Transferability of the BG1Luc ER TA was demonstrated based on results of the interlaboratory validation study that are detailed above.
- The primary practical considerations associated with the BG1Luc ER TA are the availability of the requisite cell line and the standard laboratory equipment necessary to conduct sterile cell culture procedures.
- The level of training, expertise, and time needed to conduct the BG1Luc ER TA should be similar to the currently accepted CERI STTA method.

Summary

- The BG1Luc ER TA is a highly sensitive method, capable of detecting a diverse set of chemical substances that exhibit *in vitro* ER agonist or ER antagonist activity.
- Accuracy of the BG1Luc ER TA for detecting *in vitro* ER TA agonist and antagonist activity was 97% and 100%, respectively
- Accuracy for the BG1Luc ER TA and CER1 STTA test methods is identical when using a common set of test substances.
- EC₅₀ values generated using the BG1Luc ER TA correlated well with EC₅₀ values found in published literature ($R^2 = 0.84$)
- There was a high level of concordance between the BG1Luc ER TA and *in vitro* ER binding (97%), and *in vivo* uterotrophic assays (92%)
- The BG1Luc ER TA demonstrates good inter- and intralaboratory reproducibility.

Peer Panel Review

NICEATM and ICCVAM will convene a public meeting of an independent scientific peer review panel to evaluate the validation status of the BG1Luc ER TA test method on **March 29-30, 2011**, at the William H. Natcher Conference Center at the headquarters of the National Institutes of Health in Bethesda, Maryland. The meeting is open to the public and there is no charge to attend. The BG1Luc ER TA validation study draft Background Review Document and supporting materials are available on the ICCVAM public website at:

<http://iccvam.niehs.nih.gov/methods/endocrine/PeerPanel11.htm>

Acknowledgements

This poster was supported by the Intramural Research Program of the National Institute of Environmental Health Sciences. ILS staff were supported by NIEHS contract N01-ES 35504. The views expressed above do not necessarily represent the official positions of any Federal agency.

References

ICCVAM. 2006. Finalized Addendum to ICCVAM Evaluation of *In Vitro* Test Methods for Detecting Potential Endocrine Disruptors. Research Triangle Park, NC:National Institute of Environmental Health Sciences. Available at http://iccvam.niehs.nih.gov/docs/endo_docs/EDAddendFinal.pdf

OECD. 2009. Test No. 455. Stably Transfected Human Estrogen Receptor- α Transcriptional Activation Assay for Detection of Estrogenic Agonist-Activity of Chemicals. In: OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects:OECD Publishing.

Takeyoshi M. 2006. Draft Report of Pre-validation and Inter-laboratory Validation For Stably Transfected Transcriptional Activation (TA) Assay to Detect Estrogenic Activity - The Human Estrogen Receptor Alpha Mediated Reporter Gene Assay Using hER-HeLa-9903 Cell Line.Chemicals Evaluation and Research Institute (CERI), Japan. Available at <http://www.oecd.org/dataoecd/49/55/37531918.pdf>.

Current validation study information available at:

<http://iccvam.niehs.nih.gov/methods/endocrine.htm>