



June 28, 2010

**Agonist Screen Report**  
Six samples received on 05/20/2010

**Introduction:**

Xenobiotic Detection Systems, Inc. (XDS) has a genetically engineered cell (BG1Luc4E2) line that contains the firefly luciferase gene under trans-activational control of the estrogen receptor. This cell line can be used for the detection and relative quantification of estrogenic agonists and antagonists. Our term for the in vitro assay is the LUMI-CELL<sup>®</sup> ER assay. Estrogen agonist activity is quantified versus a standard curve of 17Beta-Estradiol.

The BG1Luc4E2 cell line has been stably transfected with a luciferase reporter gene that is responsive to the exposure of the cells to estrogen or chemicals that are estrogen agonists. The cell line was grown in estrogen free DMEM for 2 days that contains 5 % Fetal Calf serum that was carbon/dextran filtered to remove endogenous estrogen activity.

**Statement of Procedure:**

During the LUMI-CELL<sup>®</sup> ER bioassay, BG-1Luc4E2 cells are cultured and 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. Before dosing cells, cells are visually inspected using an inverted microscope, and the visual observations scores recorded. Only plates in which the cells in all wells give a score of 1 are used for dosing. Test compounds and control solutions are transferred to 96 well culture plates containing the BG1Luc4E2 cells and incubated in 5% CO<sub>2</sub> incubator at 37 °C for 19 to 24 hr for optimal expression of luciferase. After incubation the media is removed and the cells were washed and examined with an inverted microscope to observe whether any observable toxicity or displacement of cells has occurred.

Comprehensive testing experiments are used to determine whether a substance possesses ER agonist activity in the LUMI-CELL<sup>®</sup> ER test method. Comprehensive testing is conducted on 96-well plates using 11 concentrations of E2 in duplicate as the reference standard. Four replicate wells for the DMSO control and four replicate wells for the methoxychlor control are included on each plate. Comprehensive agonist testing for substances consists of either an 11 point 1:2 serial dilution or an 11 point 1:5 serial dilution.

**Samples from Health Child Healthy World:**

Six samples were received from Christopher Gavigan with Health Child Healthy World on 05/20/2010. The samples were logged in and assigned XDS identification numbers. The sample log in/chain of custody form is included at the end of this report (appendix 1).

Three of the samples (tetra-pak containers) were extracted with 20 mls methanol from 20 X 7 cm surface area by shaking overnight. One ml of each extract was dried by centrifugation. The samples were resuspended in

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one ml of DMEM and serially diluted in DMEM and 1%DMSO. For the other three samples (coconut water), the coconut water was first diluted in 2x DMEM followed by a 1:2 serial dilution in 1X DMEM so that the initial testing volume was 100  $\mu$ l. These solutions were then transferred in triplicates to 96 well culture plates containing the BG1Luc4E2 cells. In addition to the dilutions of samples, a standard curve of Beta-estradiol, as well as Methoxychlor (positive QC) and DMSO (negative QC) were assayed. The plates were incubated to induce optimal expression of luciferase activity in a humidified CO<sub>2</sub> incubator. Following the incubation the media was removed and the cells were microscopically observed for viability. The luciferase assay was run to quantify the induction of luciferase activity.

The samples were analyzed and compared to Methoxychlor (positive QC) and DMSO (negative QC). All controls met QC requirements. The estrogenic activity was estimated by regression analysis from the standard curve of Beta-estradiol for agonist activity.

Cell viability: Microscopic examination of the cells following exposure to the sample extracts did not reveal any indication of toxicity to the cells from the sample.

### **Acceptance Criteria for Agonist Testing**

- Induction: Plate induction, as measured by dividing the averaged highest E2 reference standard RLU value by the averaged DMSO control RLU value, must be greater than three-fold.
- Reference standard results: The E2 reference standard concentration-response curve should be sigmoidal in shape and have at least three values within the linear portion of the concentration-response curve.
- DMSO control results: DMSO control RLU values must be between 0 and 7188.
- Positive control results: Methoxychlor control RLU values must be above the DMSO mean plus three times the standard deviation from the DMSO mean.
- An experiment that fails any single acceptance criterion will be discarded and repeated.

### **Data Analysis**

Luminescence data is measured in relative light units (RLUs). LUMI-CELL<sup>®</sup> ER uses an Excel<sup>®</sup> spreadsheet to collect and adjust the RLU values obtained from the luminometer. The Excel<sup>®</sup> spreadsheet subtracts background luminescence (average DMSO solvent control RLU value) from test substance, reference standard and control RLU values. Plate induction is calculated by dividing the average highest E2 RLU value by the average DMSO control RLU value. Test substance, reference standard, and control RLU values are then adjusted such that the maximal TA response induced by 17 $\beta$ -estradiol (E2) controls is 10,000 RLUs. After adjustment and the exclusion of cytotoxic concentrations, values are transferred to GraphPad Prism-4.0<sup>®</sup> statistical software for data analysis and graphing.

All chemicals classified as positive should have a response curve consisting of a base line, followed by a positive slope with non-overlapping error bars, concluding in a plateau or peak. The slope line must contain at least three points. The amplitude (difference between the baseline and the peak) of the curve must be at least 2000 RLU. The response should be greater than the mean RLU value plus three times the standard deviation for the DMSO. Any response below this threshold is considered negative for agonist activity. Any isolated increase in response seen at only one concentration is considered to be irrelevant and must be excluded.

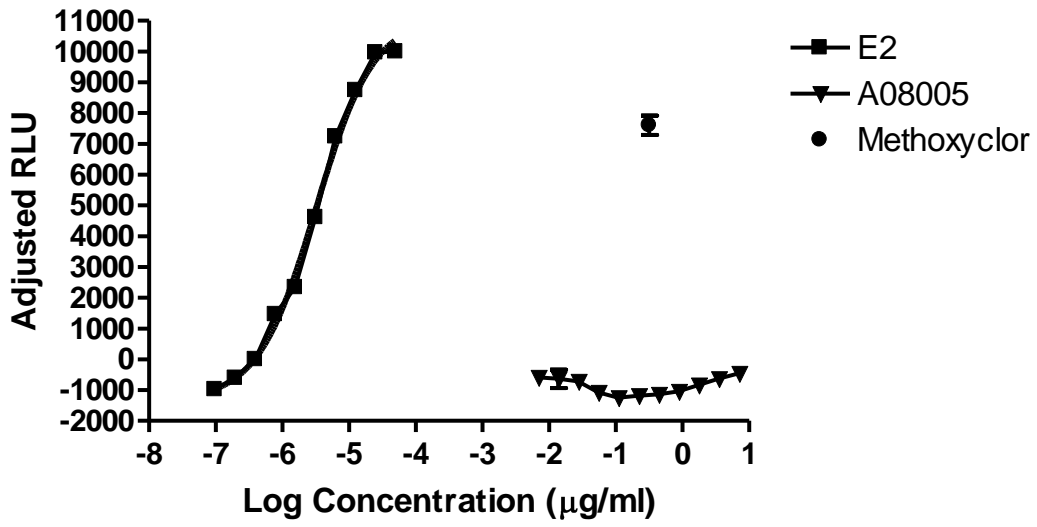
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**Summary of Results:**

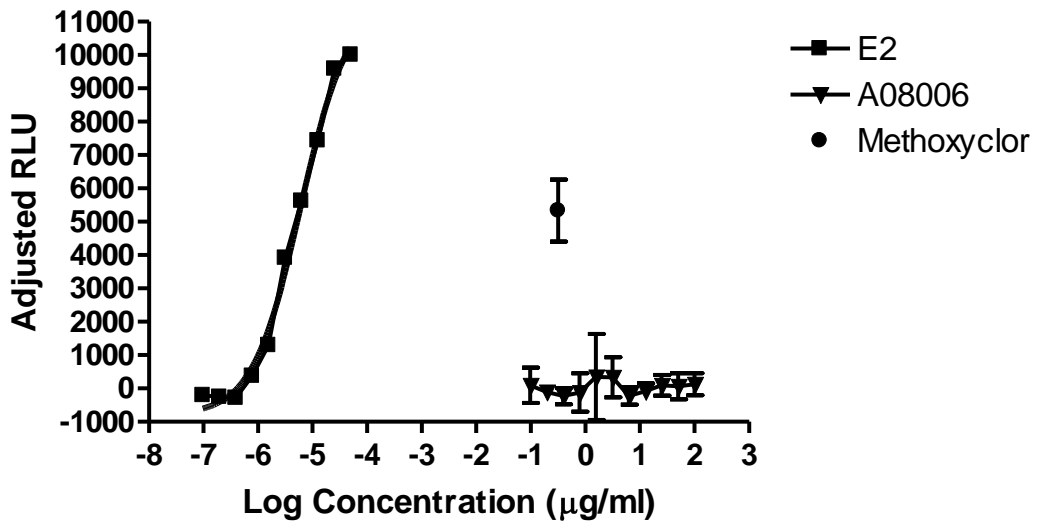
All samples were found to be negative for estrogenic activity.

<b>XDS ID Number</b>	<b>Client ID Number</b>	<b>Sample Matrix</b>	<b>Sample Aliquot</b>	<b>Extraction Solvent</b>	<b>Positive/Negative</b>
A08005	1	Coconut water	100 µl	100% MeOH	Negative
A08006	2	Coconut water	100 µl	100% MeOH	Negative
A08007	3	Coconut water	100 µl	100% MeOH	Negative
A08008	4	Tetra-pak	140cm <sup>2</sup>	None	Negative
A08009	5	Tetra-pak	140cm <sup>2</sup>	None	Negative
A08010	6	Tetra-pak	140cm <sup>2</sup>	None	Negative

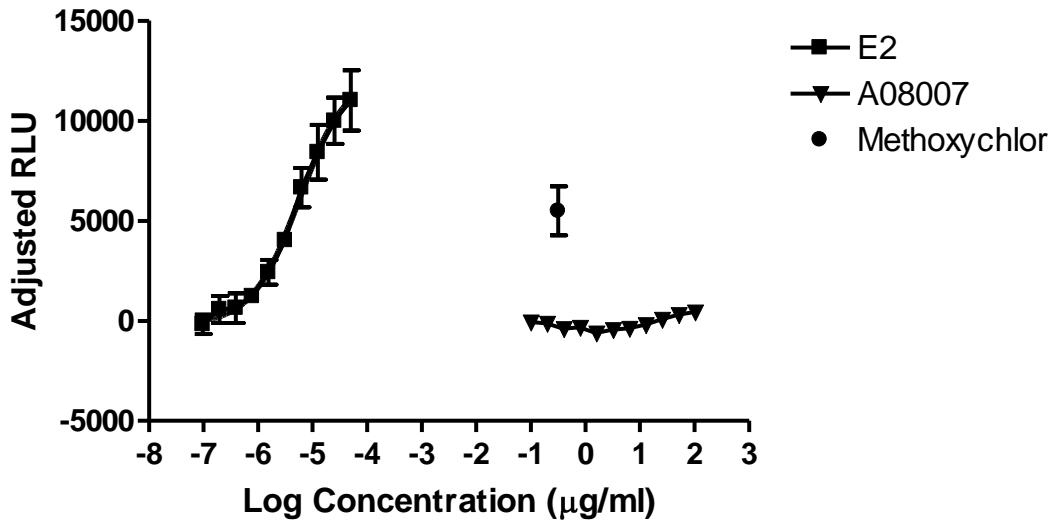
### A08005 Agonist Results



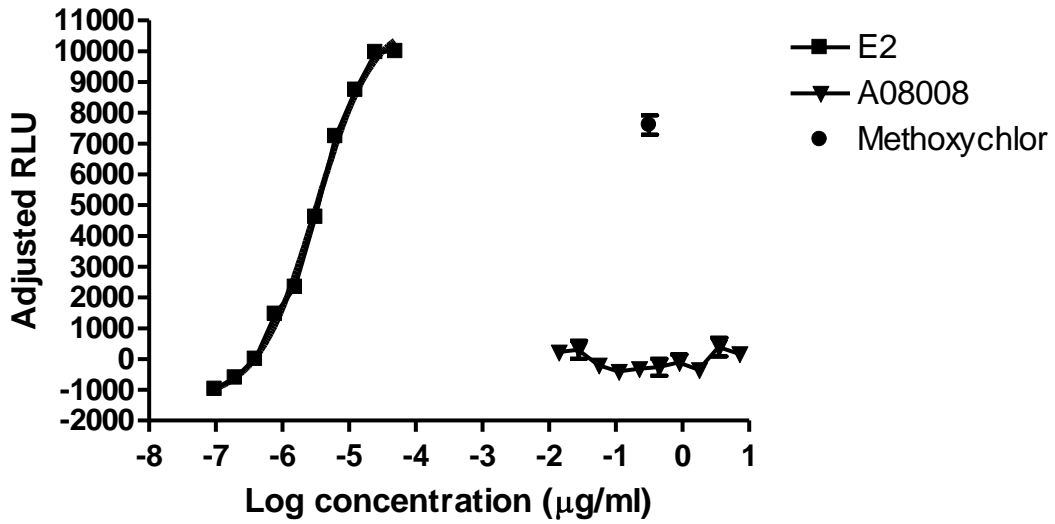
### A08006 Agonist Results



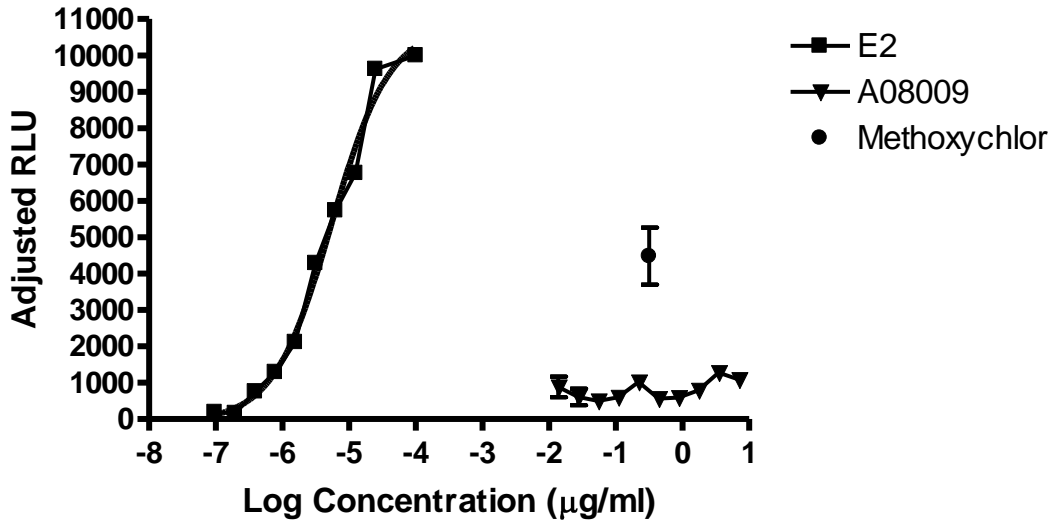
### A08007 Agonist Results



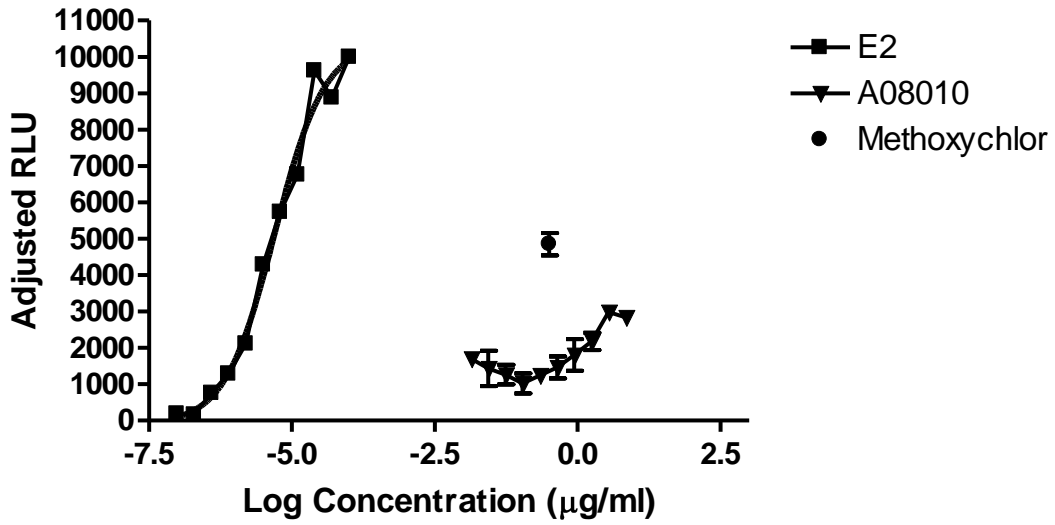
### A08008 Agonist Results



### A08009 Agonist Results



### A08010 Agonist Results



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## **Summary**

Six samples were received from Christopher Gavigan with Health Child Healthy World on 05/20/2010. The samples were logged in and assigned XDS identification numbers. Agonist comprehensive assays were performed using a 1:2 dilution. All samples tested failed to induce activation of the estrogen receptor in a concentration- dependent fashion, and are therefore negative for agonist activity.

If you have any questions regarding these results, or the methods that were used, please contact us at either (919)-688-4804 or by e-mail at [info@dioxins.com](mailto:info@dioxins.com).

*Ruth McMillan*

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Ruth McMillan  
Director of Research

06/28/2010  
Date

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## REFERENCES

ICCVAM. 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 Pub. No. 03-4503 Research Triangle Park, NC: National Institute of Environmental Health Sciences.

Rogers JM, Denison MS. 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 Vitro Mol Toxicol* 2000 Spring;13(1):67-82.

OECD. 1998. OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring Number 1: OECD principles on Good Laboratory Practice. [as revised in 1997]. ENV/MC/CHEM[98]17. Paris: OECD

Nonlinear regression analysis was performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com).

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**AGONIST REPORT APPROVAL**

This report has been reviewed and approved by the following:

**Research Director**

These tests were conducted to the standards of:

<u>Ruth McMillan, Ph.D.</u> _____	_____	_____
Research Director Xenobiotic Detection Systems	Signature	Date

**Review**

<u>Ying Gu</u> _____	_____	_____
Quality Assurance Officer Xenobiotic Detection Systems	Signature	Date

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**Appendix 1.**

<b>XDS ID#</b>	<b>Sample ID#</b>	<b>Sample Matrix</b>	<b>Customer ID</b>	<b>shipment #</b>	<b>turnaround time (day)</b>	<b>Analysis Requested</b>	<b>Arrival Date</b>
A08005	Sample-1	Coconut Water	Healthy Child Healthy World	1084	30	Estro. Ago Comp	5/20/2010
A08006	Sample-2	Coconut Water	Healthy Child Healthy World	1084	30	Estro. Ago Comp	5/20/2010
A08007	Sample-3	Coconut Water	Healthy Child Healthy World	1084	30	Estro. Ago Comp	5/20/2010
A08008	Sample-4	Plastic container	Healthy Child Healthy World	1084	30	Estro. Ago Comp	5/20/2010
A08009	Sample-5	Plastic container	Healthy Child Healthy World	1084	30	Estro. Ago Comp	5/20/2010
A08010	Sample-6	Plastic container	Healthy Child Healthy World	1084	30	Estro. Ago Comp	5/20/2010