Submission of XDS's LUMI-CELL ä ER High-Throughput System for Screening Estrogen-Like Chemicals for Review by ICCVAM

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and
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Abbreviations:

1. LUMI-CELL™ ER - LUMI-CELL™ Estrogen Receptor
2. EDC - endocrine disruptor chemicals
3. EDSTAC - Endocrine Disruptor Steering and Testing Advisory Committee
4. HTPS - High-Through Put Screening
5. EDSP - Endocrine Disruptor Screening Program
6. ICCVAM - Interagency Coordinating Committee on the Validation of Alternative Methods
7. XDS - Xenobiotic Detection Systems
8. EREs - Estrogen-response-elements
9. MMTV - mouse mammary tumor viral
10. MEM – Minimal Essential Media

11. QSAR's - Quantitative Structure-Activity Relationships
12. LF – lactoferin
13. DMSO - Dimethyl Sulfoxide
14. QC – Quality Control
15. "Plate-to-Plate" - 3 separate experimental sets (dilutions of the compound of interest to create a dose response curve) were generated and plated on 3 separate 96 well plates.
16. "Well-to-Well" - 3 separate replicates of the same compound concentration were plated on the same plate.
17. GLP – Good Laboratory Practices
18. RLU - Relative Light Units
19. EC50 – 50% of the highest activity
20. mmol/ml – mM or milli Molar (i.e. mol/l or M (Molar))
21. mean – Average of a number set
22. std dev – Standard Deviation (i.e. standard deviation of a mean)
23. % std dev – Standard Deviation divided by the Mean
24. Count – the number of experiments conducted and used in calculations
25. std error – Standard Error
26. % std error – Standard Error divided by the Mean
27. midpoint – The middle of the linear portion of a dose response curve
28. RBA – Relative Binding Affinity
29. MCRG - Mammalian Cell Reporter Gene
30. ER – Estrogen Receptor
31. AR – Androgen Receptor
32. TA – Transactivation
33. N/A – Not Applicable
34. ND – Not Determined
35. P – Positive
36. PP – Presumed Positive
37. PN – Presumed Negative
38. "+" – Positive
39. "-" – Negative
40. "X" – No Data available
41. "?" – Data not clear
42. CAS RN – Chemical Abstracts Service Registry Number
43. Coefficient of Variation - Standard Error divided by the Mean
44. Relative Induction to Estradiol – The RLU induction relative to that of 17β-estradiol
45. pg – pico grams
46. ug or µg – micro grams
47. DES – Diethylstilbestrol
48. BPA – Bisphenol A

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1.0 Introduction and Rationale for the Proposed Test Method

1.1 Introduction

1.1.1 Describe the historical background for the proposed test method, from original concept to present. This should include the rationale for its development, and overview of prior development and validation activities, and, if applicable, the extent to which the proposed test method is mechanistically and functionally similar to a validated test method with established performance standards.

The association of exposure to endocrine (hormone) disruptor chemicals (EDCs) and adverse health effects in human and wildlife populations has led to worldwide concern. Some of the health effects that have led to this concern include global increases in testicular cancer, regional declines in sperm counts, altered sex ratios in wildlife populations, increases in the incidence of breast cancer and endometriosis, and accelerated puberty in females that are expected to result from exposure to chemicals that adversely affect steroid hormone action (Colborn, vom Saal et al. 1993; Sakr 1993; Adami 1994; Birnbaum 1994; Colborn 1995; LeBlanc 1995; Adami, Bergstrom et al. 1996; Fisch 1996). These observations have focused intense national and international attention on the role of environmental chemicals known as endocrine disruptors (Gray, Kelce et al. 1997; Gray 1998; DeVito, Biegel et al. 1999). The concern over these effects in both human and wildlife populations led to passage of the Food Quality Protection Act and Safe Drinking Water Act by the U.S. Congress (Food Quality Protection Act 1996; Safe Drinking Water Act 1996). These acts mandated the U.S. EPA to investigate the effects of environmental chemicals on the reproductive capacity of both wildlife species and humans. To fulfill this mandate the EPA organized the Endocrine Disruptor Steering and Testing Advisory Committee (EDSTAC); a group of scientists from industry, academia and government; to define a consensus course of action to evaluate potential adverse reproductive effects of a wide range of environmental and industrial chemicals. EDSTAC proposed a tiered testing approach with High-Through Put Pre-Screening (HTPS) reporter gene assays, which would be used to pre-sort chemicals and assist in defining research priorities, provided that these systems are technically feasible and validated. The EDSTAC report was submitted to Congress in August 2000 and resulted in the formation of the Endocrine Disruptor Screening Program (EDSP) within the EPA. The EDSTAC report proposed that EPA pursue the standardization and validation of Tier I and Tier II screening assays for endocrine disruptors. These bioassays would specifically examine the ability of a chemical to act like a hormone (agonist) and/or to block the action of a hormone (antagonist) at the level of gene expression. Some cell lines have been developed in an attempt

to fill this role, the majority of these bioassays are not very sensitive or applicable to HTPS protocols. This latter point is extremely important especially considering the tremendous number of chemicals mandated to be tested in addition to many environmentally relevant chemicals and contaminants.

In April 2000 the EPA asked the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) to evaluate programs and systems directed toward ER and AR endocrine disruptor *in vitro* binding and transcriptional activation. In response to this request, ICCVAM assembled an expert committee made up of academic, governmental and industry experts who came up with a list of 78 compounds which are recommended for testing for validation of ER and AR endocrine disruptor *in vitro* binding and transcriptional activation test methods (ICCVAM 2002).

Xenobiotic Detection Systems (XDS) in collaboration with Dr. Michael S. Denison (University of California - Davis), has developed a stable recombinant cell line (BG1Luc4E2), which produces both alpha and beta estrogen receptors, and is sensitive and useful in detecting estrogen active chemicals in a high through put screen (HTPS) format. XDS is submitting the mechanistic basis for the assay, protocols that are reliable in estimating estrogenic activity of chemicals and mixtures, and data on chemicals we have evaluated for estrogen activity. XDS is submitting this information for review by ICCVAM for its potential as a validated regulatory method in response to the Federal Register Notice (Vol. 66, No. 57/Friday, March 23, 2001) as a HTPS method for estrogen active compounds. We are in the process of developing other recombinant cell based assays that would be useful in fulfilling the federally mandated need for analytical systems that can identify chemicals with endocrine disruptor activity.

Receptor-Dependent Mechanism of Action of Estrogen-Steroid Hormones and Effects of EDCs.

The molecular mechanism of action of estrogen-steroid hormones is based on their ability to bind to and activate specific nuclear receptor proteins in responsive cells (Carson-Jurica, Schrader et al. 1990; Beato, Herrlich et al. 1995). A drawing depicting the molecular mechanism of estrogen activation of gene expression and biochemical events that occur following exposure of cells to estrogen or estrogenic chemicals is shown in Figure 1. Estrogen or chemicals that act as agonists for the estrogen receptor bind to the receptor and then the receptor dimerizes to the ligand-activated form of the receptor that can bind to DNA sequences (Estrogen-response-elements, EREs), that are upstream of estrogen responsive genes. Binding of the ligand activated receptor complex results in initiation of transcription of the down stream-associated genes under control of the EREs. Environmental EDCs can adversely affect hormone action by exerting an effect on one or more steps in these ligand- and receptor-dependent signal transduction pathways. Chemicals can bind to these receptors and directly activate the receptor or inhibit (antagonize) the binding and activation of the receptor by its endogenous ligand (estrogen).

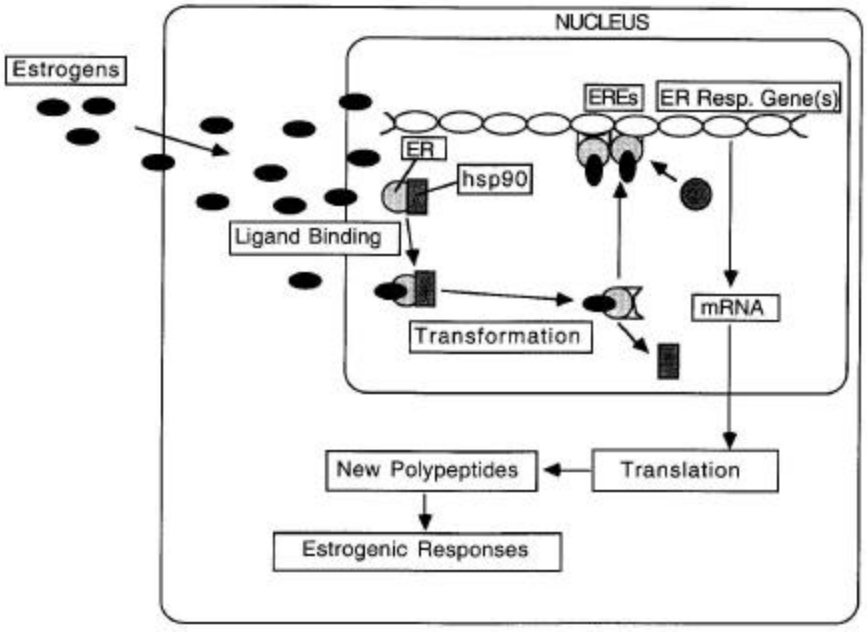


Figure 1. Molecular mechanism of estrogen hormone action. See text for details.

Describe the purpose, including the mechanistic basis, of the proposed test method.

The test method was developed by producing a recombinant cell line that contains a reporter construct that expresses luciferase activity in response to exposure of the cells to estrogen or estrogen-like compounds. Shown in Figure 2 is the plasmid pGudLuc7.ERE used to produce a recombinant cell line. This plasmid contains 4 copies of a synthetic oligonucleotide containing the estrogen response element upstream of the mouse mammary tumor viral (MMTV) promoter and the firefly luciferase gene. BG1 a human ovarian carcinoma, which expresses both the endogenous alpha and beta estrogen receptors, was transfected with the reporter gene construct and stable transfectants selected by growth in MEM containing 0.4 mg/ml geneticin (G418) until colonies inducible for luciferase activity were cloned (Rogers and Denison 2000). A stably transfected cell line designated BG1Luc4E2 was cloned from this procedure and expresses luciferase activity in response to estrogen and estrogen-like chemicals. The cell line BG1Luc4E2, has demonstrated stable induction of luciferase activity in response to exposure of the cells to estrogen for over 5 years. The conditions to grow these cells and measure the estrogen-inducible expression of luciferase activity in a high-throughput screen format are defined in this document.

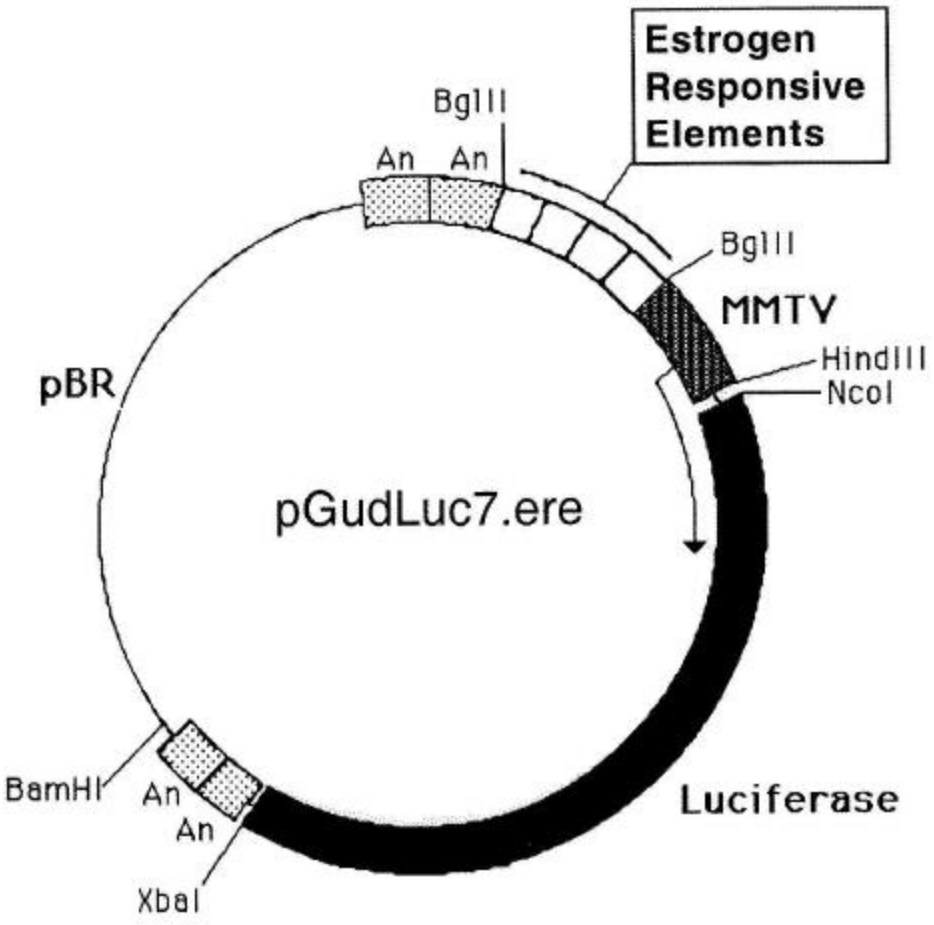


Figure 2. Plasmid pGudLuc7.ERE used to produce recombinant cell line BG1Luc4E2.

When possible, describe what is known about the similarities and differences of modes and mechanisms of action in the test system as compared to the species of interest (e.g., humans for human health-related toxicity testing).

There are other mechanisms by which chemicals may demonstrate endocrine disruptor activity in addition to directly competing for ligand binding to the estrogen receptor. EDCs can stimulate metabolic degradation or synthesis of the endogenous hormone ligand or receptor itself and/or indirectly activate or inhibit activation of the receptor by affecting receptor phosphorylation (*i.e.* by stimulating or repressing protein kinases or phosphatases known to be important in receptor function) (Spink, Lincoln et al. 1990; Spink, Eugster et al. 1992; Weigel and Zhang 1998). Additional targets for EDCs include chemical-dependent alterations in the expression of a given receptor(s) and/or the level, or function, of a critically important nuclear receptor coactivator or corepressor necessary for receptor functionality. Thus, it is possible for xenobiotic chemicals to alter normal endocrine homeostasis and hormone action both directly and indirectly by a variety of different mechanisms in different cell types. However, the primary mechanism for chemicals to act as endocrine disruptors is through acting as an agonist or an antagonist of the hormone at the receptor level altering gene expression. Irrespective of the above actions of an EDC, effects of all of these targets will result in a change in ER-dependent gene expression. Thus, the cell line BG1Luc4E2 is a useful tool for HTPS analysis of chemicals for potential activity as estrogenic agonists or antagonists of gene expression. XDS has termed the bioassay using BG1Luc4E2 as the LUMI-CELL™ ER test.

1.1.2 Summarize and provide the results of any peer review conducted with the test summarize and ongoing planned reviews.

There have been 5 papers written: 2 peer reviewed published paper on the production of the BG1Luc4E2 (Denison, Phelan et al. 1998 and Rogers and Denison 2000), one peer review paper comparing the XDS transcriptional assay with immature mouse uterotrophic responses in assessing the estrogenic activity of phytochemicals (Jefferson, Padilla-Banks et al. 2002) and two papers and several abstracts on the LUMI-CELL™ ER test (Gordon, Chu et al. 2003, 2004), (see the attached papers in Appendix H). Briefly, the LUMI-CELL™ ER bioassay (termed the ER Transcriptional assay in the Jefferson 2002 paper) demonstrated estrogen agonist activity for all the compounds tested except Taxifolin (Jefferson, 2002). The compounds demonstrating estrogen agonist activity were 17β-estradiol, DES, Zeralanol, Zeralenone, Coumesterol, Genistein, Biochanin A, Daidzein, and Naringenin. Other in vivo bioassay systems that were used to assess estrogen agonist activity were the Uterotrophic assay (measurement of uterine wet weight increase in immature mice), increase in uterine epithelial cell height, uterine Gland number increases, and induction of estrogen responsive protein lactoferin (LF assay). The Uterotrophic assay was able to detect estrogenic activity in 7 of the 10 compounds. The uterine cell height assay was able to detect estrogenic activity in 9 of the 10 compounds. The uterine Gland number assay was able to detect estrogenic activity in all 10 of the compounds tested, making it very consistent with the LUMI-CELL™ ER bioassay. Immunohistochemical analysis of lactorferin (LF) induction was able to detect estrogenic activity in 7 of the 10 compounds detected by the LUMI-CELL™ ER bioassay (Jefferson, Padilla-Banks et al. 2002). This data demonstrates the unique sensitivity of the LUMI-CELL™ ER bioassay in evaluating estrogenic

activity of these phytoestrogens and agreement with an in vivo model to evaluate estrogen agonists. Taxifolin was either a non-active or very weakly active in all of the above assays.

XDS has been in contact with members of NICEATM (Dr. William Stokes and Dr. Raymond Tice) to keep them informed on progress we have made in development of the LUMI-CELL™ ER test. Updates of the development included a site visit to the XDS laboratories and a number of meetings to review data and evaluate needs of an estrogenic agonist and antagonist assay of chemicals for potential endocrine disrupting activity.

1.1.3 Clearly indicate any confidential information associated with the test method; however inclusion of confidential information is discouraged.

Confidential information is included in this submission. The software that has been developed for automated analysis and the use of Hill equation modeling of receptor mediated gene expression is novel and under copyright submission. Information on these data analysis systems is included in Figures 4-8 of the submission (see Appendix A). Raw data will also be provided on CD in Excel file format.

1.2 Regulatory rationale and applicability

1.2.1 Describe the current regulatory testing requirement(s) for which the proposed test method is applicable.

The proposed method is suggested as a primary HTPS assay for chemicals that display estrogenic or anti-estrogenic activity. A major portion of the EDSTAC report to congress suggested that reporter gene technology may be useful for priority setting in screening chemicals that are potential endocrine disruptors [Endocrine Disruptor Screening Program, August 2000]. It was also suggested that in vitro screening would provide relative potency and dose response data that could be used to set doses in animal tests that follow screening. This could have a significant impact on reducing the number of animals used in animal testing protocols in Tier 1 and Tier 2 tests for endocrine disruptors. ICCVAM's expert panel has recommended 78 compounds to be tested during the validation process of ER and AR endocrine disruptor *in vitro* binding and transcriptional activation methods. The pharmaceutical industry has used HTPS reporter gene technology for identifying chemicals with properties that may be useful as drug candidates. The EPA awarded a contract to evaluate reporter gene technology as a screen for endocrine active chemicals. EPA concluded from this preliminary evaluation of reporter gene technology that the technology was not sufficiently sensitive or robust for identifying EDCs for regulatory purposes [Endocrine Disruptor Screening Program, August 2000]. XDS has developed extremely sensitive reporter gene systems to analyze for trace contamination of chemicals in the environment and should be useful for evaluating EDCs. This submission of data contains information on the HTPS format use of the LUMI-CELL™ ER bioassay. It is necessary for ICCVAM and the regulatory agencies to evaluate the robustness and sensitivity of reporter gene technology to be useful for regulatory purposes.

1.2.2 Describe the intended regulatory use(s) (e.g., screen, substitute, replacement, or adjunct) of the proposed test method and how it will be used to substitute, replace or complement any existing regulatory requirement(s).

The method is intended as a screen to identify chemicals that may possess estrogenic activity and for priority setting in the tiered approach for identifying endocrine disruptor active chemicals. The EDSTAC report suggests a tiered approach for evaluating chemicals for endocrine disruptor activity with HTPS of estrogen, androgen and thyroid hormones to be used in priority setting if these systems can be validated. The LUMI-CELL™ ER reporter gene system should be one of these systems that provide data for the evaluation of chemicals for estrogenic activity. Each EDC has a different ability to induce the estrogen receptor at a given concentration. The LUMI-CELL™ ER bioassay is capable of giving relative potencies of the estrogenic compounds. This would be similar (in concept only) to the World Health Organization (WHO) Toxic Equivalents (TEQ) values for Dioxin-like compounds. The EPA has suggested that Quantitative Structure-Activity Relationships (QSAR's) can be used in their priority setting for evaluating chemicals for endocrine disruptor activity. Data from our estrogen reporter system can be used as information to populate the database of QSAR's for the evaluation of the estrogenic activity of chemicals. Results of tests with the LUMI-CELL™ ER reporter gene system can also be used to compare results of other Tier 1 and 2 systems that are being evaluated such as the Uterotrophic Screen, The Hershberger Screen, The Rodent Pubertal Female Screen, The Rodent Pubertal Male Screen, Fish Reproduction Screen, The Frog Metamorphosis Screen, Estrogen and Androgen Receptor Reporter Gene Screens and Other In Vitro Screens, Mysid Shrimp (Invertebrate) Reproduction Test, and Mammalian 2-Generation Reproduction Test [Endocrine Disruptor Screening Program, August 2000]. Studies have been initiated to evaluate the LUMI-CELL™ ER in vitro system for its efficacy in identifying estrogen agonists versus the Uterotrophic Screen and other in vivo endpoints of estrogen activity (Jefferson, Padilla-Banks et al. 2002). The Uterotrophic screen has been proposed as one of the primary assays to identify estrogenic chemicals.

1.2.3 Where applicable, discuss the similarities and differences in the endpoint measured in the proposed test method and the currently used *in vivo* reference test method and, if appropriate, between the proposed test method and a comparable validated test method with established performance standards.

The LUMI-CELL™ ER reporter gene system can be performed much more rapidly and economically than the Tier 1 and Tier 2 systems being evaluated by the EPA listed above. The EPA has awarded a contract to the Battelle Corporation to validate these in vivo methods, but no studies have been published or validation parameters reported to our knowledge. There is a good correlation between the LUMI-CELL™ ER assay and the Uterotrophic assay (Thigpen, Locklear et al. 2001; Jefferson, Padilla-Banks et al. 2002). XDS has generated some data comparing the responsiveness of the LUMI-CELL™ ER reporter gene system to the mouse Uterotrophic Screen for evaluating the estrogenic activity of various feed substances. These preliminary analyses are single blinded studies being conducted with Dr. Julius Thigpen at the National Institutes of Environmental Health Sciences. Preliminary results appear promising that the LUMI-CELL™ ER reporter gene system is predictive for estrogen active contaminants in feeds that cause a

response in the mouse Uterotrophic Screen. Comparison of the LUMI-CELL™ ER reporter gene system should be undertaken with the other tests EPA is evaluating (listed above in section **1.2.1**), particularly since these tests are much more complex, many require the use of animals, and are much more costly.

There is also a paper discussed in section 1.1.2 comparing the XDS transcriptional assay, with immature mouse uterotrophic responses in assessing the estrogenic activity of phytochemicals (Jefferson, Padilla-Banks et al. 2002) (see the attached papers in Appendix H). Briefly, the LUMI-CELL™ ER bioassay (termed the ER Transcriptional assay in the Jefferson 2002 paper) demonstrated estrogen agonist activity for all the compounds tested except Taxifolin. The compounds demonstrating estrogen agonist activity were 17β-estradiol, DES, Zeralanol, Zeralenone, Coumesterol, Genistein, Biochanin A, Daidzein, and Naringenin. Other in vivo bioassay systems that were used to assess estrogen agonist activity were the Uterotrophic assay (measurement of uterine wet weight increase in immature mice), increase in uterine epithelial cell height uterine Gland number increases, and induction of estrogen responsive protein lactoferin (LF assay). The Uterotrophic assay was able to detect estrogenic activity in 7 of the 10 compounds. The uterine cell height assay was able to detect estrogenic activity in 9 of the 10 compounds. The uterine Gland number assay was able to detect estrogenic activity in all 10 of the compounds tested, making it very consistent with the LUMI-CELL™ ER bioassay. Immunohistochemical analysis of lactorferin (LF) induction was able to detect estrogenic activity in 7 of the 10 compounds detected by the LUMI-CELL™ ER bioassay (Jefferson, Padilla-Banks et al. 2002). This data demonstrates the unique sensitivity of the LUMI-CELL™ ER bioassay in evaluating estrogenic activity of these phytoestrogens and agreement with an in vivo model to evaluate estrogen agonists. Taxifolin was either a non-active or very weakly active in all of the above assays.

1.2.4 Describe how the method fits into the overall strategy of hazard or safety assessment. If a component of a tiered assessment process, indicate the weight that should be applied relative to other measures.

The LUMI-CELL™ ER reporter gene system is a mechanistically based ER-receptor bioassay system to identify chemicals that possess estrogen activity. The test system allows for specificity to be evaluated, since luciferase activity is not induced unless the estrogen receptor has been activated. A number of assays have been proposed using estrogen driven growth of cells as a screen for estrogen activity (Soto, Sonnenschein et al. 1995; Soto, Michaelson et al. 1998). However, effects of chemicals on a vast array of biochemical pathways can affect cell growth independent of the estrogen response. The specificity and sensitivity of dose response data that compares the relative estrogen activity of chemicals is one of the assets of the LUMI-CELL™ ER reporter gene system. Each EDC has a different ability to induce the estrogen receptor at a given concentration. The LUMI-CELL™ ER bioassay is capable of giving relative potencies of the estrogenic compounds. This would be similar (in concept only) to the World Health Organization (WHO) Toxic Equivalents (TEQ) values for Dioxin-like compounds.

1.2.5 Describe the intended range of materials amenable to the test and/or the limits of the proposed test method according to chemical class or physico-chemical factors.

The range of materials that can be tested is limited only by their solubility in Dimethyl Sulfoxide (DMSO) or other solvents compatible with the cell line that do not produce toxicity. The solvent DMSO can solubilize a wide range of compounds having both hydrophobic and hydrophilic characteristics. The one characteristic that limits the test system is that the chemical, solvent, or extract being tested should not be toxic to the cell system. Cell toxicity would result in a potential false negative response for estrogenic activity of the test chemical. However, the large dynamic range for induction of luciferase activity in the LUMI-CELL™ ER reporter gene system allows for dilution of the chemical or extract to a concentration at which toxicity is minimal and estrogenic activity of the compound may still be evaluated.

1.3 Scientific basis for the proposed test method.

1.3.1 Describe the purpose and mechanistic basis of the proposed test method.

The primary purpose of the LUMI-CELL™ ER bioassay is to screen chemicals for potential estrogenic activity. Eventually expanding to test feed, food and consumables for contamination for potential estrogenic activity. The mechanistic basis for this test method was described in section 1.1.1. Each compound has a different ability to induce the estrogen receptor at a given concentration. The LUMI-CELL™ ER bioassay is capable of giving relative potencies of the estrogenic compounds. This would be similar (in concept only) to the World Health Organization (WHO) Toxic Equivalents (TEQ) values for Dioxin-like compounds.

1.3.2 Describe what is known and not known about the similarities and differences of modes and mechanisms of action in the proposed test method as compared to the species of interest (e.g., humans for human health related toxicity testing).

The proposed test method uses human ovarian carcinoma cell line BG-1, which has endogenous alpha and beta estrogen receptors. The plasmid construct described in section **1.1.1** has 4 copies of the vitelogenin estrogen receptor response element in series placed in front of the reporter gene. The mechanism is very similar in humans for activation of the estrogen receptor and then regulation of gene expression on a wide variety of genes under control of the estrogen receptor.

1.3.3 Describe the intended range of substances amenable to the proposed test method and/or the limits of the proposed test method according to chemical class or physicochemical standards.

As described in section **1.2.4**, the range of materials that can be tested is limited only by their solubility in Dimethyl Sulfoxide (DMSO) or other solvents compatible with the cell line that do not produce toxicity. The solvent DMSO can solubilize a wide range of compounds having both hydrophobic and hydrophilic characteristics. The one characteristic that limits the test system is that the chemical, solvent, or extract being tested should not be toxic to the cell system. Cell toxicity would result in a potential false negative response for estrogenic activity of the test

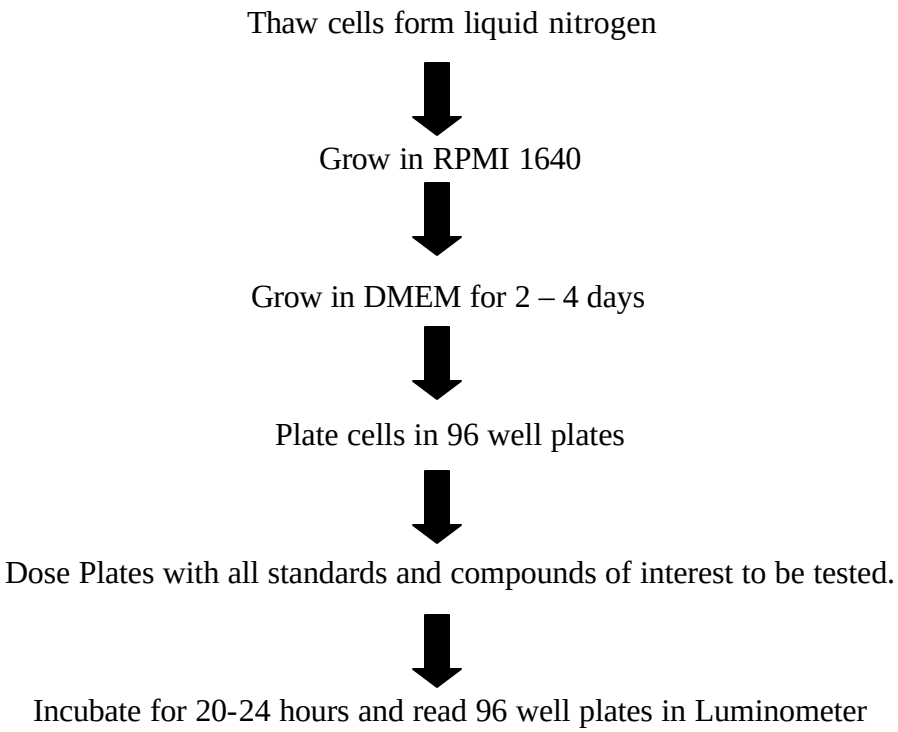
chemical. However, the large dynamic range for induction of luciferase activity in the LUMI-CELL™ ER reporter gene system allows for dilution of the chemical or extract to a concentration at which toxicity is minimal and estrogenic activity of the compound may still be evaluated.

2.0 Test Method Protocol Components

2.1 Provide and overview of how the proposed test method is conducted. If appropriate, this would include the extent to which the protocol for the proposed test method adheres to established performance standards.

See Appendix A – Detailed description of Performance of the LUMI-CELL™ ER bioassay.

Brief flow chart explanation



2.2 Provide a detailed description and rationale, if appropriate, for the following aspects of the proposed test method:

2.2.1 Materials, equipment, and supplies needed:

Equipment:

Equipment Item name	Fisher Scientific[1] Cat. #	Price, US $
Class II biological safety hood and stand	16-108-99	$7,250.00
Cell culture incubator, with CO_2 and temp. control	11-689-4	$4,197.00
Centrifuge, low speed, tabletop	04-978-50	$915.00
with swinging bucket rotor	05-103B	$430.00
Drummond diaphragm pipettor	13-681-15	$180.00
Microscope, inverted	12-561-INV	$4,400.00
Microscope	12-561-3M	$750.00
Hemocytometer, cell counter	02-671-5	$105.00
Hand tally counter	07-905-6	$27.72
Micropipettor, 0.5-10 µL range	21-377-97	$199.00
Micropipettor, 40-200 µL range	21-377-99	$199.00
Refrigerator/freezer	13-986-106A	$1,715.00
Vortex – mixer	12-814	$207.00
Vacuum pump	01-092-29	$316.00
with liquid trap (side arm erylenmeyer flask		
Multipipettor, repeating - syringe type	21-380-8	$390.00
Centrifuge concentrator	16-315-45	$5,595.00
with vacuum pump		
with cold trap		
Shaker for 96 well plates	14-271-9	$790.00
Liquid Nitrogen dewar	11-675-92	$1,154.00
or -70 celcius freezer	13-989-187	$7,350.00
Luminometer Berthold		$19,920.00
and dedicated computer		$1,679.00
Combustion test kit, CO_2 monitoring	10-884-1	$341.25
13mm test tube racks	14-809-22	$14.36
13mm test tube racks for dosing	14-810-54A	$16.99
16 mm test tube racks	14-809-24	$14.36
50 ml test tube racks	14-809-28	$15.71
sonicating water bath	15-335-30	$505.50

The recombinant cell line BG1Luc4E2, licensing arrangements can be made with XDS for use of this cell line.

Supplies:

Cell Culture

9" Pasteur pipettes
pipette bulbs, 2 ml capacity, pack of 72
15 ml plastic centrifuge tubes, sterile
50 ml plastic centri. Tubes
13/100 test tubes
Phosphate buffered saline
RPMI and DMEM medium
Trypsin
pen/strep solution
Fetal serum
RPMI Fetal medium
Lysis Solution
Substrate Solution
75 cm2 tissue culture flasks
96 well plates
Backing Tape
70 % ethanol, for cleaning and as coolant for cold trap
latex gloves
p200 pipette tips, sterile
2 ml sterile pipettes-plastic, case of 500
10 ml sterile pipettes, plastic, case of 200
1.0 ml multipipettor syringes, case of 100
10.0 ml multipipettor syringes, case of 100
sodium hydroxide
DMSO
Minimal Essential Medium
Estrogen stripped fetal calf serum

2.2.2 Dose-selection procedures, including the need for any dose range-finding studies or acute toxicity data prior to conducting the test, if applicable.

The dose selection for 17β-estradiol standard is based upon the responsiveness of our genetically engineered BG1Luc4E2 cells to estrogen. The cells are extremely sensitive to estrogen and estrogen-like chemicals demonstrating a significant agonistic response to as little as 0.04 pg of 17β-estradiol. The BG1Luc4E2 cells respond with a dose dependent induction of luciferase activity up to a maximal concentration of 40 pg of 17β-estradiol. A Tamoxifen / 17β-estradiol mixture was used in the antagonistic response test and demonstrates significant responses to Tamoxifen in the range from 1.95×10^3 pg – 2.0×10^6 pg (with a constant 10 pg 17β-estradiol concentration). See Appendix A for a more detailed description.

A screening testing for both agonistic and antagonistic estrogenic activity of a chemical is performed by initially performing a dose range finding experiment with the chemical. For the

agonist response, ten milligrams of a pure chemical for testing of estrogenic activity is weighed out into glass vial and dissolved in one-milliliter of DMSO. A 10 fold dilution series of the chemical is then produced by adding 10 microliters of the test compound to 90 microliters of DMSO in a 13 mm glass tube and repeating this procedure for six dilutions creating a dilution series of 1 mg/ml down to 1 ng/ ml. Four microliters of these solutions is then added to 400 microliters of media (final concentrations of 10 micrograms/ml down to 10 picrograms/ml) and applied to the BG1Luc4E2 cells to evaluate induction of luciferase activity. Using this screening format, 8 compounds can be evaluated per plate of BG1Luc4E2 cells. If a test chemical is positive for induction of luciferase activity (at three fold induction over the mean plus the standard deviation of the background), a second experiment using a two fold dilution series at the concentrations that are active is performed. The concentrations to be re-evaluated are determined by evaluating if a higher response is seen at one concentration and a lower response is seen at the next lower concentration. That area (plus some above and below) is re-analyzed using 2 fold dilutions. These dilutions are carried out at the high end until the top of the dose response is discovered, to the low end where there is no response. An example of the two-fold analysis activity of diethylstilbestrol was included in the example analysis provided from 50 pg/ml down to a concentration of 1.56 pg/ml (See Figure 3).

The dosing method for the antagonist response was conducted in much the same way as the agonist response with some small changes. Ten milligrams of a pure chemical for testing of antagonist estrogenic activity is weighed out into glass vial and dissolved in one-milliliter of DMSO. A 10 fold dilution series is of the chemical was again produced by adding 10 microliters of the test compound to 90 microliters of DMSO in a 13 mm glass tube and repeating this procedure for six dilutions creating a dilution series of 1 mg/ml down to 1 ng/ml. Four microliters of these solutions along with 10 pg/ml 17β-estradiol is then added to 400 microliters of media (final concentrations of 5 µg/ml down to 5 pg/ml of the compound and 10 pg/ml 17β-estradiol in each tube) and applied to the BG1Luc4E2 cells to evaluate the reduction in induction of luciferase activity. Using this screening format, 8 compounds can also be evaluated for antagonistic activity per plate of BG1Luc4E2 cells. If a test chemical is positive for reduction of luciferase activity (a 3 fold reduction under the standard deviation of the 10 pg 17β-estradiol), a second experiment using a two fold dilution series at the concentrations that are active is performed. Tamoxifen was used as the standard for the antagonistic response. Tamoxifen gave responses in the range from 1.95×10^3 pg – 2.0×10^6 pg. IICI 182,780 was not used as the standard for antagonistic response due to cost and not being readily available (i.e. only 100 mg per customer per year).

2.2.3 Endpoint(s) measured;

The endpoint measured is the induction of luciferase activity in a human ovarian carcinoma, BG-1 that has been genetically engineered with a reporter gene construct that expresses the enzyme luciferase in response to exposure of the BG1Luc4E2 cell line to estrogen or estrogen-like chemicals. The light produced can be easily quantified with a luminometer and comparison with a standard of 17β-estradiol induction of luciferase activity. Each compound has a different ability to induce the estrogen receptor at a given concentration. The LUMI-CELL™ ER bioassay is capable of giving relative potencies of the estrogenic compounds. This would be similar in concept to the World Health Organization (WHO) Toxic Equivalents (TEQ) values for Dioxin-like compounds.

2.2.4 Duration of exposure;

The duration of exposure to 17β-estradiol to induce maximal expression of the luciferase reporter gene in our BG1Luc4E2 bioassay is 20 hours. A significant induction of estrogen dependent expression of luciferase activity can be measured as early as two hours after exposure of the cells with half maximal induction occurring at eight hours following exposure of the BG1Luc4E2 cells (Rogers and Denison 2000).

2.2.5 Known limits of use;

The only known limits of use of the LUMI-CELL™ ER bioassay for measuring estrogen dependent induction of luciferase activity is if the chemical or environmental extract is toxic to the cellular system. Toxicity could potentially inhibit induction of estrogen-dependent induction of luciferase activity. However, overt toxicity is assessed in the system by visual observation of the cells before measurement of luciferase induction, and through a cell viability test. The cell viability test will consists of either a Tripan Blue test or Promega's CellTiter-Glo™ Luminescent Cell Viability Assay. If there is no response, the concentration dosed has proven to be toxic to the cells (see results in **Appendix D,** for a data summary; **Appendix J,** for QC summary charts; and Excel file "**Appendix – E Raw Data for Plate-to-Plate Agonist Data**" – cell viability tab, for raw data for Appendix E). However, the sensitivity and large dynamic range of the LUMI-CELL™ ER bioassay system allows for dilution of the sample test compound to limit toxicity and yet estimate potential induction of estrogen-dependent luciferase expression.

2.2.6 Nature of the response assessed;

The response that is measured is the enzymatic activity of luciferase that is induced in our genetically engineered cells (BG1Luc4E2) that express this enzyme in response to exposure to estrogen and estrogen-like chemicals. The enzyme activity is assessed by the production of light in a luminometer following addition of enzyme reagents. Each compound has a different ability to induce the estrogen receptor at a given concentration. The LUMI-CELL™ ER bioassay is capable of giving relative potencies of the estrogenic compounds. This would be similar (in concept only) to the World Health Organization (WHO) Toxic Equivalents (TEQ) values for Dioxin-like compounds.

2.2.7 Appropriate vehicle, positive, and negative controls and the basis for their selection;

The vehicle used for application of chemicals is DMSO. The response from the vehicle is the negative control for chemicals and solvent for extraction of environmental samples is the vehicle in testing environmental extracts. The positive controls include an eleven point 17β-estradiol dose response curve, which is the hormone ligand for the estrogen receptor (**Appendix C**, for example of 17β-estradiol curve; **Appendix D**, for data summary; and Excel file "**Appendix – E Raw Data Plate-to-Plate Agonist Data**" – beta curve tab, for raw data), 4 DMSO controls and one no DMSO (just media), as well as 2 to 8 positive response QCs (**Appendix J**, for QC summary charts). The following compounds are used as QCs for the LUMI-CELL™ ER bioassay and were selected based on historical data provided by ICCVAM and their consistent response in this assay: diethlstilbesterol (DES) (1.23×10^{-5} µg/ml); Bisphenol A (7.81×10^{-2}

μg/ml); Estrone (7.81 x 10^{-2} μg/ml); Ethelene Estradiol (6.25 x 10^{-2} μg/ml); Feneramol (12.5 μg/ml); Kaemoferol (7.81 x 10^{-2} μg/ml); Methoxychlor (1.56 μg/ml); Norethredrel (3.13 x 10^{-5} μg/ml). DES, Bisphenol A, and/or Estrone are used as the standard QCs for the plates, however the others can be added as needed for specific assays. The acceptable range for QC is 2 standard deviations from the mean and are depicted by the line in the middle as the mean and the 2 lines outside the mean as the acceptable range (i.e. the distance of 2 standard deviations). The QC performance charts are provided here in **Appendix J**.

2.2.8 Acceptable range of vehicle, positive and negative control responses and the basis for the acceptable ranges;

QC control charts have been developed for all of the QC compounds mentioned in the above section. A limit of 2 standard of deviations from the mean has been established to evaluate the acceptability of the QC data and the plate data. The acceptable range for the vehicle (DMSO and No DMSO (i.e. Media)) response is the same as all the other QCs, 2 times the standard deviation of the mean (section **2.2.7**, See also **Appendix J**). Also a minimum induction of 3 has been established for the evaluation of the 17β-estradiol dose response curve. The minimum induction is based on dividing the highest response (i.e. Highest RLU response) by the lowest RLU response. This insures that the curve covers an adequate area to ensure that the response of the compound (or substance) will be adequately seen within the dose response curve (see Figure 11).

2.2.9 Nature of the data to be collected and the methods used for data collection;

The data collected are measurements of the light induction produced by the luciferase enzyme and are measured as relative light units detected by a luminometer. The data are stored as electronic files in a computer system that is backed up daily. They are secured in the laboratory and follow methods described in EPA method 2185: Good Automated Laboratory Practices.

2.2.10 Type of media in which data are stored;

The data are stored electronically in a Windows NT network. The network hard disk is backed up every 24 hours on a Compaq workstation. Data printouts are also kept in laboratory notebooks.

2.2.11 Measures of variability;

In the screening mode of the assay replicate analysis are not performed, however the use of a varying doses of compound allows an estimate if the response demonstrates a trend. However, in confirmation assays, triplicate analysis can be performed on both "plate to plate" variability and "well-to-well" variability, and statistical model testing is performed on this data. Testing of compounds was done in the confirmation assay mode and the data is available in **Appendix D**, for Plate-to-Plate Agonist data summary and Excel file "**Appendix – E Raw Data Plate-to-Plate Agonist Data**", (the raw data for Appendix D summary); **Appendix F**, for Plate –to-Plate Antagonist data summary and Excel file "**Appendix – G Raw Data Plate-to-Plate Antagonist Data**", (the raw data for the Plate-to-Plate Antagonist summary); **Appendix H**, for Well-to-Well

Agonist data summary, and Excel file "**Appendix I – Raw Data for Well-to-Well Agonist Summary Data**", (for Appendix F raw data).

2.2.12 Statistical or non-statistical method(s) used to analyze the resulting data (including methods to analyze for a dose-response relationship). Justify and described the method(s) employed;

The data that is generated from the 17β-estradiol standard is modeled using a four parameter Hill equation. The Hill equation is a mathematical model that generates the best fit for receptor mediated induction of gene expression (Kohn, Lucier et al. 1993; Kohn, Sewall et al. 1996; Kohn, Walker et al. 2001). A Q-test is used to look for outliers in the data (see Section 2.8).

2.2.13 Decision criteria or the prediction model used to classify a test chemical (e.g., positive, negative, or equivocal), as appropriate;

There have been two initial criteria adopted for assigning a positive (or Active) designation for a chemical in the LUMI-CELL™ ER estrogen screen. The first criteria applied is to demonstrate that the chemical induces luciferase activity that is greater (statistically significant) than the mean plus 3 times the standard deviation of the DMSO blank at an applied concentration (see **Appendix D**, data summary and **Appendix E**, raw data). (Note: Three standard deviations is a normal statistical criterion for discarding negative data and has been used in EPA methods such as EPA Method 8290, 1613B etc.) The second and more stringent criteria applied is to demonstrate that the chemical induces luciferase activity at a number of concentrations in a two-fold dilution re-analysis, demonstrating dose-dependent induction of luciferase to where an EC 50 can be calculated and a relative response to 17β-estradiol can be assigned (see **Appendix D**, data summary). A compound meeting the first criteria but not the second would be classified as a weak positive (or weak activator). A negative designation for activity in the LUMI-CELL™ ER bioassay estrogen screen is assigned when no induction of luciferase activity (statistically significant) is detected at any concentration over the mean plus 3 times the standard deviation of the DMSO blank (see **Appendix D**, data summary; and **Appendix E** raw data). An EC 50 is only calculated when the top and bottom of the dose response curve have been elucidated. Each compound has a different ability to induce the estrogen receptor at a given concentration. The LUMI-CELL™ ER bioassay is capable of giving relative potencies of the estrogenic compounds. This would be similar (in concept only) to the World Health Organization (WHO) Toxic Equivalents (TEQ) values for Dioxin-like compounds.

2.2.14 Information that will be included in the test report.

Information in test reports include the standard curve generated by a two-fold dilution series of the positive control chemical 17β-estradiol, background determinations of solvent carrier (DMSO and Media), QCs, cell viability (if applicable), and test substance results and limit of detection (if applicable) (see: **Appendix C**, and example of the 17β-estradiol curve; **Appendix D**, data summary; and Excel file "**Appendix – E Raw Data for Plate-to-Plate Agonist Data**" – beta curve tab, for raw data and **Appendix J,** QC Charts). The report can also include all of the calculations including modeling of the 17β-estradiol response using a four parameter Hill equation, and response of compound range finding at six different 10 fold dilutions from 10 micrograms/ml down to 10 pg/ml in our LUMI-CELL™ ER bioassay (see **Appendix A**).

Appendix D includes relative induction and relative efficacy, which gives an estimate of potency compared to 17β-estradiol response.

2.3 Explain the basis for selection of the test method system. If an animal model is being used, this should include the rationale for selecting the species, strain or stock, sex, acceptable age range, diet, and other applicable parameters.

The LUMI-CELL™ ER bioassay is an *in vitro* system using a genetically engineered cell line and is a mechanistically based ER-receptor bioassay system to identify chemicals that possess estrogen activity. This test method should greatly reduce, refine and in some cases replace animal use in discovery of estrogenic endocrine potency.

2.4 If the test method employs propriety components, describe what procedures are used to ensure their integrity (in terms of reliability and accuracy) from "lot-to-lot" and over time. Also describe procedures that are used to verify the integrity of the proprietary components.

The integrity of the proprietary LUMI-CELL™ ER bioassay is maintained by several means. The First is the standard 17β-estradiol dose response curve. The cells must respond in a standard sigmoidal shaped curve with an induction of grater than three. (The minimum induction is based on dividing the highest response (i.e. Highest RLU response) by the lowest RLU response. This insures that the curve covers an adequate area to ensure that the response of the compound (or substance) will be adequately seen within the dose response curve.) Also, a minimum of 7 additional positive and negative QCs, are used in each plate to evaluate the cells integrity. Two are positive QCs (usually DES, Bisphenol A, and/or Estrone (see **Appendix J** for QC performance charts)) and 5 negative controls (4 DMSO and 1 no DMSO (i.e. just media)). These QCs are checked against established QC charts described in section **2.2.7** and **Appendix J**. The BG1Luc4E2 cell line is also stored in liquid nitrogen, which preserves the integrity of the cell system.

2.5 Describe the basis for the number of replicate and repeat experiments; provide the rationale if studies are not replicated or repeated.

In this study triplicate analysis was preformed on all samples. Samples were analyzed in a "Plate-to-Plate" format where each analysis was done on a completely different experimental setup (**Appendix D**, Agonist Plate-to-Plate data summary) as well as on a "Well-to-Well" format, where 3 of the same samples was analyzed three times on the same plate from the same experimental setup (**Appendix H**, Agonist Well-to-Well data summary). But as described in section 2.2.11, in the screening mode of the assay replicate analysis are not performed, however the use of a varying doses of compound allows an estimate if the response demonstrates a trend. However, in confirmation assays, triplicate analysis can be performed on both "Plate-to-Plate"

variability and "Well-to-Well" variability, and statistical model testing may be performed on this data.

2.6 Discuss the basis for any modifications to the proposed that were made based on results from validation studies.

Validation studies are currently in progress with the LUMI-CELL™ ER bioassay analysis system. Modification of protocols will be advanced after sufficient testing demonstrates that modifications improve the systems.

2.7 If applicable, discuss any differences between the protocol for the proposed test method and that for a comparable validated test method with established performance standards.

XDS Inc. is not aware of any validated test method for detection of estrogenic endocrine disruptors. However a paper published by Jefferson et al. (2002) (briefly summarized in sections **1.1.2** and **1.2.3**) demonstrated considerable consistency between the LUMI-CELL™ ER bioassay and the mouse uterotrophic assay, Cell height assay, Gland number assay, and LF protein assay. (see attached paper in **Appendix K**).

2.8 Explain the basis for the decision criteria established for the test.

The decision criteria that we have initially established to identify estrogen agonists by the LUMI-CELL™ ER reporter gene system are explained in section **2.1.14** above. These criteria allow for some indication that a dose dependent induction of luciferase activity is occurring in the system. This should be one of the criteria for establishing whether a chemical is an endocrine active compound. A second criterion should be that a significant response over background is generated. We set a cut-off point of 3-fold increase over the mean plus standard deviation of the background as potential noise in the system. This level for discriminating noise or background is accepted for other EPA validated analytical systems such as Method 8290 for analysis of dioxin chemical contamination. The scientific community has not identified criteria for classifying chemicals for endocrine disruptor activity with any certainty at this time. XDS is submitting this system as a mechanistically based ER-receptor bioassay system as a HTPS for Tier 1 priority setting in further evaluating chemicals for their potential as estrogen agonists. Estrogen antagonist activity can also be assessed with the system but HTPS methodology has not yet been extensively tested on chemicals.

An outlier test (Q test) was also used on the comprehensive (triplicate) analysis to determine if any of the data was an outlier. The following is an example of a Q test, and please note the Q test does note replace experience and commonsense.

Formula:

Outlier-Nearest Neighbor

Range (highest-lowest**)**

1. Q test is not used on all numbers, only those that are in question.
2. For the Q test to work there must be a minimum of three numbers.
3. Since all samples are run in triplicate, there should always be three separate numbers per sample.

Calculating using the Q test method:

1. Arrange the data in decreasing order (lowest to highest)
2. Calculate the difference between the data in question and its nearest neighbor. Once calculated, divide it by the range.
3. The range is the difference between the highest number and the lowest number.
4. If the result is higher than the tabulated values of Q at the 90% confidence level (see Chart 1), then the number can be discarded.

Chart 1:

Number Of Observations	Q
2	**-**
3	**0.94**
4	**0.76**
5	**0.64**
6	**0.56**
7	**0.51**
8	**0.47**
9	**0.44**
10	**0.41**

Example1:

196,355,169

1. In decreasing order: 169,196,355
2. There is a difference of 159 between the suspect number (355) and its closet neighbor (196)
3. The range is 186(355-169)
4. So 159/186=. 85.
5. This number can not be discarded because its value isn't higher than the Q test limit of .94

Example2:

665,124,122

1. Decreasing order: 122,124,665
2. Difference is 541
3. Range is 543
4. 541/543=. 996

Since the difference is higher than the Q test limit of 0.94, this data can be discarded.

3.0 Substances Used for Validation of the Proposed Test Method (See Appendix B – Characterization of substances tested)

3.1 Describe the rationale for the chemicals or products selected for use in the validation process. Include information on the suitability of chemicals selected for testing, indicating any chemicals that were found to be unsuitable.

Chemicals tested in the LUMI-CELL™ ER bioassay system included chemicals that have been reported to possess estrogenic activity as well as chemicals that have not been reported to have estrogen agonist activity. The study report conducted by the ICCVAM expert committee has established a list of 78 compounds to be tested for ER and AR transcriptional activation assays (ICCVAM 2002). In this screening mode, data could be generated in the system for validation of both positive and negative results in the LUMI-CELL™ ER bioassay system for identifying estrogenic chemicals. In analyzing a wide variety of chemicals it may also be established that the LUMI-CELL™ ER bioassay system has the potential of identifying novel estrogenic compounds or mixtures.

3.2 Discuss the rationale for the number of chemicals that were tested.

One hundred and twenty four chemicals were tested in the LUMI-CELL™ ER BG1Luc4E2 bioassay system for this submission. 56 of these chemicals were recommended by ICCVAM for validation of ER binding and transcriptional activation. Of the 56 chemicals tested, which were recommended by ICCVAM, all of the 28 compounds having historical data for a positive response demonstrated estrogenic activity in the LUMI-CELL™ ER bioassay. Out of the 124 chemicals tested by LUMI-CELL™ ER bioassay system, 65 demonstrated estrogenic activity, while 59 showed no activity. Of the 68 chemicals tested, which were not included in the ICCVAM requirements for validation, 28 were found to possess' estrogenic activity, while 40 showed no activity.

3.3 Describe the chemicals/products evaluated. For each chemical or product, including the following information:

3.3.1 Chemical or product name, if a mixture, provide information on all components;

See **Appendix B** – Characterization of chemicals tested.

The mixtures of chemicals that were tested included 7 Arochlors, a series of chemicals, which are defined mixtures of polychlorinated biphenyls with different degrees of chlorination of the isomers.

3.3.2 CASRN

See **Appendix B** – Characterization of chemicals tested.

3.3.3 Chemical and product classes;

See **Appendix B** – Characterization of chemicals tested.

3.3.4 Physical/chemical characteristics (e.g., water and lipid solubility, pH, pKa, etc.). Any characteristics thought or know to impact the test method accuracy and/or reliability should be clearly described.

See **Appendix B** – Characterization of chemicals tested.

3.3.5 Stability of test substance in test medium.

See **Appendix B** – Characterization of chemicals tested.

Most of these chemicals are pesticides or complex polyaromatic hydrocarbons.

3.3.6 Concentrations tested;

The concentrations tested were a 10 fold dilution series of six different concentrations beginning at 10 µg/ml down to 10 pg/ml for range finding (some were tested at lower concentrations depending on response). The compounds were then re-examined in the regions which demonstrated an agonist or antagonistic response using 2 fold dilutions until a sigmoidal dose response curve was detected. Some of the positive chemicals (Diethylstilbesterol, Zearalenone, Coumesterol, Genestein, Bisphenol A, Estrone, Ethelene Estradiol, Feneramol, Kaemoferol, Methoxychlor, Norethredrel, and Diadzein) were tested more thoroughly to develop dose response characteristics and relative potency determinations. See **Appendix B** for the exact concentrations tested for each compound.

3.3.7 Purity, including the presence and identity of contaminants and stabilizing additives;

See **Appendix B**

All of the chemicals are greater than 95% pure and generally were greater than 99% pure.

3.3.8 Supplier/source.

The suppliers for chemicals are listed below. The majority of chemicals were purchase either from the Aldrich Chemical Co., P.O. Box 355 Milwaukee, WI and Sigma Chemical Corporation, P.O. Box 14508, St. Louis, MO 68178. Some of the chemicals were purchase from Chem Service Inc., 660 Tower Lane, P.O. Box 599, West Chester, PA 19381-0599

3.4 Describe the coding procedures used in the validation studies.

Independent validation studies have not been conducted yet, and therefore coding procedures have not been used. However a paper published by Jefferson et al. (2002) (briefly summarized in sections **1.1.2** and **1.2.3**) demonstrated considerable consistency between the LUMI-CELL™ ER bioassay and the mouse uterotrophic assay, Cell height assay, Gland number assay, and LF protein assay. (see attached paper in **Appendix H**).

3.5 For the methods that are mechanistically and functionally similar to a validated test method with established performance standards, discuss the extent to which the recommended reference chemicals were tested in the proposed test method. In situations where a listed reference chemical was unavailable, the criteria used to select a replacement chemical should be described. To the extent possible, when compared to the original reference chemical, the replacement chemical should be from the same chemical/product class and produce similar effects in the *in vivo* reference test method. In addition, if applicable, the replacement chemical should have been tested in the mechanistically and functionally similar validated test method. If applicable, the rationale for adding additional chemicals and the adequacy of data from the *in vivo* reverence test method or the species of interest should be provided.

XDS Inc. is not aware of any validated test method for detection of estrogenic endocrine disruptors. The reference compound used in this test method was 17β-estradiol (see **Appendix C**, an example of the 17β-estradiol curve and **Appendix D**, data summary). The only known direct comparison of the LUMI-CELL™ ER bioassay, system to known animal studies, was done by Jefferson et al. (2002). Please refer to section **1.1.1** (or **1.2.3**) and the attached paper in **Appendix K**.

4.0 *In Vivo* Reference Data Used for an Assessment of the Accuracy of the Proposed Test Method.

The lack of reference data to establish guidelines for assessing what data constitutes information on the potential of a chemical to act as an endocrine disruptor is one of the most difficult areas to overcome in this field of research. We feel that the data provided by our LUMI-CELL™ ER bioassay system could be used as reference data to evaluate other systems for the estrogenic activity of chemicals. The system provides a rapid HTPS to evaluate and scale the potential estrogenic activity of chemicals and is based on the molecular mechanism of action of estrogenic chemicals. One method that has been suggested as a reference method for estrogenic activity is the mouse uterotrophic assay. We have initiated studies with Dr. Julius Thigpen of the National Institute of Environmental Health Sciences to compare the data generated by our LUMI-CELL™ ER bioassay system and the mouse uterotrophic assay in extracts of feed samples but that data is coded at this time and can not be presented in this filing of information to ICCVAM at this time.

The only known direct comparison of the LUMI-CELL™ ER bioassay system to known animal studies, was done by Jefferson et al. (2002). Please refer to section **1.1.2** (or **1.2.3**) and the attached paper in **Appendix K**.

4.1 Provide a clear description of the protocol(s)used to generate data from the *in vivo* reference test method. If a specific guideline has been followed, it should be provided. Any deviations should be indicated, including the rationale for the deviation.

XDS's LUMI-CELL™ ER bioassay is an *in vitro* assay. Also there is no known validated test method (*in vitro* nor *in vivo*) to be used as a reference test method for detection of estrogenic endocrine disruptors. However a paper published by Jefferson et al. (2002) (briefly summarized in sections **1.1.2** and **1.2.3**) demonstrated considerable consistency between the LUMI-CELL™ ER bioassay and the mouse uterotrophic assay, Cell height assay, Gland number assay, and LF protein assay. (see attached paper in **Appendix K**).

4.2 Provide the *in vivo* reference test method data used to assess the accuracy of the proposed test method. Individual human and/or animal reference test data, if available, should be provided. Provide the source of the reference data, including the literature citation for published data, or the laboratory study director and year generated for unpublished data.

XDS's LUMI-CELL™ ER bioassay is an *in vitro* assay. Also there is no known validated test method (*in vitro* nor *in vivo*) to be used as a reference test method for detection of estrogenic endocrine disruptors. The main sources of data used to determine accuracy of the test method have been through individual published reports and the list of compounds reposted by ICCVAM with historical data on estrogenic response (ICCVAM 2002). However a paper published by Jefferson et al. (2002) (briefly summarized in sections **1.1.2** and **1.2.3**) demonstrated

considerable consistency between the LUMI-CELL™ ER bioassay and the mouse uterotrophic assay, Cell height assay, Gland number assay, and LF protein assay. (see attached paper in **Appendix K**).

4.3 If not included in the submission, indicate if original records are available for the *in vivo* reference test method data.

XDS's LUMI-CELL™ ER bioassay is an *in vitro* assay. Also there is no known validated test method (*in vitro* nor *in vivo*) to be used as a reference test method for detection of estrogenic endocrine disruptors. The only known *in vivo* comparison to the LUMI-CELL™ ER bioassay, was done by Jefferson et al. 2002 (see sections **1.1.2**, and **1.2.3** and attached papers in **Appendix K**).

4.4 Indicate the quality of the *in vivo* reference test method data, including the extent of GLP compliance and any use of coded chemicals.

There is no known validated test method (*in vitro* nor *in vivo*) to be used as a reference test method for detection of estrogenic endocrine disruptors. No coded compounds have been tested as of yet. GLP guidelines were followed in the production of the LUMI-CELL™ ER bioassay. The only known *in vivo* comparison to the LUMI-CELL™ ER bioassay, was done by Jefferson et al. 2002 (see sections **1.1.2**, and **1.2.3** and attached papers in **Appendix K**).

4.5 Discuss the availability and use of relevant toxicity information from the species of interest (e.g., human studies and reported toxicity from accidental or occupational exposure for human health-related toxicity testing).

XDS's LUMI-CELL™ ER bioassay is an *in vitro* assay.

4.6 Discuss what is known or not known about the accuracy and reliability of the *in vivo* reference test method.

There is no known validated test method (*in vitro* nor *in vivo*) to be used as a reference test method for detection of estrogenic endocrine disruptors. However a paper published by Jefferson et al. (2002) (briefly summarized in sections **1.1.2** and **1.2.3**) demonstrated considerable consistency between the LUMI-CELL™ ER bioassay and the mouse uterotrophic assay, Cell height assay, Gland number assay, and LF protein assay. (please see attached paper in **Appendix K**).

5.0 Test Method Data and Results

5.1 Describe the proposed test method protocol used to generate each submitted set of data. Any differences from the proposed test method protocol should be described, and a rationale or explanation for the difference provided. Any protocol modifications made during the development process and their impact should be clearly stated for each data set.

Methods for HTPS of chemicals by our LUMI-CELL™ ER bioassay were performed as described in section 2 and **Appendix A**, defining the method for HTPS we are currently using. The data presented in **Appendix D, F, H** and **J** data summaries, used the same protocol described in **Appendix A**. Briefly, the cells were grown in DMEM for 4 days prior to plating. These plates were then incubated 24 hours prior to dosing with the desired compound, incubated an additional 20 hours and then analyzed.

5.2 Provide all data obtained using the proposed test method. This should include copies of original data from individual animals and/or individual samples, as well as derived data. The laboratory's summary judgment as to the outcome of each test should be indicated. The submission should include data (and explanations) from all studies successful or not.

In this submission for ICCVAM review we are submitting data summaries for the HTPS of the chemicals we have evaluated in the LUMI-CELL™ ER bioassay for estrogen activity. Test data summaries for each chemical screened are represented by dose response curves of the screened chemicals depicting activation of LUMI-CELL™ ER bioassay to express luciferase activity and are attached in **Appendix D – J**. A detailed description of the performance of the LUMI-CELL™ ER bioassay can be found in: **Appendix C**, an example of the 17β-estradiol curve; **Appendix D**, Agonist Plate-to-Plate data summary; **Appendix F**, Antagonist Plate-to-Plate data summary; **Appendix H**, Agonist Well-to-Well data summary; and **Appendix J**, QC performance charts, for both positive and negative controls. All raw data files are appended to a final submission of our HTPS LUMI-CELL™ ER bioassay for estrogen activity as Excel files. The Data Summary Appendix has its corresponding Raw Data Appendix, and they are:

"**Appendix D – Plate-to-Plate Agonist Data Summary**" is a data summary for the raw data "**Appendix E – Raw Data for Plate-to-Plate Agonist Summary Data**"; is the raw data for Appendix D

"**Appendix F – Plate-to-Plate Antagonist Data Summary**" is a data summary for the raw data "**Appendix G – Raw Data for Plate-to-Plate Antagonist Summary Data**", is the raw data for Appendix F.

"**Appendix H – Well-to-Well Agonist Data Summary**" is the data summary for the raw data "**Appendix I – Raw Data Well-to-Well Agonist Summary Data**", is the raw data for Appendix H.

5.3 Describe the statistical approach used to evaluate the data resulting from the studies conducted with the proposed test method.

The statistical approach for evaluating data was described in section **2.2.12** and **Appendix A**. The data transformation from raw data to data summary tables is outlined in Figures 9 – 15. In **Appendix D**, **F**, and **G** compounds are ranked as active only if there is a statically significant increase over the mean plus 3 times the standard deviation of the DMSO blanks. The compounds were ranked as weak active if there was an increase above the 3 times the standard deviation plus the mean, but not statically significant. All others were ranked as non-active. In **Appendix D**, **F**, and **G**, the relative induction of each chemical was calculated. This is calculated by dividing the average EC50 Molar concentration for each compound by the EC50 Molar concentration for 17β-estradiol. This will give 17β-estradiol a relative induction of 1, and show how potent all other compounds are at their EC50 relative to 17β-estradiol.

5.4 Provide a summary, in graphic or tabular form, of the results. The suggested tabular format for providing data for use in assessment of accuracy is provided in Appendix B.

See **Appendix B** (Characterization of Substances Tested) of this report.

A useful bioassay should provide a quantitative estimate of the relative estrogenic potency of a chemical or chemical mixture. Accordingly, we reanalyzed all active compounds in our LUMI-CELL™ ER system to derive EC50 values of their activity. An EC50 value is 50% of the maximum response obtained when a complete dose response curve is generated. The EC 50 values have been calculated from multiple replicates on multiple plates of dose response curves. One hundred and twenty four chemicals were tested in the LUMI-CELL™ ER BG1Luc4E2 bioassay system for this submission. 53 of these chemicals were recommended by ICCVAM for validation of ER binding and transcriptional activation. Of the 53 chemicals tested, which were recommended by ICCVAM, all of the 28 compounds having historical data for a positive response demonstrated estrogenic activity in the LUMI-CELL™ ER bioassay (ICCVAM 2002). Out of the 124 chemicals tested by LUMI-CELL™ ER bioassay system, 65 demonstrated estrogenic activity, while 59 showed no activity. Of the 68 chemicals tested, which were not included in the ICCVAM requirements for validation, 28 were found to possess' estrogenic activity, while 40 showed no activity. (see **Appendix D**, data summary)

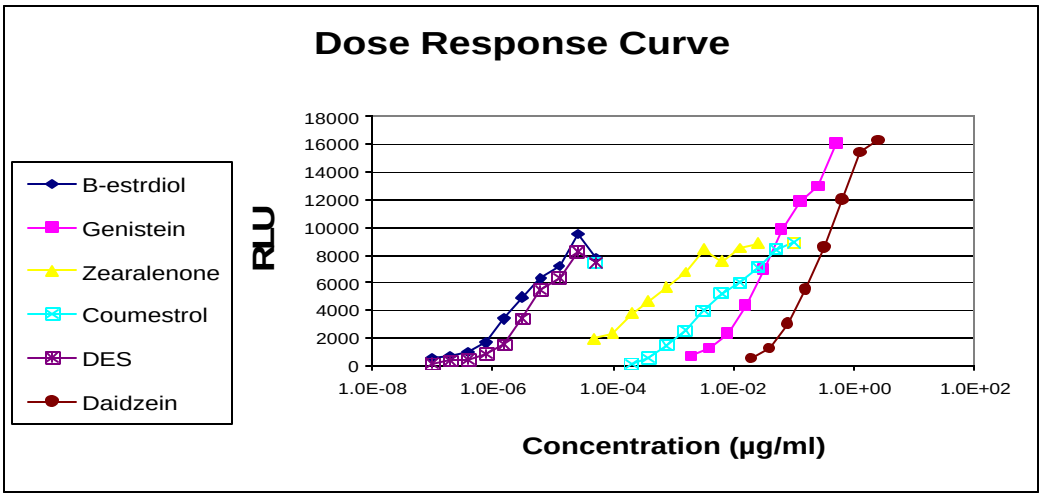


Figure 3. Repeat tests with dose response curves to evaluate EC50 response for generation of relative potency.

Typical dose response characteristics of the LUMI-CELL™ ER analysis are show in Figure 3. These experiments reveal that each chemical could induce luciferase activity in BG1Luc4E2 cells in a dose-dependent manner and the differences in relative potency of a given chemical is determined by comparison of its dose-response curve to that obtained using 17β-estradiol. Comparison of the EC_{50} values for each curve (concentration of chemical that induces luciferase to 50% of maximal) allows estimation of the relative potency of a test chemical relative to that of 17ß-estradiol. The induction potency estimates calculated from this comparison are presented in Appendix D. These results reveal that the relative potency values for these selected EDCs range from 1.5 (for DES) to about 44,000-times (for Diadzein) lower than that of 17ß-estradiol. These results demonstrate the utility of our bioassay for estimating the relative potency of other estrogenic chemicals (*i.e.* xenoestrogens). The response of Genistein and Daidzein are higher than 17ß-estradiol and are known Protein Kinase C activators that appear to attenuate the response of the Estrogen receptor. The receptor is likely attenuated by phosphorylation as a result of Genistein and Daidzein activating Protein Kinase C. This phenomena has been termed receptor cross talk and occurs in a number of the steroid hormone receptors as well as with the Ah Receptor. This increases the maximal activity but has little effect on the estimation of EC 50 values for the compounds.

5.5 For each set of data, indicate whether coded chemicals were tested, whether experiments were conducted without knowledge of the chemicals being tested, and the extent to which experiments followed GLP guidelines.

The laboratory is run using standard operating procedures and follows all Good Laboratory practices in producing data and analytical systems. The laboratory has been audited by the Belgium Government for compliance to Good Laboratory practices since the Scientific Institute of Public Health of Belgium purchased XDS bio-analytical system for analysis of dioxin and dioxin-like chemicals. XDS is open to GLP audit by any of the US regulatory agencies and would welcome an audit and accreditation. The current experiments were not conducted in a

blind coded manner. XDS has a current study being conducted on the LUMI-CELL™ ER system in collaboration with Dr. Julius Thigpen of the National Institute of Environmental Health Sciences on measuring and comparing the estrogenic activity of feed extracts. XDS would welcome double blind evaluation of the LUMI-CELL™ ER HTPS if any of the regulatory agencies would be interested in testing the system.

5.6 Indicate the "lot-to-lot" consistency of the test substances, the time frame of the various studies, and the laboratory in which the study or studies were done. A coded designation for each laboratory is acceptable.

Lot to lot consistency is conducted by comparison to the positive and negative QCs described in sections **2.2.7** and **2.4** (also see data in **Appendix J**, the QC summary charts). Inter-laboratory variability of the analysis system is currently being undertaken with the laboratory of Dr. Leo Goeyens of the Belgium Scientific Institute of Public Health and with Dr. Fujio Kayama of the Jichi Medical School of Japan and Mr. Yamamoto of the Hiyoshi Corporation of Japan. Studies have not been completed at this date.

5.7 Indicate the availability of any data not submitted for external audit, if requested.

All data analyzed at the XDS laboratory are available for audit. The current work was funded by a Phase I SBIR and Phase II SBIR grant from the National Institutes of Environmental Health Sciences, (Grant Number 1 R43 ES10533-01) "Cell Bioassays to Detect Endocrine Disruptors" and (Grant Number ES10533-03) "Recombinant Bioassays to Detect Endocrine Disruptors". We appreciate the funding supplied by NIEHS and support of the Dr. Jerry Heindel in aiding development of the LUMI-CELL™ ER bioassay.

6.0 Test Method Accuracy

Test method performance for estrogen active chemicals is difficult in that there is not an accepted and validated test procedure that we are aware of. We are pursuing ICCVAM submission of our data to begin the process of having our analysis system evaluated for performance and hope to work with ICCVAM and the other regulatory agencies to accomplish this goal.

6.1 Describe the accuracy (e.g., concordance, sensitivity, specificity, positive and negative predictivity, false positive and negative rates) of the proposed test method compared with the reference test method. Explain how discordant results in the same or multiple laboratories from the proposed test were considered when calculating accuracy.

There is no known validated test method (*in vitro* nor *in vivo*) to be used as a reference test method for detection of estrogenic endocrine disruptors. However a paper published by Jefferson et al. (2002) (briefly summarized in sections **1.1.2** and **1.2.3**) demonstrated considerable consistency between the LUMI-CELL™ ER bioassay and the mouse uterotrophic assay, Cell height assay, Gland number assay, and LF protein assay. Sense there is no validated test method false positive and false negative rates can not be established. But there is considerable consistency between the LUMI-CELL™ ER bioassay and the data published in the Jefferson et. al. (2002) paper. More specifically, the LUMI-CELL™ ER bioassay and the Gland number assay showed 100% consistency. While the other assays (uterotropic assay, Cell height assay and LF assay all showed false negatives in that they did not demonstrate activity for all of the compounds tested (Jefferson, Padilla-Banks et al. 2002) (please see attached paper in **Appendix K**). A Q-test is used to look for outliers in the data (see Section 2.8).

6.2 Discuss results that are discordant with results from the *in vivo* reference method.

There is no known validated test method (*in vitro* nor *in vivo*) to be used as a reference test method for detection of estrogenic endocrine disruptors. Nine of the compounds tested by Jefferson et. al. (2002) were also tested by XDS and came up with very consistent results. When Jefferson et. al. (2002) compared the LUMI-CELL™ ER bioassay to the Gland number assay they showed 100% consistency. The Uterotrophic assay was able to detect activity in 7 of 9 compounds tested, and was not able to detect Daidzein nor Naringenin. The Cell height assay was able to detect activity in 8 of 9 compounds tested, and was not able to detect Daidzein. The LF protein assay was able to detect activity in 7 of 9 compounds tested, and was not able to detect activity in Biochanin A nor Daizedin. (see attached paper in **Appendix K**).

6.3 Discuss the accuracy of the proposed test method compared to data or recognized toxicity from the species of interest (e.g., humans for human health-related toxicity testing), where such data or toxicity classifications are available. This is essential when the method is measuring or predicting an endpoint for which there is no preexisting method. In instances where the proposed test method was discordant reference test method, describe the frequency of correct predictions of each test method compared to recognized toxicity information from the species of interest.

Most of the historical data on compound response has come from the ICCVAM publication "Current Status of Test Methods for Detecting Endocrine Disruptors: In Vitro Estrogen Receptor Transcriptional Activation" (ICCVAM 2002). One hundred and twenty four chemicals were tested in the LUMI-CELL™ ER BG1Luc4E2 bioassay system for this submission. 53 of these chemicals were recommended by ICCVAM for validation of ER binding and transcriptional activation. Of the 53 chemicals tested, which were recommended by ICCVAM, all of the 28 compounds having historical data for a positive response demonstrated estrogenic activity in the LUMI-CELL™ ER bioassay (ICCVAM 2002). Out of the 124 chemicals tested by LUMI-CELL™ ER bioassay system, 65 demonstrated estrogenic activity, while 59 showed no activity. Of the 68 chemicals tested, which were not included in the ICCVAM requirements for validation, 28 were found to possess' estrogenic activity, while 40 showed no activity. (see **Appendix D**, data summary)

6.4 State the strengths and limitations of the method, including those applicable to specific chemical classes or physical-chemical properties.

The strengths of the LUMI-CELL™ ER system have been highlighted in previous sections (particularly introduction and sections **1.2.1, 1.2.2, 1.2.3**, and **1.2.4**). Briefly, one of strengths of the LUMI-CELL™ ER system is that it is a mechanistically based bioassay system that measure function of the estrogen receptor system and the effects of chemicals on this system. The assay is rapid, economical, and provides relative potency of chemicals due to the large dynamic range of the system as demonstrated in the data in section **5** (particularly section **5.4** and **Appendix C**, an example of the 17β-estradiol curve; **Appendix D**, Agonist Plate-to-Plate data summary; **Appendix F**, Antagonist Plate-to-Plate data summary; **Appendix H**, Agonist Well-to-Well data summary; and **Appendix J**, QC performance charts). The luciferase endpoint is easy to measure (production of light) and quantify and it is specific since this gene is not normal to the cell making background expression controllable. The only limitation of the method is that it requires the cells to be alive to respond and can not test acutely toxic chemicals, which are toxic at concentration thought to be active.

6.5 Describe the salient issues of data interpretation, including why specific parameters were selected for inclusion.

Parameters such as inclusion of 7 QC points in addition to the 11 point, 17β-estradiol dose response curve were included to preserve the integrity of the system (see **Appendix C**, an example of the 17β-estradiol curve; **Appendix D**, Agonist Plate-to-Plate data summary; and **Appendix J**, QC performance charts).

6.6 In cases where the proposed test method is mechanistically and functionally similar to a validated test method with established performance standards, the results obtained with both test methods should be compared with each other and with the *in vivo* reference test method and/or toxicity information from the species of interest.

There is no known validated test method (*in vitro* nor *in vivo*) to be used as a reference test method for detection of estrogenic endocrine disruptors. The only known *in vivo* comparison to the LUMI-CELL™ ER bioassay, was done by Jefferson et al. 2002 (see sections **1.1.2**, **1.2.3** and **6.2** and attached papers in **Appendix K**).

7.0 Test Method Reliability (Repeatability/Reproducibility)

7.1 Discuss the selection rationale for the substances used to evaluate the reliability (intra-laboratory repeatability and intra- and inter-laboratory reproducibility) of the proposed test method as well as the extent to which the chosen set of chemicals represents the range of possible test outcomes.

The basis for test chemical selection was in 2 parts, those recommended by ICCVAM (ICCVAM 2002) and other chemicals which were selected since they have been reported as potential estrogen active chemicals. Most of the other chemicals are known environmental contaminants and evaluation of their potential as estrogen agonists is needed. Many compounds with historical data for positive and negative for both agonistic and antagonistic response were used in theses studies. Most of the historical data on these compounds came from the ICCVAM publication "Current Status of Test Methods for Detecting Endocrine Disruptors: In Vitro Estrogen Receptor Transcriptional Activation" (ICCVAM 2002).

7.2 Provide analyses and conclusions reached regarding inter- and intra-laboratory repeatability and reproducibility. Acceptable methods of analyses include those described in ASTM E691-92 (6) or by coefficient of variation analysis.

Coefficient of variation analysis has been conducted for both the agonist plate-to-plate and well-to-well variability as well as the antagonist variability of LUMI-CELL™ ER bioassay. This data is available in: **Appendix C**, an example of the 17β-estradiol curve; **Appendix D**, Agonist Plate-to-Plate data summary; **Appendix F**, Antagonist Plate-to-Plate data summary; **Appendix H**, Agonist Well-to-Well data summary; and **Appendix J**, QC performance charts.

Intra-laboratory validation has not been done at this time but studies are underway with the investigators mentioned in section **5.5** (Dr. Goeyens of the Scientific Institute of Public Health of Belgium and Dr. Kayama of Jichi Medical School and Mr. Yamamoto of the Hiyoshi Corporation of Japan).

Also the only known *in vivo* comparison to the LUMI-CELL™ ER bioassay was done by Jefferson et al. 2002 (see sections **1.1.2**, **1.2.3** and **6.2** and attached papers in **Appendix K**).

7.3 Summarize historical positive and negative control data, including number of experiments, measures of central tendency, and variability.

The history of all positive and negative controls is maintained in QC charts (see **Appendix J**). The QCs have been described in sections **2.2.7, 2.2.8** and **Appendix A**. Briefly the acceptable range for QC is 2 standard deviations from the mean and are depicted by the line in the middle as the mean and the 2 lines outside the mean as the acceptable range (i.e. the distance of 2 standard deviations).

7.4 In cases where the proposed test method is mechanistically and functionally similar to a validated test method with established performance standards, the reliability of the two test methods should be compared and any differences discussed.

There is no known validated test method (*in vitro* nor *in vivo*) to be used as a reference test method for detection of estrogenic endocrine disruptors. The only known *in vivo* comparison to the LUMI-CELL™ ER bioassay was done by Jefferson et al. 2002 (see sections **1.1.2, 1.2.3** and **6.2** and attached papers in **Appendix K**).

8.0 Test Method Data Quality

8.1 State the extent of adherence to national and international GLP guidelines (7-12) for all submitted data, including that for the proposed test method, the *in vivo* reference test method, and if applicable, a comparable validated test method. Information regarding the use of coded chemicals and coded testing should be included.

The laboratory as discussed above follows all GLP guidelines and audit of the laboratory for GLP compliance has been done by the Belgium Government. Coded studies are underway but not yet completed. The only known *in vivo* comparison to the LUMI-CELL™ ER bioassay, was done by Jefferson et al. 2002 (see sections **1.1.2, 1.2.3** and **6.2** and attached papers in **Appendix K**).

8.2 Summarize the results of any data quality audits, if conducted.

No data quality audits have been conducted to this point.

8.3 Discuss the impact of deviations from GLP guidelines or any noncompliance detected in the data quality audits.

No deviations from the GLP guidelines have occurred, and no data quality audits have been conducted to this point.

8.4 Address the availability of laboratory notebooks or other records for an independent audit. Unpublished data should be supported by laboratory notebooks.

All records and notebooks are available for viewing upon request from independent auditors.

9.0 Other Scientific Reports and Reviews

9.1 Summarize all available and relevant data from other published or unpublished studies conducted using the proposed test method.

Appended is a peer reviewed scientific publication by Rogers and Denison on the BG1Luc4E2 system (Rogers and Denison 2000) and a published paper for the Dioxins 2003 conference (Gordon, Chu et al. 2003), 2 abstracts, one submitted to SOT 2004 (Gordon, Chu et al. 2004) the other to e.hormone 2003 (Gordon, Chu et al. 2003) and a paper currently being reviewed for submission (Gordon, Chu et al. 2004). The only known *in vivo* comparison to the LUMI-CELL™ ER bioassay, was done by Jefferson et al. 2002 (see sections **1.1.2, 1.2.3** and **6.2** and attached papers in **Appendix K**).

9.2 Comment on and compare the conclusions published in independent peer- reviewed reports or other independent scientific reviews of the proposed test method. The conclusions of such scientific reports and reviews should be compared to the conclusions reached in this submission. Any ongoing evaluations of the proposed test method should be described.

The only known *in vivo* comparison to the LUMI-CELL™ ER bioassay was done by Jefferson et al. 2002 (see sections **1.1.2, 1.2.3** and **6.2** and attached papers in **Appendix K**).

9.3 In cases where the proposed test method is mechanistically and functionally similar to a validated test method with established performance standards, the results of studies conducted with the validated test method subsequent to the ICCVAM evaluation should be included and any impact on the reliability and accuracy of the proposed test method should be discussed.

There is no known validated test method (*in vitro* nor *in vivo*) to be used as a reference test method for detection of estrogenic endocrine disruptors. The only known *in vivo* comparison to the LUMI-CELL™ ER bioassay, was done by Jefferson et al. 2002 (see sections **1.1.2, 1.2.3** and **6.2** and attached papers in **Appendix K**).

10.0 Animal Welfare Considerations (Refinement, Reduction, and Replacement)

10.1 Describe how the proposed test method will refine (reduce or eliminate pain or distress), reduce, and/or replace animal use compared to the current methods used.

The LUMI-CELL™ ER bioassay is an *in vitro* system and could be used to reduce, refine and replace the number of animal tests now being conducted, if it is validated and found to be predictive of estrogen agonists and antagonists. The system is at the early stages of validation and need to be further explored on how well it could replace animal systems.

10.2 If the proposed test method requires the use of animals, the following items should be addressed:

10.2.1 Describe the rationale for the need to use animals and describe why the information provided by the proposed test method requires the use of animals (i.e., cannot be obtained using non-animal methods).

XDS's LUMI-CELL™ ER bioassay in an *in vitro* assay and does not use animals in testing.

10.2.2 Include a description of the sources used to determine the availability of alternative test methods that might further refine, reduce, or replace animal use for this testing. This should, at a minimum, include the databases searched, the search strategy used, the search date(s), a discussion of the results of the search, and the rationale for not incorporating available alternative methods.

XDS's LUMI-CELL™ ER bioassay in an *in vitro* assay, therefore would be used to refine, reduce and replace animals used in testing. Since there is no other validated test for the detection of estrogenic endocrine disruptors, this method would fit these requirements completely. The only known *in vivo* comparison to the LUMI-CELL™ ER bioassay, was done by Jefferson et al. 2002 (see sections **1.1.2, 1.2.3** and **6.2** and attached papers in **Appendix K**).

10.2.3 Describe the basis for determining that the number of animals used is appropriate.

XDS's LUMI-CELL™ ER bioassay in an *in vitro* assay and does not use animals in testing.

10.2.4 If the proposed test method involves potential animal pain and distress, discuss the methods and approaches that have been incorporated to minimize and, whenever possible, eliminate the occurrence of such pain and distress.

XDS's LUMI-CELL™ ER bioassay in an *in vitro* assay and does not use animals in testing.

11.0 Practical Considerations

11.1 Discuss the following aspects of test method transferability. Include an explanation of how this compares to the transferability of the reference test method and, if applicable, to a comparable validated test method with established performance standards.

XDS is unaware of an accepted reference test method. One of the many methods suggested is the mouse uterotrophic assay that requires specialized animal facilities, large numbers of animals, and highly trained individuals to evaluate results. The only known *in vivo* comparison to the LUMI-CELL™ ER bioassay, was done by Jefferson et al. 2002 (see sections **1.1.2, 1.2.3** and **6.2** and attached papers in **Appendix K**).

11.1.1 Discuss the facilities and major fixed equipment needed to conduct a study using the test method.

The equipment and supplies need to perform the test are detailed in section **2**. The facilities required are a functioning laboratory. Our current facility is a 1600 square foot laboratory but all analysis can be performed in single room (i.e. 10 foot by 20-foot tissue culture facility).

11.1.2 Discuss the general availability of other necessary equipment and supplies.

All equipment necessary for the LUMI-CELL™ ER bioassay is readily available from the suppliers listed in section **2**.

11.2 Discuss the following aspects of proposed test method training. Include an explanation of how this compares to the level of training required to conduct the *in vivo* reference test method and, if applicable, a comparable validated test method with established performance standards.

11.2.1 Discuss the required level of training and expertise needed for personnel to conduct the proposed test method.

There is a certain level of training needed to conduct the proposed test method. But, this training can easily be conducted by XDS staff. For cross lab validation the training would be minimal if the four labs currently using XDS's CALUX® method are used.

11.2.2 Indicate any training requirements needed for personnel to demonstrate proficiency and describe any laboratory proficiency criteria that should be met.

Persons should be adept at cell culture and organic extractions.

11.3 Cost Considerations - Discuss the cost involved in conducting a study with the proposed test method. Discuss how this compares to the cost of the *in vivo* reference test method and, if applicable, with that of a comparable validated test method with established performance standards.

There is no known validated test method (*in vitro* nor *in vivo*) to be used as a reference test method for detection of estrogenic endocrine disruptors. However the cost considerations are limited to the equipment and supplies listed in section **2**. The cost per sample is $200.00 for a screen (i.e. single analysis) and $350.00 for comprehensive analysis (i.e. triplicate analysis) at a 21 working day turnaround. Price can vary with number of samples and turnaround time.

11.4 Time Considerations - Indicate the amount of time needed to conduct a study using the proposed test method and discuss how this compares with the *in vivo* reference test method and, if applicable, with that of a comparable validated test method with established performance standards.

Once the cell line is established and growing (see **Appendix A**, detailed description of performance of the LUMI-CELL™ ER bioassay), studies can be conducted in as little as 48 hours. There is no known validated test method (*in vitro* nor *in vivo*) to be used as a reference test method for detection of estrogenic endocrine disruptors. However, current in vivo studies take anywhere from several weeks, with the uterotrophic assays, to years, with the 2-generation studies, to conduct.

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13.0 Supporting Materials

13.1 Provide the complete, detailed protocol for the proposed test method.

See **Appendix A** - detailed description of performance of the LUMI-CELL™ ER bioassay

13.2 Provide the detailed protocol(s) used to generate reference data for this submission and any protocols used to generate validation data that differ from the proposed protocol.

There was no data generated which differs from the protocol in **Appendix A**, detailed description of performance of the LUMI-CELL™ ER bioassay.

13.3 Provide copies of all relevant publications, including those containing data from the proposed test method, the *in vivo* reference test method, and if applicable, a comparable validated test method with established performance standards.

The only known *in vivo* comparison to the LUMI-CELL™ ER bioassay, was done by Jefferson et al. 2002 (see sections **1.1.2, 1.2.3** and **6.2** and attached papers in **Appendix K**). Copies of all publications are attached here as appendices.

13.4 Include all available non-transformed original data for both the proposed test method, the *in vivo* reference test method, and if applicable, a comparable validated test method with established performance standards.

See **Appendix C**, an example of the 17β-estradiol curve; **Appendix D**, Agonist Plate-to-Plate data summary; **Appendix F**, Antagonist Plate-to-Plate data summary; **Appendix H**, Agonist Well-to-Well data summary; and **Appendix J**, QC performance charts.

13.5 If appropriate performance standards for the proposed test method do not exist, performance standards for consideration by NICEATM and ICCVAM may be proposed. Examples of established performance standards can be located on the ICCVAM/NICEATM web site at http://iccvam.niehs.nih.gov.

Performance standards are available from ICCVAM (ICCVAM 2002)and were used in this study. QC performance charts can be found in **Appendix J**.

Appendix A: Detailed Description of Performance of the LUMI-CELL® ER bioassay

Mass culture of BG1Luc4E2 cell line: The cell line BG1Luc4E2 has remained stably transfected with the reporter plasmid for over 5 years. Early clones of the cells are stored in liquid nitrogen in 1 ml ampoules. First locate the cells in the liquid nitrogen dewer. Cells are thawed quickly by first releasing the gasses in the tube by slightly loosening the cap and then tightening it again. The tube is then thawed by holding in ones hand and rolling between palms. Do not thaw slowly, the cells will not survive a slow thawing process. The cells are then placed in a 50 ml centrifuge tube (Note: you may want to rinse the cryostorage vial with 1x PBS, 2 times), and add ~ 20 ml RPMI media (8% FCS, 1% Penn/Strep). Centrifuged at 1000 x g for 8 - 15 min. (Time for centrifugation is dependent on the centrifuge. Check after 8 or 10 min. to see if there is a good pellet. If not centrifuge an additional 5 min.). Note the time to form a good pellet in a logbook. The media is then removed and the cells re-suspended in 5 ml RPMI and incubated in a small (25 cm^2) flask until 80% confluent. NOTE: This may take 2 – 3 days.

After cells have grown to 80% confluence (2 – 3 days), transfer the cells to a medium (75 cm^2) flask. This is done by first removing the old media and rinsing the cells with 3 ml 1x PBS. This is done to remove all FCS, which will inactivate the trypsin. Then adding 1 - 2 ml 1x trypsin (without phenol red) to the small flask and incubating at 37° C for 5 – 10 min. Bump the side of the flask lightly to dislodge any cells still sticking to the flask, and check under an inverted microscope to be sure the cells are dislodged. Rinse the cells with 5 ml PBS and add to a 50 ml centrifuge tube (an additional 5 ml rinse may be needed if cells are still stuck to the flask). Add ~20 ml RPMI media and centrifuge at 1000 x g for 8 - 15 min. (see above for centrifugation times). Re-suspend the pellet in 10 ml RPMI media and add to medium (75 cm^2) flask. Allow the cells to grow until they are ~80% confluent.

The cells are then transferred to a large (175 cm^2) flask. This is done by removing the old media and rinsing the cells with 5 ml 1 x PBS. This time add 2 ml of 1x trypsin to the medium flask and incubating at 37° C for 5 – 10 min. Rinse the cells with 5 ml PBS and add to a 50 ml centrifuge tube (an additional 5 ml rinse may be needed if cells are still stuck to the flask). Add ~20 ml RPMI media and centrifuge at 1000 x g for 8 - 15 min. (see above for centrifugation times). Re-suspend the pellet in 20 ml RPMI media and add to a large (175 cm^2) flask. Allow the cells to grow until they are ~80% confluent. Note: If you are not going to use large (175 cm^2) flask and use just medium (75 cm^2) flask, transfer to two medium (75 cm^2) flasks and skip to the start of the DMEM phase and adjust volumes appropriately (i.e. use 2 ml trypsin, 10 ml media, ect.).

After the cells have reached 80% confluence the cells are split into 2 large flasks. This is done identically to the previous step, except for the amount of trypsin and the re-suspension. Use 3 ml of trypsin (instead of 2ml as before) when transferring the cells. And the pellet is re-suspended in 40 ml of RPMI with 20 ml is added to each of 2 large flasks.

1. **DMEM Media stock solutions (VWR Catalog #'s):**

    a. DMEM media without phenol Red and without L-Glutamine (DMEM, Mediatech - Catalog #: 45000-336)

- Note: DMEM with phenol red and/or L-Glutamine will likely contain estrogenic materials.

b. G418 Sulfate (50 mg/ml) (1x20ml) – Catalog #: 45000-630
c. L-Glutamine (29.2 mg/ml) (6x100ml) - Catalog #: 45000-676
d. Pen/Strep (5000 µg/ml) (6x100ml) - mediatech# 30001CI – Catalog #: 45000-650
e. Fetal Bovine Serum – Charcoal/Dextran Treated, triple 0.1 µm sterile filtered, Catalog #: SH30068.03

2. DMEM make-up: 1% Pen/Strep : 2% L. Glutamine : 5% Striped FBS

a. Add 5 ml 1x Pen/Strep into 500 ml DMEM.
b. Add 10 ml L-Glutamine to the same 500 ml DMEM.
c. Add 24 ml Striped Fetal Bovine Serum to same 500 ml DMEM.

Next is the start of the DMEM phase. The 2 large flasks are now transferred into 4 large flasks (2 for DMEM, used in plating; and 2 for RPMI, used in growing more cells). The first step is to removing the old media and rinsing the cells with 5 ml 1 x PBS. Then trypsonize with 3 ml of trypsin, and centrifuge as before. Important: Re-suspend the pellet (for the RPMI grown cells) in 4 ml of DMEM media. Add 20 ml DMEM to 2 large flasks and 20 ml RPMI to another 2 large flasks. Add 150 µl G418 to the DMEM flasks and 220 µl G418 to the RPMI flasks. Note: For medium (75 cm^2) flasks use 10 ml media and add 80 µl G418 to the DMEM flasks and 120 µl G418 to the RPMI flasks. Add 1 ml of the pelleted cells (which are now in 4 ml DMEM) to each of the four large (175 cm^2) flasks. Allow the cells to grow until they are ~80% confluent, which takes about 2 – 3 days. At which point the cells in the DMEM should be ready to plate.

Re-Seeding cells and Preparing for Plating. After the cells have grown for 2 – 3 days in the DMEM or RPMI with G418, remove all 4 flasks from the incubator (DMEM and RPMI flasks). Remove old media and rinse cells with 5 ml 1x PBS. Add 3 ml 1x trypsin to each flask and incubating at 37° C for 5 – 10 min. Rinse the DMEM cell flasks with 5 ml 1x PBS and add to a 50 ml centrifuge tube. Add ~20 ml DMEM media and centrifuge at 1000 x g for 8 - 15 min. (see above). Do the same for the RPMI flasks (including adding ~20 ml DMEM), making sure not to mix the two cells grown in two different media. Important: Re-suspend all pellets (for the RPMI and DMEM) grown cells in 4 ml DMEM media. Add 20 ml DMEM to 2 large flasks and 20 ml RPMI to another 2 large flasks. Add 150 µl G418 to the DMEM flasks and 220 µl G418 to the RPMI flasks. Add 1 ml of the RPMI grown cells (which are now in 4 ml DMEM) to each of the large (175 cm^2) flasks. Allow the cells to grow until they are ~80% confluent. This should take 2 – 3 days and repeat this step for up to 3 months where new cells should be brought up. (Note: You need to re-suspend the pellets in DMEM so that when re-seeding the flasks you don't contaminate the DMEM flasks with estrogenic material in the RPMI media.) The pellet of cells from the DMEM media cultures is then re-suspended in 20 ml DMEM (cell are re-suspended in 10 ml DMEM if the cells are grown in medium (75 cm^2) flasks). The cells are counted and volume adjusted with DMEM media to give 200,000 cells/ml. 200 µl of this solution is then plated on a 96 well plate in each well. The plates must incubate 20 – 24 hours before use but not longer than 48 hours before use.

Counting and Plating Cells. After the cells are re-suspended in 20ml DMEM, (For cells grown in Medium (75 cm^2) flasks re-suspend in 10 ml DMEM), make sure that the cell/media solution is well mixed and using a pipette take an aliquot of 15µl. Place the cover slip on the hemocytometer so that it rests on the two grey supports. Add the 15µl to the "v" shaped curve on the hemocytometer. Make sure that the solution covers the whole surface area, and let cells settle before counting. Using 100x magnification on the Microscope, place the hemocytometer in plate clamps and view the counting grid. Counting grid consists of four sections (upper left, upper right, lower left and lower right). Each section consists of four by four grids. Starting at the top left and moving clockwise, count all cells in each of the four by four grids. Some cells will be touching the outside boards of the square, only count those that touch the top and right boarders of the square. Determine the average of the four grids.

Volume of each square is 10^{-4}ml, therefore:
Cells/ml=(average number per grid) x 10^4ml. x 1/(starting dilution).

Examples for how to calculate volume adjustments:

Starting dilution: 20ml
Total count of cells for all four grids: 175
Average of four grids: 43.75
Average / 20 (equivalent of 20 x $10^{4)}$= 2.188
2.188 x starting dilution (20ml)= 43.75
Add 23.75 ml for a total of 43.75 ml.
On average 20mls needed for one 96 well plate.

Starting dilution: 10ml
Total count of cells for all four grids: 275
Average of four grids: 68.75
Average / 20 (equivalent of 20 x $10^{4)}$= 3.43
3.43 x starting dilution (10ml)= 34.375
Add 24.4ml for a total of 24.4ml.
On average 20mls needed for one 96 well plate.

Next, start by removing a 96 well plate from its sterile package. Using an eppendorf repeater pipettor (or equivalent), pipette 200µl of cell/media solution to each well. Label plate with date and time of plating, cell type, and initials of technician. Incubate plate(s) at 36-38^o C in 5% CO_2 for 20 – 24 hours, but no longer than 48 hours before dosing.

Standard Curves and QCs:

To determine the Agonistic response, dilutions of 17β-estradiol and test compounds are prepared in DMSO. A standard solution of 10 ng/ml of 17β-estradiol in DMSO is used to prepare dilutions of this standard to produce an 11 point standard curve. Four µl of DMSO is added to ten, of the eleven 13 mm glass tubes. Four µl of the 10 ng/ml standard solution of 17β-estradiol is added to both the first tube (not containing DMSO) and the second tube containing the 4 µl of DMSO in the tube. The second tube is vortexed and four µl transferred to the next tube in the series. This is repeated for each of the 10 tubes creating a two fold dilution series. Stock solutions of each dilution may also be prepared in advance in large volumes. Then simply take 4 µl of each dilution and place in each of the 13 mm tubes in succession. These stock solutions should be made monthly.

For the Antagonistic response, a standard solution of 5 µg/ml of Tamoxifen in DMSO is used to prepare dilutions of this standard. Four µl of DMSO is added to ten, of the eleven 13 mm glass tubes. 4 µl of the 5 µg/ml standard solution of Tamoxifen is added to both the first tube (not containing DMSO) and second tube containing the 4 µl of DMSO in the tube. The second tube is vortexed and four µl transferred to the next tube in the series. This is repeated for each of the 10 tubes creating a two fold dilution series. 4 µl of a 5 ng/ml 17β-estradiol standard solution is added to each tube, vortexed, and 4

µl removed from each tube to keep the total volume of DMSO at 4 µl. Four µl of all appropriate positive and negative control QCs are also added to separate tubes to ensure the integrity of the system. For this system that would include 4 DMSO QCs, 1 no DMSO QC (i.e. just media) and 2 or 3 positive response QCs (DES, Bisphenol A, and/or Estrone (if needed)). Also, if appropriate, the cell viability assay is used. The cell viability assay includes adding 4 µl of 5 ng/ml or 0.5 ng/ml 17β-estradiol standard solution to the highest concentration of the test substance being analyzed (and to ½ and $1/10^{th}$ the highest concentration of the test substance being analyzed if antagonism is suspected). To each tube, 400 µl of Estrogen free DMEM media is added to the DMSO solution and the tube vortexed vigorously. Similar dilution series are produced for test compounds or extracts being analyzed for estrogenic activity by the BG1Luc4E2 cells.

Preparing Compounds for testing:

Agonist Range Finding: Prepare a 10mg/ml solution of the compound of interest. (Note: If using Molar the range for starting concentrations is 1×10^{-4} M – 1×10^{-10} M (with the average being ~2.19×10^{-4} M)). Prepare five 10-fold serial dilutions of the compound. Add 4 µl of each, the original 10mg/ml, and the 5 serial dilutions to 6 different 13 mm tubes. To each tube, 400 µl of Estrogen free DMEM media is added to the DMSO solution and the tube vortexed vigorously.

Agonist Dose Response Curve: After Range Finding, determine the top response concentration. Start an 11 point 2- fold dilution curve at a slightly higher concentration than the highest response from the Range Finding. (i.e. if the highest point was the 0.01 mg/ml dilution, start your 2 fold dilution curve at about 0.5 mg/ml). The 11 point 2-fold dilution curve is started by adding four µl of DMSO is added to ten of the eleven 13 mm glass tubes. Four µl of the highest concentration of the compound to be tested is added to both the first tube (not containing DMSO) and the second tube containing the 4 µl of DMSO in the tube. The second tube is vortexed and four µl transferred to the next tube in the series. This is repeated for each of the 10 tubes creating an 11 point 2-fold dilution series. (Note: After analysis if the top or bottom of the dose response curve is not elucidated, the concentrations will have to be extended higher and/or lower). To each tube 400 µl of Estrogen free DMEM media is added to the DMSO solution and the tube vortexed vigorously.

Antagonist Range Finding: Prepare a 10mg/ml solution of the compound of interest in DMSO. Prepare five 10-fold serial dilutions of the compound in DMSO. Add 4 µl of each the original 10mg/ml and the 5 serial dilutions to 6 different 13 mm tubes. Add 4 µl of 5 ng/ml 17β-estradiol to each tube, vortex and remove 4 µl from each tube. To each tube 400 µl of Estrogen free DMEM media is added to the DMSO solution and the tube vortexed vigorously.

Antagonist Dose Response Curve: After Range Finding, determine the top response concentration. Start an 11 point 2- fold dilution curve at a slightly higher concentration than the highest response from the Range Finding. (i.e. if the highest point was the 0.01 mg/ml dilution, start your 2 fold dilution curve at about 0.5 mg/ml). The 11 point 2-fold dilution curve is started by adding four µl of DMSO is added to ten 13 mm glass tubes. Four µl of the highest concentration of the compound to be tested is added to both the first tube (not containing DMSO) and the second tube containing the 4 µl of DMSO in the tube. The second tube is vortexed and four µl transferred to the next tube in the series. This is repeated for each of the 10 tubes creating an 11 point 2-fold dilution series. (Note: After analysis if the top or bottom of the dose response curve is not elucidated, the concentrations will have to be extended higher or

lower). Add 4 µl of 5 ng/ml 17β-estradiol to each tube, vortex and remove 4 µl from each tube. To each tube 400 µl of Estrogen free DMEM media is added to the DMSO solution and the tube vortexed vigorously.

Cell Viability: An additional assay using either Tripan Blue or Promega's CellTiter-Glo Luminexcent Cell Viability Assay to determine the level of cell toxicity. This assay is only used in cased when antagonism is suspected.

Dosing Procedure unknown samples (feed, blood, water and other substrates):

Agonist Feed (and other dry samples): After extraction (in MeOH) prepare dilutions of 1:100, 1:500, 1:1000, and 1:10,000 of the unknown solution in MeOH. (Note: Higher or lower dilutions may be needed for some samples). Add 4 µl DMSO to four 13 mm tubes. Add the diluted sample (as above) to each of the 4 tubes. Bring volume up to 1 ml hexane in each of the 4 tubes and each of the standard curve tubes and the QC tubes. Vacuum centrifuge the samples to dryness (i.e. speedvac for 6 min. Check tubes, if not dry speedvac for 2 more min. Check tubes again and if they are cold on the bottom speedvac for an additional 2 min. Note: All speedvacs are different therefore drying times will vary). To each tube, 400 µl of Estrogen free DMEM media is added to the DMSO solution and the tube vortexed vigorously.

Agonist Blood Samples: Add 4 µl DMSO to a 13 mm tubes. Add 5 µl of the blood sample directly to each 13 mm tube. To each tube, 400 µl of Estrogen free DMEM media is added to the DMSO solution and the tube vortexed vigorously.

Agonist Water Samples: Add 4 µl DMSO to a 13 mm tubes. Add 5 µl of the water sample directly to each 13 mm tubes. To each tube, 400 µl of Estrogen free DMEM media is added to the DMSO solution and the tube vortexed vigorously.

Antagonist Feed (and other dry samples): After extraction (in MeOH) prepare dilutions of 1:100, 1:500, 1:1000, and 1:10,000 of the unknown solution. (Note: Higher or lower dilutions may be needed for some samples). Add 4 µl of 5 ng/ml 17β-estradiol (in DMSO) to each tube. Add the diluted sample (as above) to each of the 4 tubes. Add 1 ml hexane to each of the 4 tubes and each of the standard curve tubes and the QC tubes. Speedvac for 6 min. Check tubes and speedvac for 2 more min. Check tubes again and if they are cold on the bottom speedvac for an additional 2 min. To each tube, 400 µl of Estrogen free DMEM media is added to the DMSO solution and the tube vortexed vigorously.

Antagonist Blood Samples: Add 4 µl of 5 ng/ml 17β-estradiol (in DMSO) to each tube. Add 5 µl of the blood sample directly to each 13 mm tubes. To each tube, 400 µl of Estrogen free DMEM media is added to the DMSO solution and the tube vortexed vigorously.

Antagonist Water Samples: Add 4 µl of 5 ng/ml 17β-estradiol (in DMSO) to each tube. Add 5 µl of the water sample directly to each 13 mm tubes. To each tube, 400 µl of Estrogen free DMEM media is added to the DMSO solution and the tube vortexed vigorously.

Dosing 96 Well Plates: Remove the 96 well plate of cells that have been incubated for 20 – 24 hours (but not longer than 48 hours), at 36-38^{o} C and 5% CO_2, from the incubator. Inspect wells

using an inverted microscope and note any wells with dead, missing, morphologically changed cells, and/or any contamination. Place a piece of absorbent paper in bio-hood. Remove plate lid and invert the plate and shake on paper to remove medium (do not let plate touch paper). After visually inspecting plate, add 200µl sample to be tested to one well. (Do not use the outer wells, outer wells may be used for range finding). When adding the sample, place the tip of the pipettor on the wall of the well and pipette slowly. This is done so that the cells layer is not disturbed. Once all samples have been added, add media to the outer wells, record date/time, and any pertinent sample information on plate and in cell culture logbook. Place plate in incubator for 20 – 24 hours before analysis.

Lysing Cells and Measurement of estrogen induced luciferase activity in BG1Luc4E2 cells:
Luciferase that is produced in the BG1Luc4E2 cells in response to exposure to estrogen accumulates in the cytoplasm of the cells over the twenty-four hour incubation. To measure luciferase the cells must be lysed and substrates for measurement of luciferase enzyme activity added and results, light emission by the enzymatic activity measured in a luminometer. This is accomplished after dosing and incubating the 96well plate at 37^{o} C for 20 – 24 hours. The plates are removed from the incubator and the media removed from the plate by inverting the plate and lightly shaking the media out of the plate over absorbent bench paper. Lightly tap the plate on the bench paper to remove excess liquid. Rinse the wells with 50 µl 1x PBS, and remove the PBS by again inverting the plate and lightly shaking the media out of the plate over bench paper. Again lightly tap the plate on the bench paper to remove excess liquid. Examine all wells under an inverted microscope. Make notes of any wells with missing, dislodged, or morphologically changed cells. Place white backing tape on bottom of the 96 well plate. Add 30 µl 1x Promega Cell Lysis Buffer to each well. Shake on an orbital shaker for 1 min.
The plate is now ready to be analyzed using the Berthold Microplate Luminometer. The measured RLU by the instrument is then exported to a dedicated computer and analyzed with software designed to provide analysis of the RLU of the 17β-estradiol standard, subtraction of blank responses and interpolation of unknown responses to the standard curve.

Shown in figures 4 through 8 are sample templates for a typical HTPS data analysis system XDS has developed for estimation of the estrogenic activity of chemicals and extracts of environmental samples. A sample template for the 96 well plate analysis is shown in Figures 4 and 5. Figure 4 depicts a typical plate for analysis of agonist activity, and it includes, the 17β-estradiol standards, 4 DMSO controls, 1 No DMSO control, 2 positive QC points; and samples (including XDS I.D.#; sample dilution; client I.D.#; and RLU result). Figure 5 depicts a typical plate for analysis of antagonist activity and includes the Tamoxifen/17β-estradiol standards, 2 cell viability QC points, 4 DMSO controls, 1 No DMSO control, 2 positive QC points; and samples (including XDS I.D.#; sample dilution; client I.D.#; and RLU result). As described earlier, we have determined that the responsiveness of the BG1Luc4E2 cells is sensitive to an edge effect in which determinations made in the outer wells of the plate are variable and result in reduced confidence of analysis of luciferase activity in these wells. Therefore, on a 96 well plate, 56 wells of the plate are useful for determination of estrogen dependent induction of luciferase activity.

	1	2	3	4	5	6	7	8	9	10	11	12
									20 hour exposure			
A												
					Sample ID 0001	Sample ID 0007	Sample ID 0013	Sample ID 0019	Sample ID 0025	Sample ID 0031	Sample ID 0037	
		4.00E+01	6.25E-01	DMSO	dilution	dilution	dilution	dilution	dilution	dilution	dilution	
B		ß-estradiol	ß-estradiol	control	Sample Name 1	Sample Name 7	Sample Name 13	Sample Name 19	Sample Name 25	Sample Name 31	Sample Name 37	
		0.00	0.00	0.00	RLU Results	RLU Results	RLU Results	RLU Results	RLU Results	RLU Results	RLU Results	
				QC	Sample ID 0002	Sample ID 0008	Sample ID 0014	Sample ID 0020	Sample ID 0026	Sample ID 0032	Sample ID 0038	
		2.00E+01	3.13E-01	5.00E+00	dilution	dilution	dilution	dilution	dilution	dilution	dilution	
C		ß-estradiol	ß-estradiol	DES QC	Sample Name 2	Sample Name 8	Sample Name 14	Sample Name 20	Sample Name 26	Sample Name 32	Sample Name 38	
		0.00	0.00	0.00	RLU Results	RLU Results	RLU Results	RLU Results	RLU Results	RLU Results	RLU Results	
				QC	Sample ID 0003	Sample ID 0009	Sample ID 0015	Sample ID 0021	Sample ID 0027	Sample ID 0033	Sample ID 0039	
		1.00E+01	1.56E-01	2.50E+04	dilution	dilution	dilution	dilution	dilution	dilution	dilution	
D		ß-estradiol	ß-estradiol	BPA QC	Sample Name 3	Sample Name 9	Sample Name 15	Sample Name 21	Sample Name 27	Sample Name 33	Sample Name 39	
		0.00	0.00	0.00	RLU Results	RLU Results	RLU Results	RLU Results	RLU Results	RLU Results	RLU Results	
				0.00E+00	Sample ID 0004	Sample ID 0010	Sample ID 0016	Sample ID 0022	Sample ID 0028	Sample ID 0034	Sample ID 0040	
		5.00E+00	7.81E-02	DMSO	dilution	dilution	dilution	dilution	dilution	dilution	dilution	
E		ß-estradiol	ß-estradiol	control	Sample Name 4	Sample Name 10	Sample Name 16	Sample Name 22	Sample Name 28	Sample Name 34	Sample Name 40	
		0.00	0.00	0.00	RLU Results	RLU Results	RLU Results	RLU Results	RLU Results	RLU Results	RLU Results	
				0.00E+00	Sample ID 0005	Sample ID 0011	Sample ID 0017	Sample ID 0023	Sample ID 0029	Sample ID 0035	Sample ID 0041	
		2.50E+00	3.91E-02	DMSO	dilution	dilution	dilution	dilution	dilution	dilution	dilution	
F		ß-estradiol	ß-estradiol	control	Sample Name 5	Sample Name 11	Sample Name 17	Sample Name 23	Sample Name 29	Sample Name 35	Sample Name 41	
		0.00	0.00	0.00	RLU Results	RLU Results	RLU Results	RLU Results	RLU Results	RLU Results	RLU Results	
				0.00E+00	Sample ID 0006	Sample ID 0012	Sample ID 0018	Sample ID 0024	Sample ID 0030	Sample ID 0036	Sample ID 0042	
		1.25E+00	DMSO	No DMSO	dilution	dilution	dilution	dilution	dilution	dilution	dilution	
G		ß-estradiol	control	control	Sample Name 6	Sample Name 12	Sample Name 18	Sample Name 24	Sample Name 30	Sample Name 36	Sample Name 42	
		0.00	0.00	0.00	RLU Results	RLU Results	RLU Results	RLU Results	RLU Results	RLU Results	RLU Results	

Figure 4: Agonist response template for 96 well plate including β-estradiol curve, 4 DMSO controls, 1 No DMSO control, 2 positive response QC points; and samples (including sample I.D.#; sample dilution; client I.D.#; and RLU result).

	1	2	3	4	5	6	7	8	9	10	11	12
									20 hour exposure			
B		2.00E+06 Tam + 10 pg ß-E 0.00	3.13E+04 Tam + 10 pg ß-E 0.00	DMSO control 0.00	Sample ID 0001 dilution Sample Name 1 RLU Results	Sample ID 0007 dilution Sample Name 7 RLU Results	Sample ID 0013 dilution Sample Name 13 RLU Results	Sample ID 0019 dilution Sample Name 19 RLU Results	Sample ID 0025 dilution Sample Name 25 RLU Results	Sample ID 0031 dilution Sample Name 31 RLU Results	Sample ID 0037 dilution Sample Name 37 RLU Results	
C		1.00E+06 Tam + 10 pg ß-E 0.00	1.56E+04 Tam + 10 pg ß-E 0.00	QC 5.00E+00 DES QC 0.00	Sample ID 0002 dilution Sample Name 2 RLU Results	Sample ID 0008 dilution Sample Name 8 RLU Results	Sample ID 0014 dilution Sample Name 14 RLU Results	Sample ID 0020 dilution Sample Name 20 RLU Results	Sample ID 0026 dilution Sample Name 26 RLU Results	Sample ID 0032 dilution Sample Name 32 RLU Results	Sample ID 0038 dilution Sample Name 38 RLU Results	
D		5.00E+05 Tam + 10 pg ß-E 0.00	7.81E+03 Tam + 10 pg ß-E 0.00	QC 2.50E+04 BPA QC 0.00	Sample ID 0003 dilution Sample Name 3 RLU Results	Sample ID 0009 dilution Sample Name 9 RLU Results	Sample ID 0015 dilution Sample Name 15 RLU Results	Sample ID 0021 dilution Sample Name 21 RLU Results	Sample ID 0027 dilution Sample Name 27 RLU Results	Sample ID 0033 dilution Sample Name 33 RLU Results	Sample ID 0039 dilution Sample Name 39 RLU Results	
E		2.50E+05 Tam + 10 pg ß-E 0.00	3.91E+03 Tam + 10 pg ß-E 0.00	0.00E+00 DMSO control 0.00	Sample ID 0004 dilution Sample Name 4 RLU Results	Sample ID 0010 dilution Sample Name 10 RLU Results	Sample ID 0016 dilution Sample Name 16 RLU Results	Sample ID 0022 dilution Sample Name 22 RLU Results	Sample ID 0028 dilution Sample Name 28 RLU Results	Sample ID 0034 dilution Sample Name 34 RLU Results	Sample ID 0040 dilution Sample Name 40 RLU Results	
F		1.25E+05 Tam + 10 pg ß-E 0.00	1.95E+03 Tam + 10 pg ß-E 0.00	0.00E+00 DMSO control 0.00	Sample ID 0005 dilution Sample Name 5 RLU Results	Sample ID 0011 dilution Sample Name 11 RLU Results	Sample ID 0017 dilution Sample Name 17 RLU Results	Sample ID 0023 dilution Sample Name 23 RLU Results	Sample ID 0029 dilution Sample Name 29 RLU Results	Sample ID 0035 dilution Sample Name 35 RLU Results	Sample ID 0041 dilution Sample Name 41 RLU Results	
G		6.25E+04 Tam + 10 pg ß-E 0.00	DMSO control 0.00	0.00E+00 No DMSO control 0.00	Sample ID 0006 dilution Sample Name 6 RLU Results	Sample ID 0012 dilution Sample Name 12 RLU Results	Sample ID 0018 dilution Sample Name 18 RLU Results	Sample ID 0024 dilution Sample Name 24 RLU Results	Sample ID 0030 dilution Sample Name 30 RLU Results	Sample ID 0036 dilution Sample Name 36 RLU Results	Sample ID 0042 dilution Sample Name 42 RLU Results	

Figure 5: Antagonist response template for 96 well plate including 17β-estradiol standards, the Tamoxifen/17β-estradiol standards, 2 cell viability QC points, 4 DMSO controls, 1 No DMSO control, 3 QC points; and samples (including XDS I.D.#; sample dilution; client I.D.#; and RLU result).

Figure 6: Displays a raw data input template for luminometer results. This is where background is subtracted and induction is calculated.

Orion
Berthold

Test Name: **XDS1**
Cell line ID: BG1

Microplate	PACKARD OPTIPLATE 96	No. of Intervals	50	Interval Time [s]	0.3
Layout	XDS 96 well (Inside)	Tot. Meas. Time/Well [s]	15	Start Measurement [s]	0
		Start Injection 1 [s]	0	Start Injection 2 [s]	15

Test Type: Well Mode
Reading Direction: vertical
Calculation Range: Start 5 Stop 50

Induction:

Raw Data

Table 1

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Raw Data - Blank

Table 2

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		0	0	0	0	0	0	0	0	0	0	
C		0	0	0	0	0	0	0	0	0	0	
D		0	0	0	0	0	0	0	0	0	0	
E		0	0	0	0	0	0	0	0	0	0	
F		0	0	0	0	0	0	0	0	0	0	
G		0	0	0	0	0	0	0	0	0	0	
H												

blank

THE MODEL:
RLU = (v*(d^n))/(d^n + k^n)

'd' is the natural logarithm of TCDD concentration
"v" is the limiting value of the RLU response as 17β-estradiol concentration increases
"k" is the dose at which the response is 50% of maximum
"n" is a parameter that determines sigmoidal shape of curve
"b" is the interecept parameter

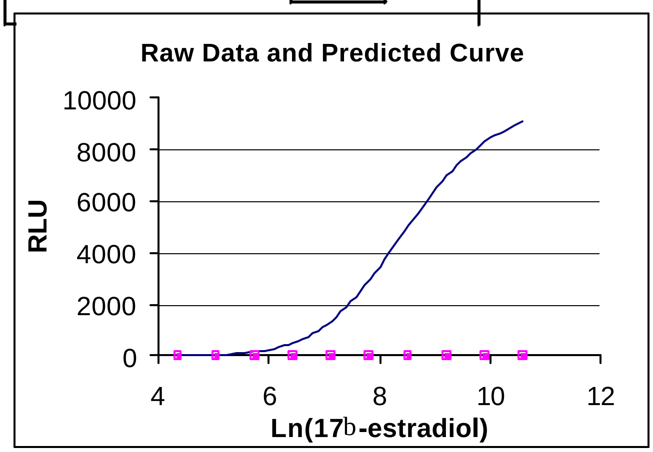
Initial Values (replaced with final estimates by 'Solver')

k	8.5
n	10.5
v	10000
b	0

To fit standard curve, paste 17β-estradiol and RLU data into the framed columns below. Then select 'Solver;' from the Tools menu. Be sure that the 'Target Cell' is set to **D43**, the 'Equal To' option is set to **min**, and the 'By Changing Cells' option is set to **B8:B11**

17β-estradiol	Ln(17β-estradiol)	RLU
40000.00	10.60	0.00
20000.00	9.90	0.00
10000.00	9.21	0.00
5000.00	8.52	0.00
2500.00	7.82	0.00
1250.00	7.13	0.00
625.00	6.44	0.00
312.50	5.74	0.00
156.25	5.05	0.00
78.13	4.36	0.00
39.06	3.67	0.00

Pred	Res	Res^2
9101.07	9101.07	########
8326.63	8326.63	########
6990.41	6990.41	########
5053.04	5053.04	########
2952.39	2952.39	########
1365.67	1365.67	########
512.80	512.80	262964.55
160.80	160.80	25857.39
42.18	42.18	1779.43
8.99	8.99	80.74
1.46	1.46	2.13
		2.37E+08

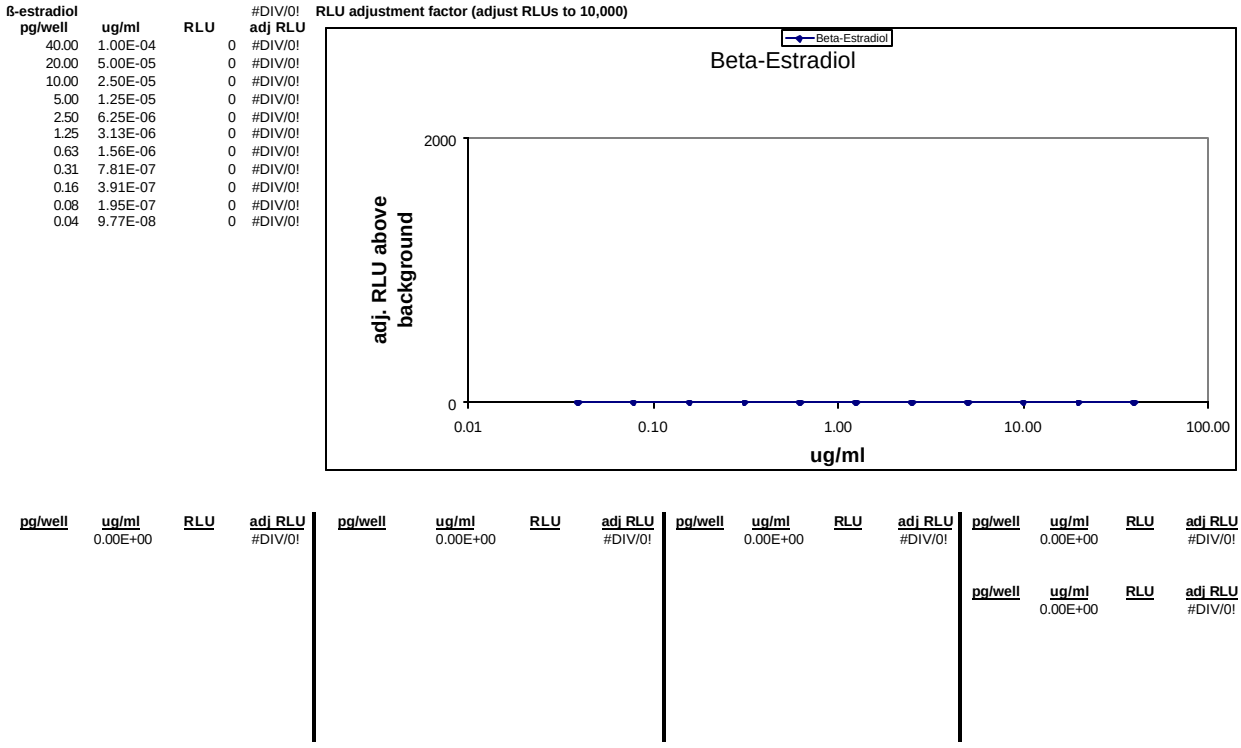


To obtain predicted Ln(17β-estradiol) concentrations from observed RLU data, paste observed RLU values into the framed cells below. These are your predicted concentrations based on the standard curve

Observed Response	Predicted Ln(17 β-estradiol)	pg
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001
0.00	0	0.001

Figure 7: Template calculation page used to generate the TEQ values using the Hill equation.

Figure 8 A: Displays the estrogen report template for known compounds.



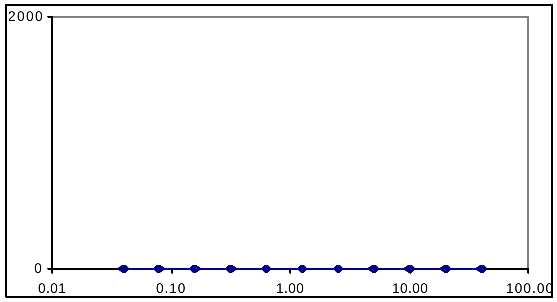
ß-estradiol			#DIV/0!	RLU adjustment factor (adjust RLUs to 10,000)
pg/well	ug/ml	RLU	adj RLU	
40.00	1.00E-04	0	#DIV/0!	
20.00	5.00E-05	0	#DIV/0!	
10.00	2.50E-05	0	#DIV/0!	
5.00	1.25E-05	0	#DIV/0!	
2.50	6.25E-06	0	#DIV/0!	
1.25	3.13E-06	0	#DIV/0!	
0.63	1.56E-06	0	#DIV/0!	
0.31	7.81E-07	0	#DIV/0!	
0.16	3.91E-07	0	#DIV/0!	
0.08	1.95E-07	0	#DIV/0!	
0.04	9.77E-08	0	#DIV/0!	

pg/well	ug/ml	RLU	adj RLU	pg/well	ug/ml	RLU	adj RLU	pg/well	ug/ml	RLU	adj RLU	pg/well	ug/ml	RLU	adj RLU
	0.00E+00		#DIV/0!		0.00E+00		#DIV/0!		0.00E+00		#DIV/0!		0.00E+00		#DIV/0!
												pg/well	ug/ml	RLU	adj RLU
													0.00E+00		#DIV/0!

Figure 8 B: Displays the estrogen report template for unknown samples.

ß-estradiol pg/well	ug/ml	RLU	#DIV/0! adj RLU
40.00	1.00E-04	0	#DIV/0!
20.00	5.00E-05	0	#DIV/0!
10.00	2.50E-05	0	#DIV/0!
5.00	1.25E-05	0	#DIV/0!
2.50	6.25E-06	0	#DIV/0!
1.25	3.13E-06	0	#DIV/0!
0.63	1.56E-06	0	#DIV/0!
0.31	7.81E-07	0	#DIV/0!
0.16	3.91E-07	0	#DIV/0!
0.08	1.95E-07	0	#DIV/0!
0.04	9.77E-08	0	#DIV/0!

RLU adjustment factor (adjust RLUs to 10,000)



CALCULATIONS

Sample	identity	fraction	RLU	TEQ, pg/well	ppt/sample	corrected	-bkg	mean	std dev	% std dev
0	0	0	0	0.00						
0			0	0.00						
0			0	0.00						
0			0	0.00						
0			0	0.00						
0			0	0.00						
0			0	0.00						
0			0	0.00						
0			0	0.00						
0			0	0.00						
0			0	0.00						
0			0	0.00						
0			0	0.00						
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0			0	0.00						
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0			0	0.00						
0			0	0.00						
0			0	0.00						

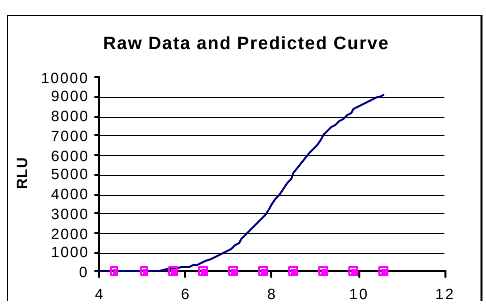


Figure 6 displays a raw data template for the luminometer output from an analysis. The relative light units (RLU) measured for each well of the test plate are shown in Table 1, while Table 2 displays the RLU corrected for the selected background wells. These data are then exported to a program designed by XDS for graphic display of the results and calculation of the estrogenic activity of samples based on extrapolation to the standard curve of 17β-estradiol.

We have determined that the output of receptor mediated gene expression systems is best estimated by a 4 parameter Hill equation. The Hill equation that we are using to extrapolate receptor mediated gene expression is shown at the top of Figure 7. Input of the RLU for samples is entered into this equation and the pg of estrogenic like activity for the sample is estimated from the model. The output is expressed as pg of estrogenic activity derived from the model.

Figure 8A displays the template for the estrogen report for known compounds. Figure 8B displays the template for the output of the analysis corrected for the amount of sample extracted for the determination. The estimated estrogenic activity of each sample from Figure 6 are corrected for the dilution of sample extract that was used in the analysis (Figure 7). This analysis also displays a non-modeled graphic display of the data. The table output provides an estimate of the estrogenic activity in pg per ml (or parts per trillion of estrogenic activity in the sample; column 6 of the output).

Raw Data Transformation

In order to follow the transformation of raw data into the data summary tables, first look up the name of the compound (from Appendix D, F, or H) of interest in the raw data summary chart in Appendix E, G, or I. The dates found associated with each data set tracks all raw data in all other charts. If you are interested in seeing the original raw data before background subtraction and factoring to 10,000 RLUs, go to the file with the appropriate year and click on that date (Note: there may be multiple files with the same date. Simply search each date file until you find the one you are looking for). If you are looking for any of the QCs (i.e. DES, BPA, or DMSO) go to Appendix J and look up the date associated with the compound of interest in each of the data set tabs.

The following section shows the transformation of a data set for Dieldren from concept of experimental set-up, to raw data, to transformed data, to data summaries and Appendixes. Here is a list of the figure legends and the following pages are the figures with explanation bubbles.

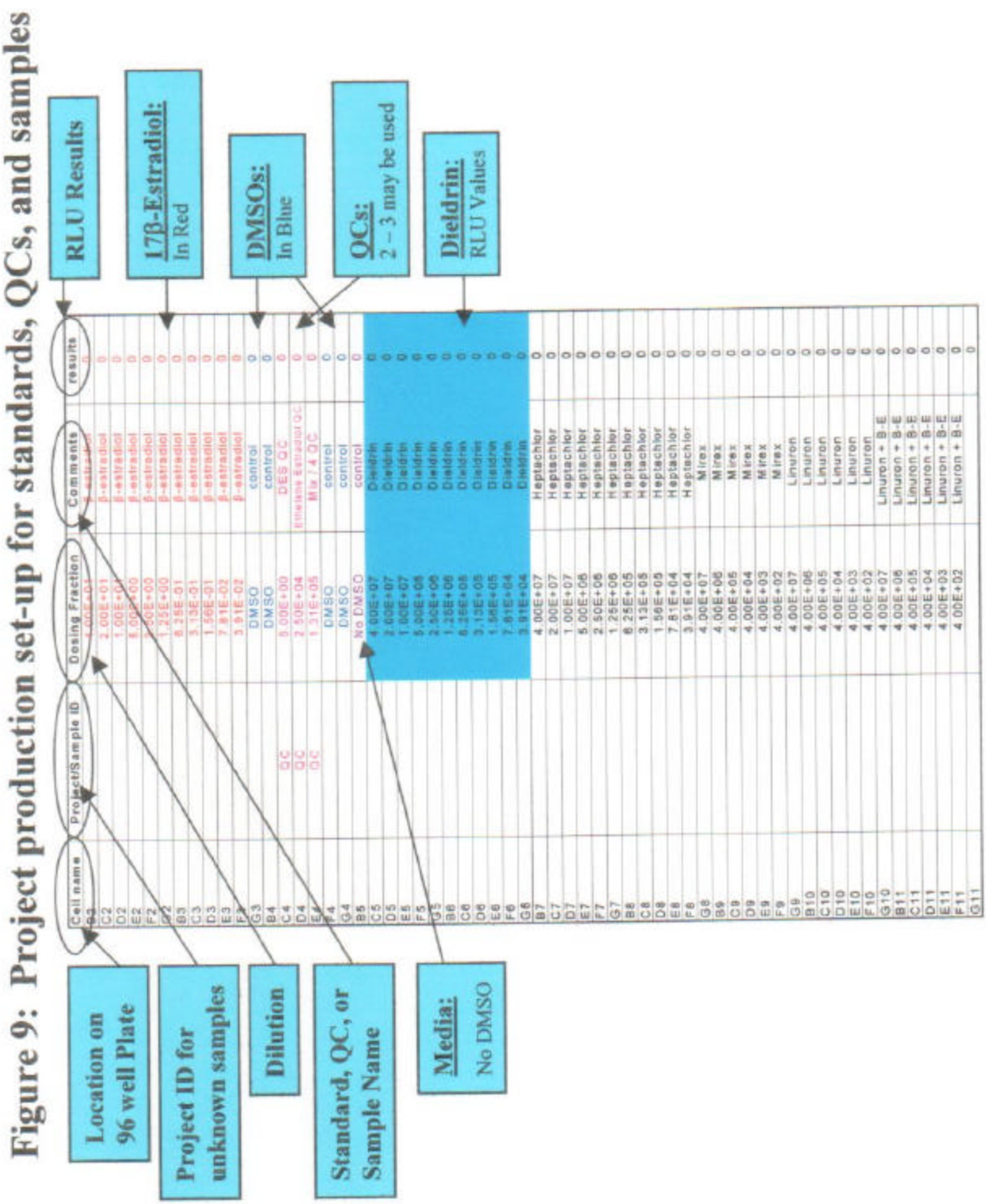


Figure 9: The project production set-up form is used to layout what samples are to be tested and at what concentration. As well as setting up the 17β-Estradiol curve and all associated positive and negative controls. ("List" tab on Excel spreadsheet).

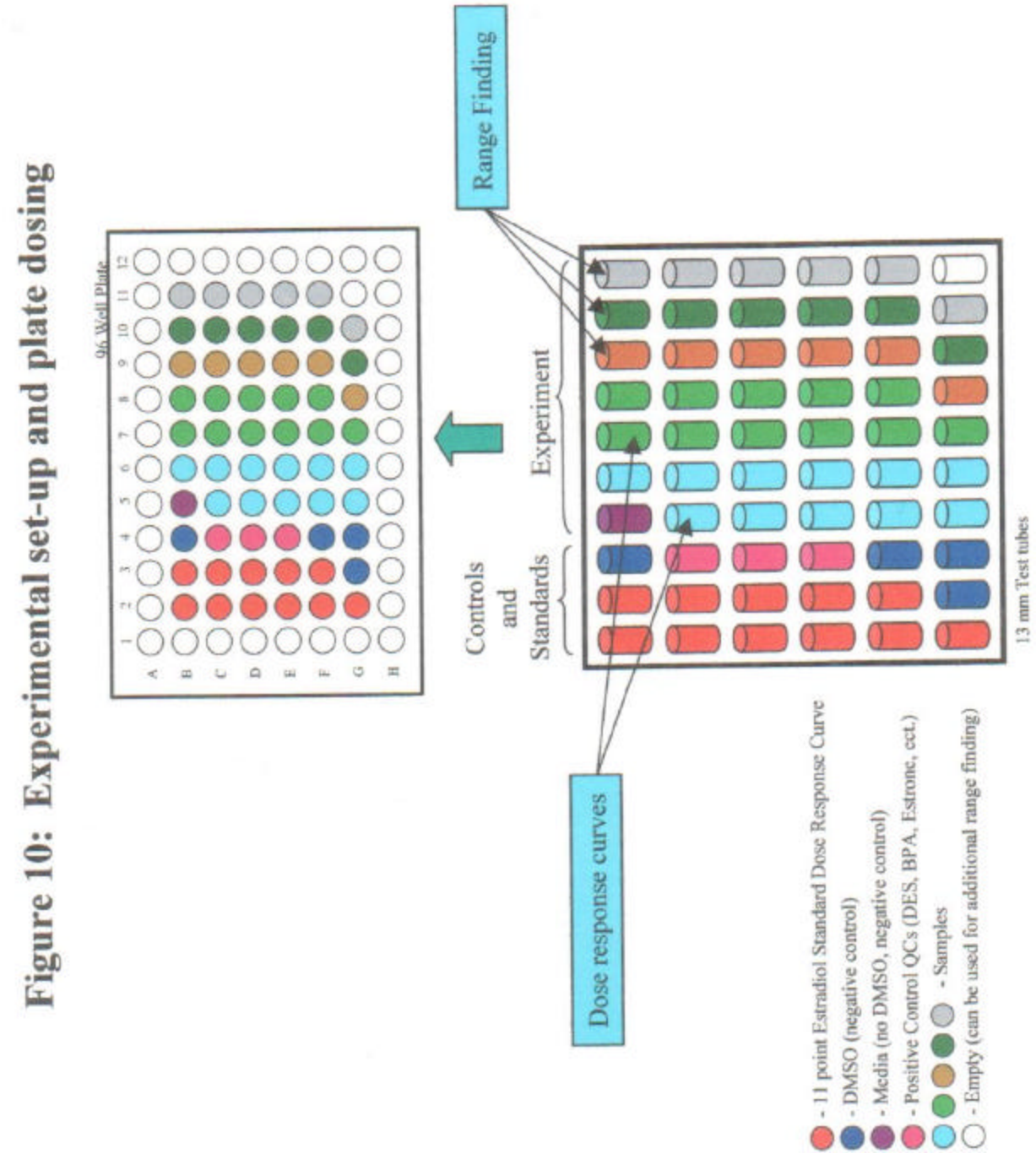


Figure 10: This figure depicts the experimental set-up in 13 mm test tubes, showing both dose response curve and range finding set-ups along with all of the standards. As well as how it is dosed into a 96 well plate.

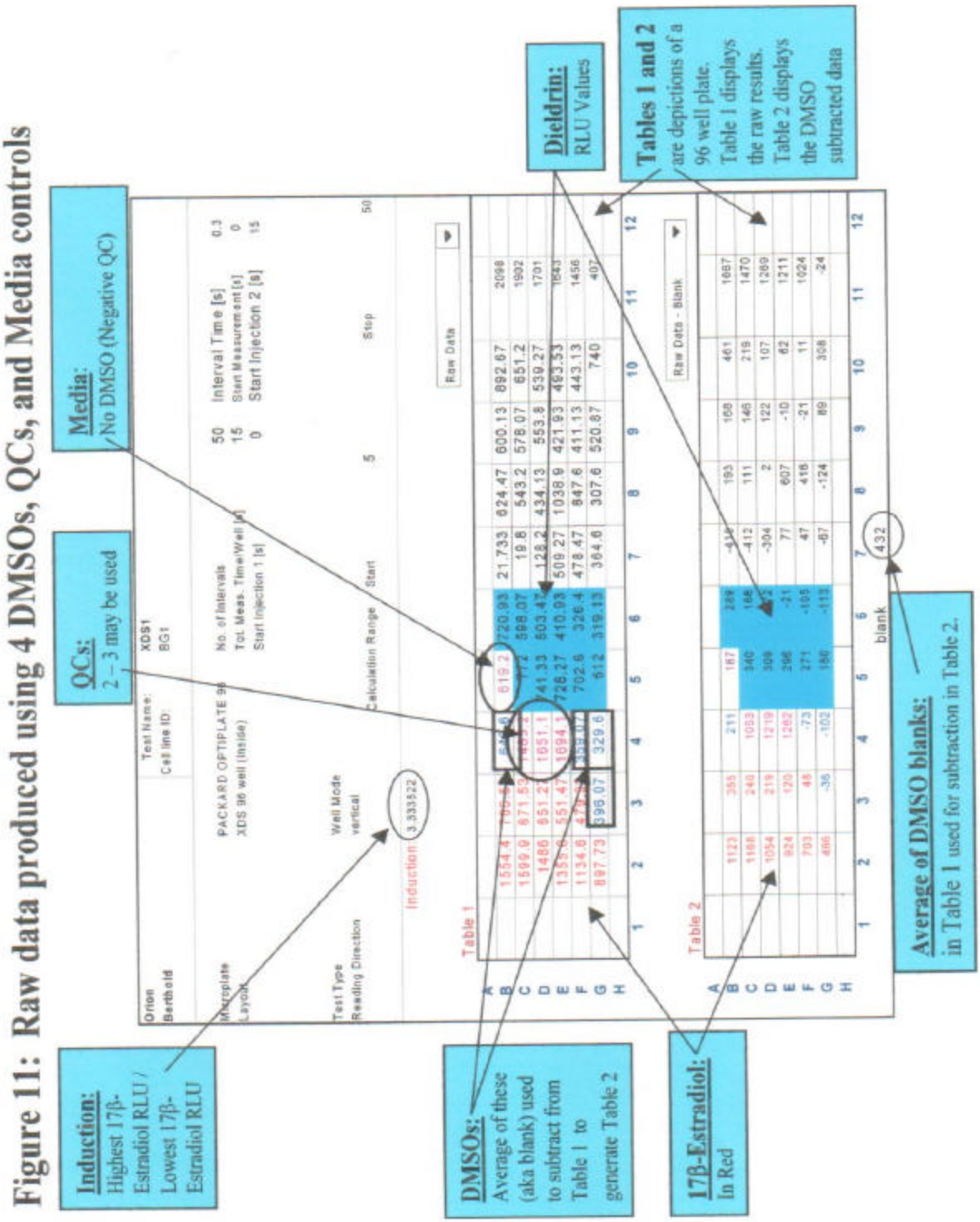


Figure 11: An actual raw data readout for Dieldren using 4 DMSOs. It shows the raw RLU data as it came off of the Luminometer in Table 1 and the background (DMSO) subtracted data in Table 2. The DMSO average is below Table 2. It also shows the induction of the plate just above Table 1. This is the same experimental data produced from the set-ups in Figures 9 and 10. ("Raw Data" tab on Excel spreadsheet).

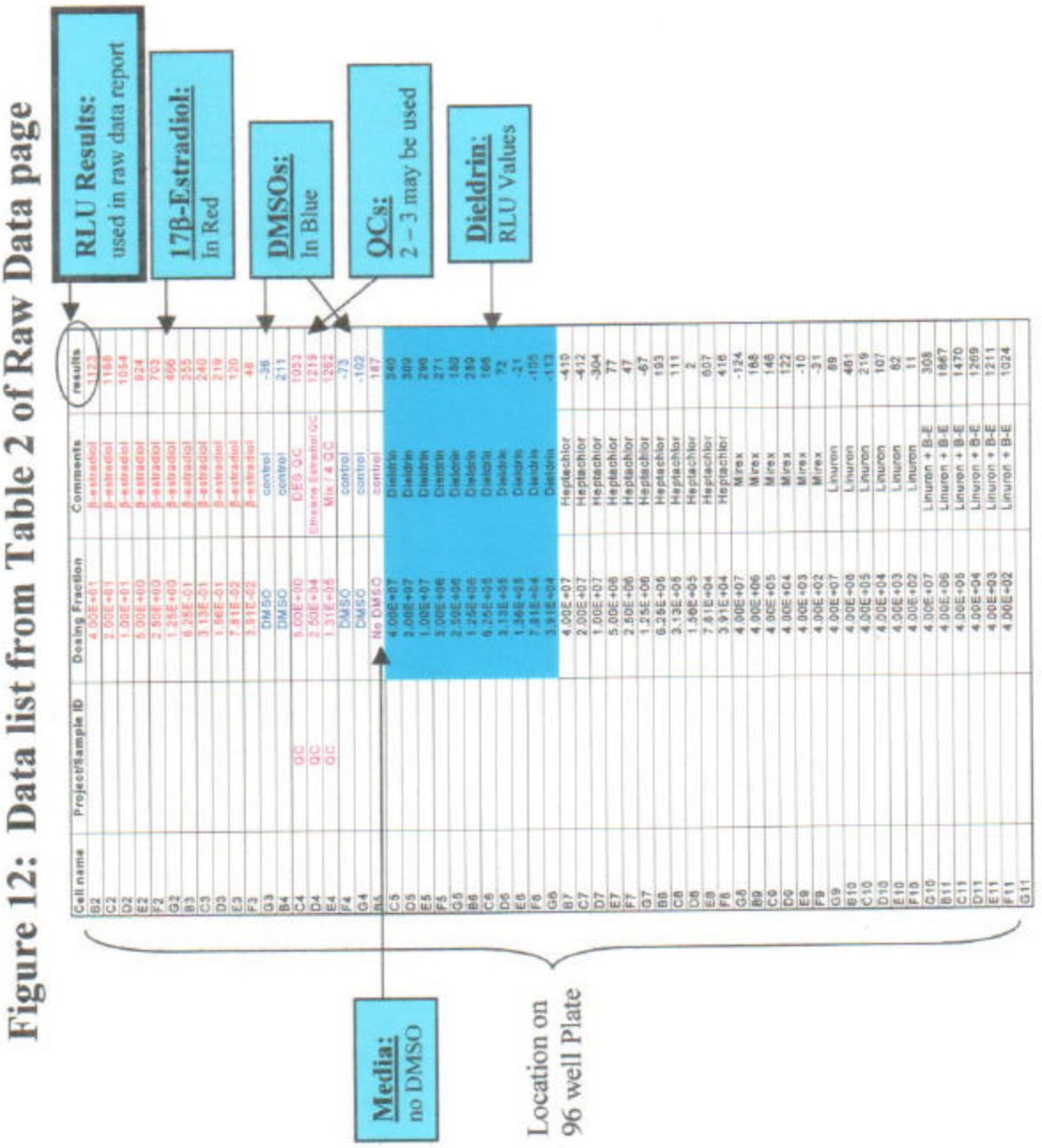


Figure 12: This is the same project production set-up form seen in Figure 9, except it now has the background subtracted RLU data in the far right column. This data comes from Table 2 in Figure 11. ("List" tab on Excel spreadsheet).

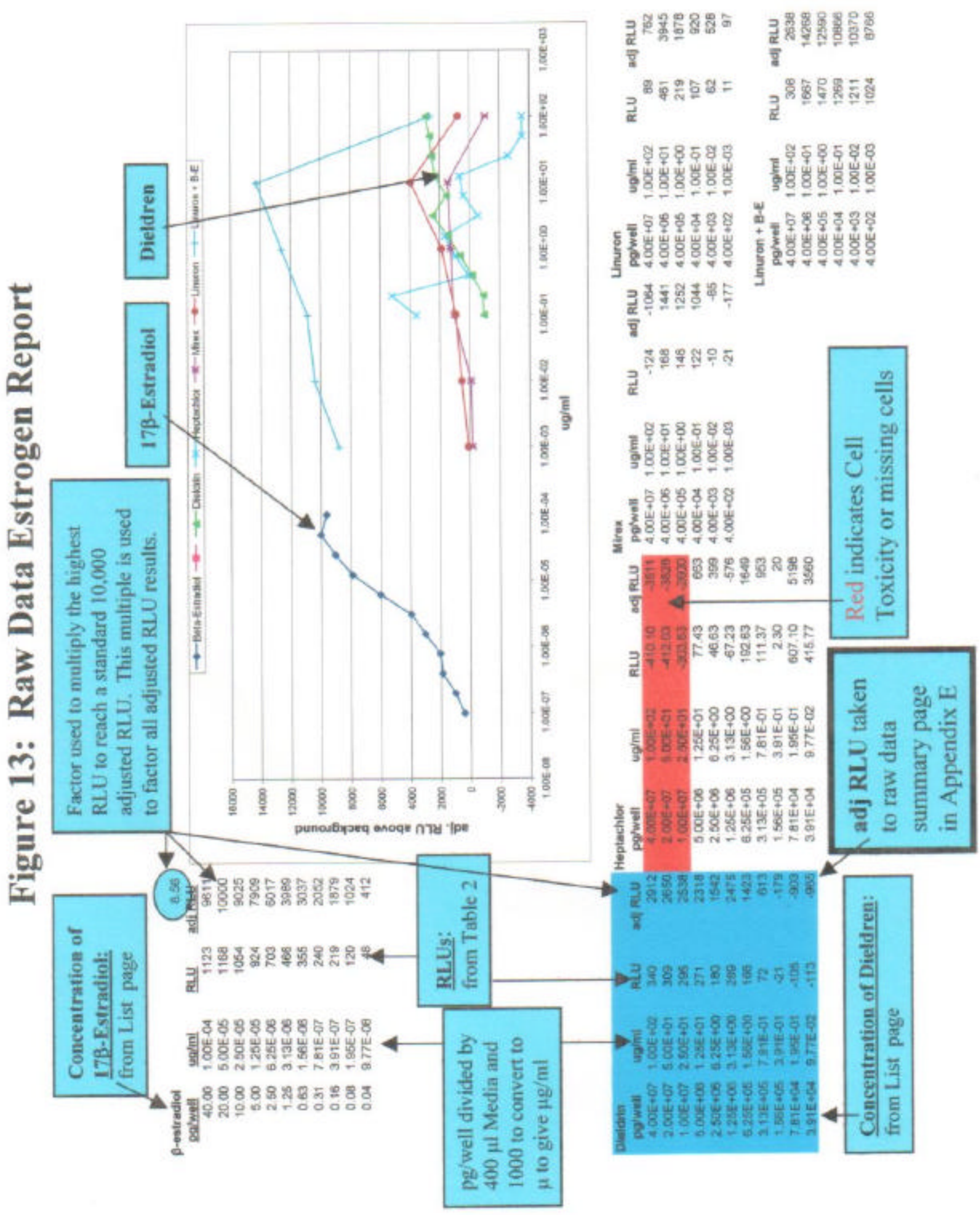


Figure 13: Depicts the Raw data report generated using the data in Figure 12. This figure depicts the data conversion from pg to µg/ml and the factor used to transform the highest point on the 17β-Estradiol curve to 10,000 RLUs. This factor is then used to factor all of the RLU data throughout the plate. ("DX Report" tab on Excel spreadsheet).

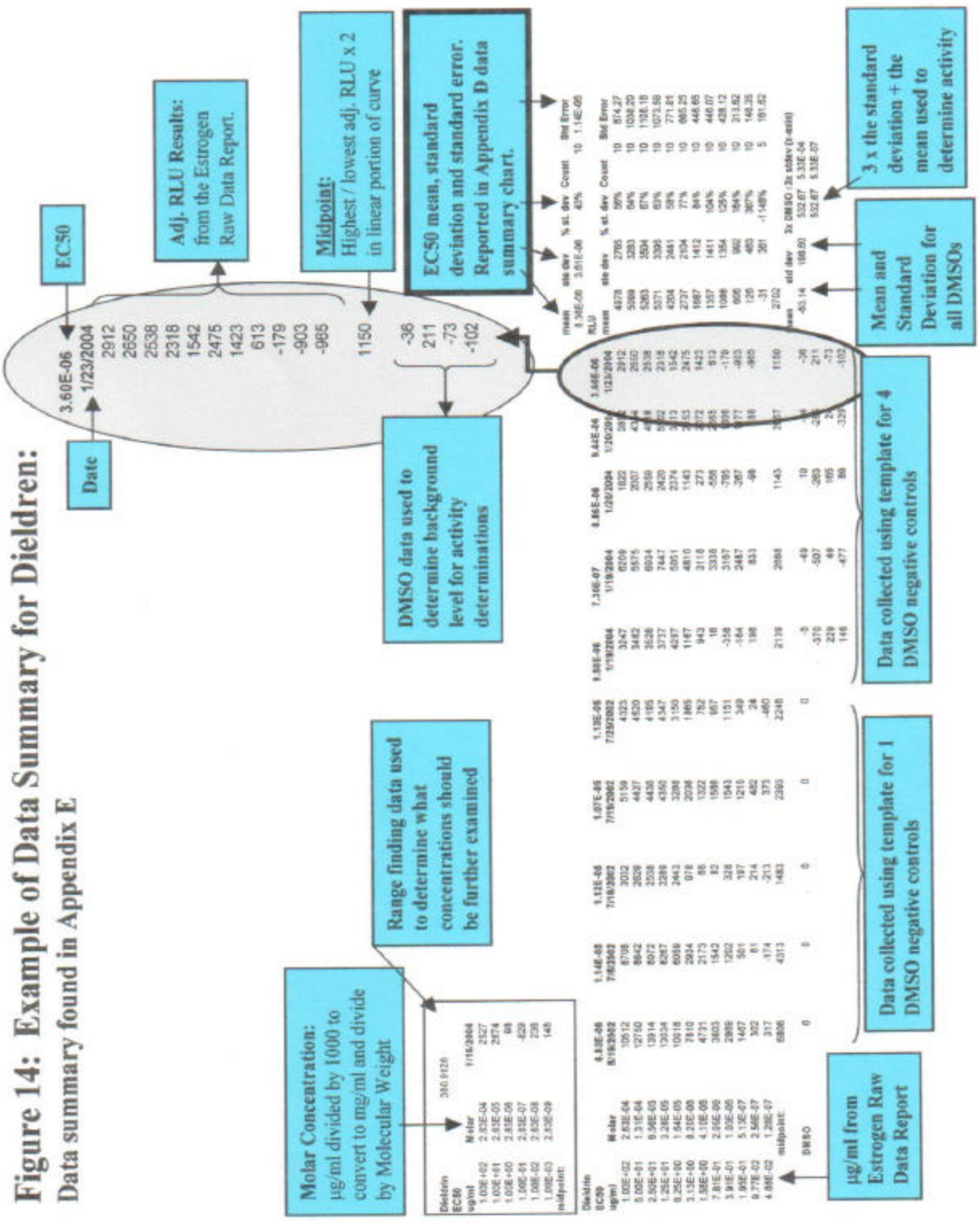


Figure 14: The raw data summary for Dieldren found in Appendix E. This figure shows range finding and dose response curves for Dieldren. It also shows experimental templates using 1 DMSO and 4 DMSOs for background. The enlarged bubble shows one data set enlarged for easier viewing. (Found in Appendix E on Excel spreadsheets).

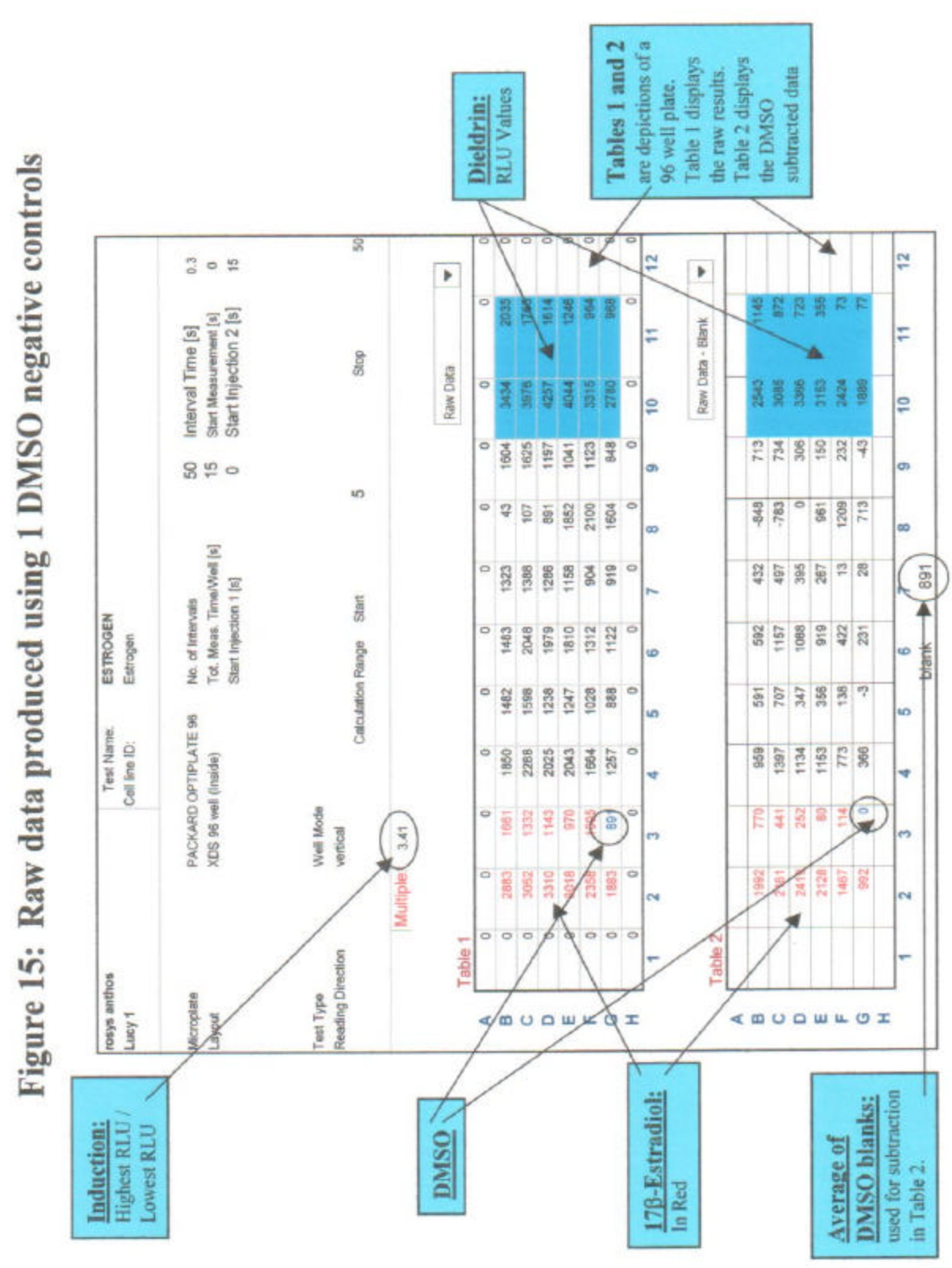


Figure 15: An actual raw data readout for Dieldren using 1 DMSO. ("Raw Data" tab on Excel spreadsheet).

Appendix B: Characterization of Substances Tested

ND - not determined

ICCVAM Recommended Compounds for Validation of ER TA Assays	CAS RN	Chemical Class	Product Class	Concentrations Tested converted from pico gram to micro gram / ml to Molar			Purity	Supplier or Source of Substances	Physical and Chemical
ER POSITIVE:				pg	mg/ml	Molar			
Apigenin	520-36-5	Flavanoid; Flavone; Phenol	Natural Product	2.00E+07 - 1.95E+04	5.00E+01 - 4.88E-02	1.85E-04 - 1.81E-07	> 95%	Sigma	Powder
Bisphenol A	80-05-7	Diphenylalkane; Bisphenol; Phenol	Chemical Intermediate	4.00E+06 - 4.77E-01	1.00E+01 - 1.19E-06	4.38E-05 - 5.22E-12	> 99%	Sigma	Powder
Butylbenzyl phthate	85-68-7	Phthalate	Plasticizer	4.00E+07 - 3.91E+04	1.00E+02 - 9.77E-02	3.20E-04 - 3.13E-07	98%	Sigma	liquid
Coumesterol	479-13-0	Coumestan; Ketone Benzopyranone; Coumarin	Natural Product	4.00E+06 - 1.22E+02	1.00E+01 - 3.05E-04	3.73E-05 - 1.14E-09	> 98%	Sigma	Powder
Daidzein	486-66-8	Flavanoid; Isoflavone; Phenol	Natural Product	4.00E+06 - 1.95E+03	1.00E+01 - 4.88E-03	3.93E-05 - 1.92E-08	> 98%	Sigma	Powder
Dexamethasone	50-02-2	Steroid, nonphenolic	Pharmaceutical	4.00E+07 - 3.91E+04	1.00E+02 - 9.77E-02	4.54E-04 - 4.43E-07	> 98%	Sigma	Powder
p,p' -DDE	72-55-9	Organochlorine; Diphenylalkene	Pesticide	4.00E+07 - 7.81E+04	1.00E+02 - 1.95E-01	7.86E-05 - 1.54E-07	> 99%	Sigma	Powder
p,p'-DDT	50-29-3	Organochlorine; Diphenylalkene	Pesticide	8.00E+06 - 3.91E+03	2.00E+01 - 9.77E-03	2.82E-04 - 1.38E-07	98%	Sigma	Powder
Dibenzo(a,h)anthacene	53-70-3	Polycyclic aromatichydrocarbon; Anthracene	Carcinogenic	4.00E+06 - 1.95E+03	1.00E+01 - 4.88E-03	3.59E-05 - 1.75E-08	97%	Chem Service	Powder
Di-n -butyl phthalate	84-74-2	Phthalate	Plasticizer	4.00E+07 - 3.91E+04	1.00E+02 - 9.77E-02	3.59E-05 - 1.75E-08	> 99%	Sigma	Powder
Diethylstilbestrol	56-53-1	Stilbene; Benzylidene; Diphenylalkene	Pharmaceutical	4.00E+01 - 1.96E-02	1.00E-04 - 4.89E-08	3.73E-10 - 1.82E-13	> 99%	Sigma	Powder
17α-Estradiol	57-91-0	Steroid, phenolic; Estrene	Steroid	4.00E+04 - 4.77E-03	1.00E-01 - 1.19E-08	3.67E-07 - 4.38E-14	> 99%	Sigma	Powder
17β-Estradiol	50-28-2	Steroid, phenolic; Estrene	Hormone	4.00E+01 - 3.91E-02	1.00E-04 - 9.77E-08	3.67E-10 - 3.59E-13	> 99%	Sigma	Powder
17alpha-Ethynyl Estradiol	57-63-6	Steroid, phenolic	Pharmaceutical Steroid	4.00E+01 - 1.95E-02	1.00E-04 - 4.88E-08	3.37E-10 - 1.65E-13	> 98%	Sigma	Powder
Estrone	53-16-7	Steroid, phenolic; Estrene	Pharmaceutical, Steroid	2.00E+03 - 2.44E-01	5.00E-03 - 6.10E-07	1.85E-08 - 2.26E-12	> 99%	Sigma	Powder
Ethyl paraben	120-47-8	Paraben; Organic acid	Pharmaceutical	4.00E+07 - 3.91E+04	1.00E+02 - 9.77E-02	6.02E-04 - 5.88E-07	> 99%	Sigma	Powder
Fenarimol	60168-88-9	Heterocycle; Pyrimidine	Pesticide	4.00E+07 - 3.91E+04	1.00E+02 - 9.77E-02	3.02E-04 - 2.95E-07	> 98%	Sigma	Powder

Appendix B:

Characterization of Substances Tested

ER POSITIVE (continued):	CAS RN	Chemical Class	Product Class	Concentrations Tested			Purity	Supplier or Source of Substances	Physical and Chemical
				pg	mg/ml	Molar			
Flavone	525-82-6	Flavanoid; Flavone	Natural Product	4.00E+07 - 3.91E+04	1.00E+02 - 9.77E-02	4.50E-04 - 4.39E-07	> 99%	Sigma	Powder
Genistein	446-72-0	Flavanoid; Isoflavone; Phenol	Natural Product	4.00E+06 - 1.95E+03	1.00E+01 - 4.88E-03	3.70E-05 - 1.81E-08	> 98%	Sigma	Powder
Kaempferol	520-18-3	Flavanoid; Flavone; Phenol	Natural Product	5.00E+06 - 9.77E+03	1.25E+01 - 2.44E-02	4.37E-05 - 8.53E-08	> 96%	Sigma	Powder
Kepone	143-50-0	Organochlorine; Chlorinated bridged cycloalkane	Pesticide	4.00E+05 - 1.95E+02	1.00E+00 - 4.88E-04	2.04E-05 - 9.95E-09	> 98%	Chem Service	Powder
Methoxychlor	72-43-5	Organochlorine; Chlorinated hydrocarbon	Pesticide	4.00E+07 - 3.91E+04	1.00E+02 - 9.77E-02	2.89E-04 - 2.83E-07	> 95%	Sigma	Powder
n-Nonylphenol	104-40-5	Alkylphenol; Phenol	Chemical Intermediate	2.00E+06 - 1.95E+03	5.00E+00 - 4.88E-03	2.27E-05 - 2.22E-08	> 98%	Sigma	Powder
Norethynodrel	68-23-5	Steroid, nonphenolic; Norpregnene	Pharmaceutical	4.00E+02 - 3.91E-01	1.00E-03 - 9.77E-07	3.35E-09 - 3.27E-12	> 98%	Sigma	Powder
4-tert-Octylphenol	140-66-9	Alkylphenol; Phenol	Chemical Intermediate	4.00E+05 - 4.77E-02	1.00E+00 - 1.19E-07	4.85E-06 - 5.78E-13	97%	Sigma	Powder
Tamoxifen	10540-29-1	Triphenylethylene; Benzylidene; Stilbene	Pharmaceutical	4.00E+05 - 1.95E+02	1.00E+00 - 4.88E-04	2.69E-06 - 1.31E-09	> 99%	Sigma	Powder
2,4,5-Trichlorophenoxyacetic acid	93-76-5	Organochlorine; Chlorinated aromatic hydrocarbon	Pesticide	4.00E+07 - 7.81E+04	1.00E+02 - 1.95E-01	3.91E-04 - 7.64E-07	97%	Sigma	Powder
Zearalenone	17924-92-4	Steroid, Resorcylic acid lactone; Phenol	Chemical Intermediate, Natural Product	4.00E+03 - 3.91E+00	1.00E-02 - 9.77E-06	3.14E-08 - 3.07E-11	> 98%	Sigma	Powder
ER NEGATIVE:									
Actinomycin D	50-76-0	Phenoxazone; Lactone; Peptide	Pharmaceutical	4.00E+07 - 4.00E-03	1.00E+02 - 1.00E-08	7.97E-05 - 7.97E-15	> 98%	Sigma	Powder
Ammonium perchlorate	7790-98-9	Organic acid; Organic salt	Pharmaceutical	4.00E+07 - 3.91E+04	1.00E+02 - 9.77E-02	8.51E-04 - 8.31E-07	> 99%	Sigma	Powder
4-Androstene	63-05-8	Steroid, nonphenolic	Hormone	4.00E+07 - 3.91E+04	1.00E+02 - 9.77E-02	3.49E-04 - 3.41E-07	> 98%	Sigma	Powder
Atrazine	1912-24-9	Aromatic amine; Triazine; Arylamine	Pecticide	4.00E+07 - 4.00E+00	1.00E+02 - 1.00E-05	4.64E-04 - 4.64E-11	> 98%	Sigma	Powder
2-sec-Butylphenol	89-72-5	Phenol	Pharmaceutical	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	6.66E-04 - 6.66E-09	> 98%	Sigma	Powder
Corticosterone	50-22-6	Steroid, nonphenolic	Steroid, nonphenolic	4.00E+07 - 3.91E+04	1.00E+02 - 9.77E-02	2.89E-04 - 2.82E-07	> 98%	Sigma	Powder

Appendix B:

Characterization of Substances Tested

ER Negative (continued):	CAS RN	Chemical Class	Product Class	Concentrations Tested			Purity	Supplier or Source of Substances	Physical and Chemical
				pg	mg/ml	Molar			
Cycloheximide	66-81-9	Piperidine; Glutaramide	Pharmaceutical	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	3.55E-04 - 3.55E-09	> 95%	Sigma	Powder
Cyproterone acetate	427-51-0	Nitrile; Diphenyl ether; Organochlorine	Pharmaceutical	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	2.40E-04 - 2.40E-09	> 98%	Sigma	Powder
Diethylhexyl phthalate	117-81-7	Phthalate	Plasticizer	4.00E+07 - 3.91E+04	1.00E+02 - 9.77E-02	2.56E-04 - 2.50E-07	> 99%	Sigma	Powder
Flutamide	13311-84-7	Amide; Anilide; Nitrobenzene	Pharmaceutical	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	3.62E-04 - 3.62E-09	> 98%	Sigma	Powder
Haloperidol	52-86-8	Butyrophenone; Ketone; Piperazine	Pharmaceutical	4.00E+07 - 3.91E-01	1.00E+02 - 9.77E-07	2.66E-04 - 2.60E-12	> 98%	Sigma	Powder
4-Hydroxytamoxifen	68047-06-3	Triphenylethylene; Benzylidene; Stilbene	Pharmaceutical	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	2.58E-05 - 2.58E-10	> 98%	Sigma	Powder
Ketoconazole	65277-42-1	Imidazole; Piperazine	Pharmaceutical	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	1.88E-04 - 1.88E-09	> 98%	Sigma	Powder
Linuron	330-55-2	Urea	Pesticide	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	4.01E-04 - 4.01E-09	> 98%	Chem Service	Powder
Medroxyprogesterone Acetate	71-58-9	Steroid, nonphenolic; Polycyclic hydrocarbon	Pharmaceutical	4.00E+07 - 3.91E+04	1.00E+02 - 9.77E-02	2.59E-04 - 2.53E-07	> 97%	Sigma	Powder
Mifepristone	8471-65-3	Steroid, nonphenolic; Polycyclic hydrocarbon	Pharmaceutical	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	2.33E-04 - 2.33E-09	> 95%	Sigma	Powder
Morin	480-16-0	Flavanoid; Flavone; Phenol	Dye	4.00E+07 - 3.91E+04	1.00E+02 - 9.77E-02	3.31E-04 - 3.23E-07	> 95%	Sigma	Powder
Nilutamide	63612-50-0	Heterocycle; Imidazole	Pharmaceutical	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	3.15E-04 - 3.15E-09	> 98%	Sigma	Powder
Phenolphthlin	81-90-3	Heterocycle; Pyrimidine	Analytical Reagent	4.00E+07 - 3.91E+04	1.00E+02 - 9.77E-02	3.12E-04 - 3.05E-07	> 98%	Sigma	Powder
Pimozide	2062-78-4	Piperidine; Benzimidazole	Pharmaceutical	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	2.17E-04 - 2.17E-09	> 98%	Sigma	Powder
Procymidone	32809-16-8	Organochlorine; Cyclic imide	Pesticide	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	3.52E-04 - 3.52E-09	> 98%	Sigma	Powder
Progesterone	57-83-0	Steroid, nonphenolic; Pregnenedione	Pharmaceutical	4.00E+06 - 4.00E+02	1.00E+01 - 1.00E-03	3.18E-05 - 3.18E-09	> 99%	Sigma	Powder
Propylthiouracil	51-52-5	Pyrimidine; Uracil	Pharmaceutical	4.00E+07 - 3.91E+04	1.00E+02 - 9.77E-02	5.87E-04 - 5.74E-07	99%	Sigma	Powder
Reserpine	50-55-5	Heterocycle; Yohimban	Pharmaceutical	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	1.64E-04 - 1.64E-09	> 99%	Sigma	Powder
Spironolactone	52-01-7	Steroid, nonphenolic; Pregnene lactone	Pharmaceutical	4.00E+07 - 3.91E+04	1.00E+02 - 9.77E-02	2.40E-04 - 2.34E-07	99%	Sigma	Powder

Appendix B: Characterization of Substances Tested	CAS RN	Chemical Class	Product Class	Concentrations Tested			Purity	Supplier or Source of Substances	Physical and Chemical
ER Negative (continued):				pg	mg/ml	Molar			
12-O -Tetradecanoylphorbol-13-acetate	16561-29-8	Phorbol ester; Terpene	Pharmaceutical	4.00E+07 - 3.91E+04	1.00E+02 - 9.77E-02	1.66E-04 - 1.62E-07	99%	Sigma	Powder
L-Thyroxine	51-48-9	Aromatic amino acid	Hormone	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	1.29E-04 - 1.29E-09	99%	Sigma	Powder
Vinclozolin	50471-44-8	Organochlorine; Cyclic imide; Carbamate	Pesticide	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	3.50E-04 - 3.50E-09	> 98%	Sigma	Powder
Environmental Contaminants Not on ICCVAM List for Validation									
Arochlor 1016	12674-11-2	Organochlorine	Industrial Chemicals	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	ND	Mixture	Chem Service	Powder
Arochlor 1221	11104-28-2	Organochlorine	Industrial Chemicals	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	ND	Mixture	Chem Service	Powder
Arochlor 1232	11141-16-5	Organochlorine	Industrial Chemicals	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	ND	Mixture	Chem Service	Powder
Arochlor 1242	53469-21-9	Organochlorine	Industrial Chemicals	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	ND	Mixture	Chem Service	Powder
Arochlor 1248	12672-29-6	Organochlorine	Industrial Chemicals	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	ND	Mixture	Chem Service	Powder
Benzo(a)anthracene	56-55-3	Natural Product	carcinogenic	8.00E+06 - 3.91E+03	2.00E+01 - 9.77E-03	8.76E-05 - 4.28E-08	99%	Sigma	Powder
Benzo(a)pyrene	50-32-8	Natural Product	carcinogenic	8.00E+06 - 3.91E+03	2.00E+01 - 9.77E-03	7.93E-05 - 3.87E-08	> 97%	Sigma	Powder
Benzo(k)fluoranthene	207-08-9	Natural Product	carcinogenic	4.00E+06 - 1.95E+03	1.00E+01 - 4.88E-03	3.96E-05 - 1.94E-08	> 95%	Chem Service	Powder
Biochanin A	491-80-5	environmental contaminant	environmental contaminant	4.00E+06 - 1.95E+03	1.00E+01 - 4.88E-03	3.52E-05 - 1.72E-08	> 97%	Sigma	Powder
α–Chlordane	57-74-9	Organochlorine	pesticides	4.00E+05 - 1.95E+02	1.00E+00 - 4.88E-04	2.44E-05 - 1.19E-08	> 95%	Chem Service	liquid
? -Chlorodane	12789-03-6	Organochlorine	pesticides	4.00E+07 - 9.77E+03	1.00E+02 - 2.44E-02	2.44E-04 - 5.96E-08	> 95%	Chem Service	liquid
Chrysene	218-01-9	environmental contaminant	Industrial byproduct	4.00E+06 - 1.95E+03	1.00E+01 - 4.88E-03	4.38E-05 - 2.14E-08	98%	Sigma	Powder
p-Cresol	106-44-5	Natural Product	Pesticide / Herbicide	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	9.25E-05 - 9.25E-10	95%	Chem Service	Powder
p-Cymene	99-87-6	Natural Product	environmental contaminant	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	7.45E-05 - 7.45E-10	99%	Sigma	Powder
DDD	72-54-8	Pesticide Metabolite	environmental contaminant	8.00E+06 - 3.91E+03	2.00E+01 - 9.77E-03	5.71E-05 - 2.79E-08	97%	Sigma	Powder
Dieldrin	60-57-1	Pesticide	environmental contaminant	4.00E+07 - 1.95E+04	1.00E+02 - 4.88E-02	2.63E-04 - 1.28E-07	> 98%	Sigma	Powder

Appendix B:
Characterization of Substances Tested

	CAS RN	Chemical Class	Product Class	Concentrations Tested			Purity	Supplier or Source of Substances	Physical and Chemical
				pg	mg/ml	Molar			
α–Endosulfan	959-98-8	Pesticide	environmental contaminant	4.00E+07 - 1.95E+04	1.00E+02 - 4.88E-02	2.46E-04 - 1.20E-07	> 98%	Sigma	Powder
β–Endosulfan	33213-65-9	Pesticide	environmental contaminant	4.00E+07 - 1.95E+04	1.00E+02 - 4.88E-02	2.46E-04 - 1.20E-07	> 98%	Sigma	Powder
Endrin	72-20-8	Pesticide	environmental contaminant	4.00E+07 - 1.95E+04	1.00E+02 - 4.88E-02	2.63E-04 - 1.28E-07	> 95%	Chem Service	liquid
Fluorene	86-73-7	Natural Product	environmental contaminant	4.00E+07 - 1.95E+04	1.00E+02 - 4.88E-02	6.02E-04 - 2.94E-07	> 99%	Sigma	liquid
Isodrin	465-73-6	environmental contaminant	environmental contaminant	4.00E+07 - 1.95E+04	1.00E+02 - 4.88E-02	2.74E-04 - 1.34E-07	> 95%	Chem Service	Powder
Lindane	58-89-9	environmental contaminant	environmental contaminant	4.00E+07 - 1.95E+04	1.00E+02 - 4.88E-02	3.44E-04 - 1.68E-07	> 95%	Chem Service	Powder
Naringenin	480-41-1	environmental contaminant	environmental contaminant	2.00E+07 - 9.77E+03	5.00E+01 - 2.44E-02	1.84E-04 - 8.97E-08	> 95%	Chem Service	Powder
2-Phenylindole	948-65-2	environmental contaminant	environmental contaminant	4.00E+07 - 1.95E+04	1.00E+02 - 4.88E-02	5.17E-04 - 2.53E-07	> 98%	Sigma	Powder
4-Phenyl Toluene	644-08-6	environmental contaminant	environmental contaminant	4.00E+07 - 1.95E+04	1.00E+02 - 4.88E-02	5.94E-04 - 2.90E-07	> 98%	Sigma	Powder
Pyrene	129-00-0	Natural Product	environmental contaminant	4.00E+06 - 1.95E+03	1.00E+01 - 4.88E-03	4.94E-05 - 2.41E-08	> 98%	Sigma	Powder
2,4,5-Trichlorophenol	95-95-4	Organochlorine	environmental contaminant	4.00E+07 - 1.95E+04	1.00E+02 - 4.88E-02	5.06E-04 - 2.47E-07	> 95%	Chem Service	Powder
α-Zearalenol	36455-72-8	Natural Product	environmental contaminant	4.00E+01 - 1.95E-02	1.00E-04 - 4.88E-08	3.10E-10 - 1.51E-13	> 98%	Sigma	Powder
Acenaphthylene	208-96-8	environmental contaminant	environmental contaminant	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	6.57E-05 - 6.57E-10	> 99%	Sigma	Powder
Aldicarb-sulfone	1646-88-4	environmental contaminant	environmental contaminant	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	4.50E-04 - 4.50E-09	> 96%	Sigma	Powder
5b-Androstane3A-OL-17-One	53-42-9	Steroid	Steroid	4.00E+06 - 4.00E+02	1.00E+01 - 1.00E-03	3.28E-05 - 3.28E-09	> 98%	Sigma	Powder
Arochlor 1254	11097-69-1	Organochlorine	Industrial Chemicals	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	ND	Mixture	Chem Service	Powder
Arochlor 1260	11096-82-5	Organochlorine	Industrial Chemicals	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	ND	Mixture	Chem Service	Powder
Carbaryl	63-25-2	environmental contaminant	environmental contaminant	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	4.97E-04 - 4.97E-09	> 96%	Sigma	Powder
Carbazole	86-74-8	environmental contaminant	intermediate in the manufacture of dyes	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	5.98E-05 - 5.98E-10	> 96%	Sigma	Powder

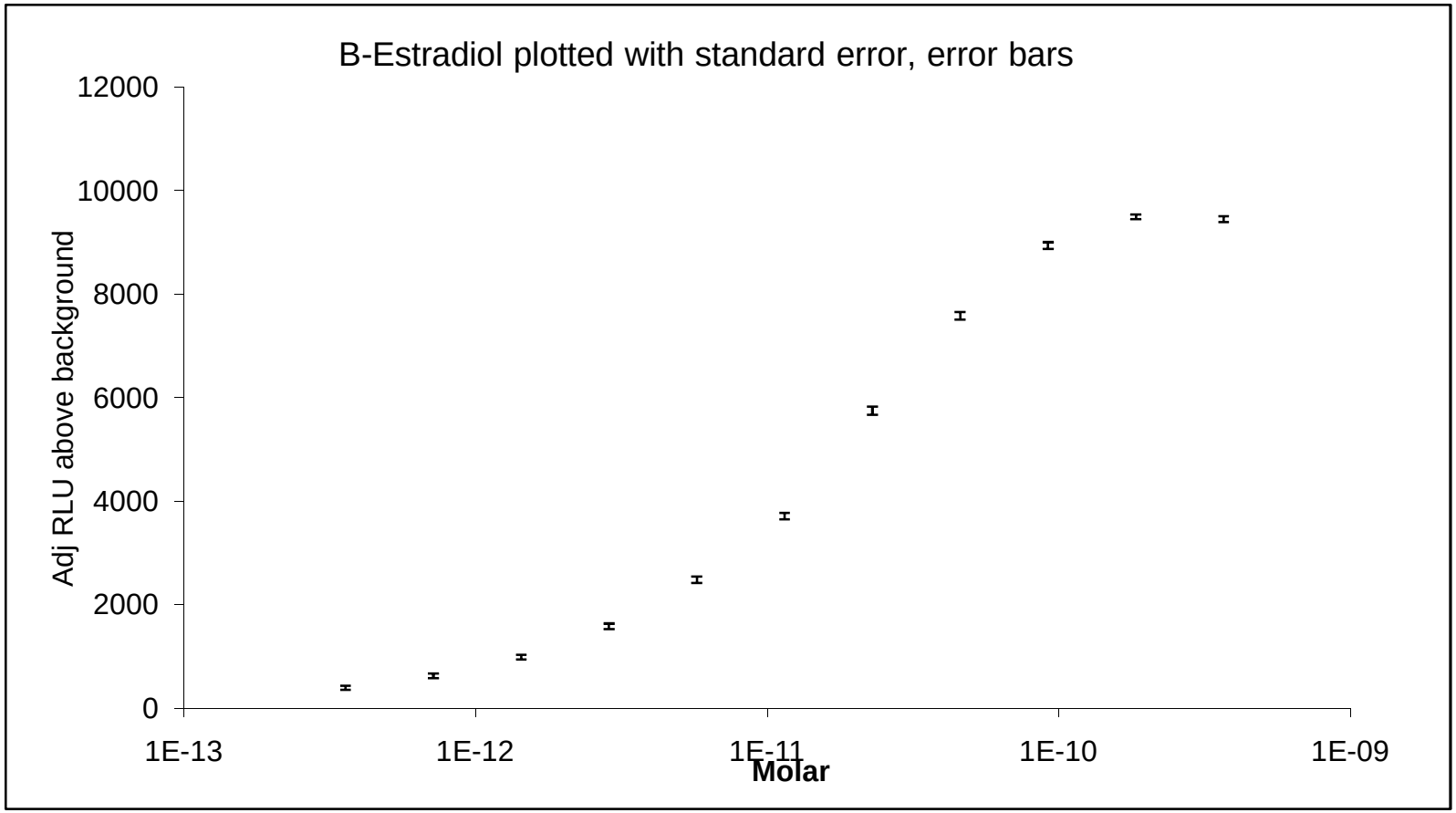
Appendix B:
Characterization of Substances Tested

	CAS RN	Chemical Class	Product Class	Concentrations Tested			Purity	Supplier or Source of Substances	Physical and Chemical
				pg	mg/ml	Molar			
Carbofuran	1563-66-2	environmental contaminant	environmental contaminant	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	4.52E-04 - 4.52E-09	> 96%	Sigma	Powder
Chlorpyrifos		environmental contaminant	environmental contaminant	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	2.85E-04 - 2.85E-09	> 96%	Sigma	Powder
Creosote	8001-58-9	Natural Product	environmental contaminant	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	ND	Mixture	Chem Service	liquid
o-Cresol	95-48-7	environmental contaminant	Pesticide / Herbicide	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	9.25E-05 - 9.25E-10	> 95%	Chem Service	Powder
Cumene	98-82-8	environmental contaminant	Solvent	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	8.32E-05 - 8.32E-10	99%	Sigma	Powder
Desethyl-Atrazin	6190-65-4	environmental contaminant	environmental contaminant	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	5.33E-04 - 5.33E-09	> 95%	Sigma	Powder
Desisopropyl-Atrazin	1007-28-9	environmental contaminant	environmental contaminant	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	5.76E-04 - 5.76E-09	> 95%	Sigma	Powder
Diazinon	333-41-5	environmental contaminant	environmental contaminant	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	3.29E-04 - 3.29E-09	> 95%	Sigma	Powder
1,4 Dioxane	123-91-1	environmental contaminant	environmental contaminant	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	1.13E-04 - 1.13E-09	> 99%	Sigma	Powder
Disulfoton	298-04-4	environmental contaminant	environmental contaminant	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	3.64E-05 - 3.64E-10	> 95%	Sigma	Powder
Endrin Aldehyde	7421-93-4	environmental contaminant	environmental contaminant	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	2.61E-05 - 2.61E-10	> 98%	Sigma	liquid
Epichlorohydrin	106-89-8	environmental contaminant	environmental contaminant	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	1.08E-04 - 1.08E-04	> 95%	Chem Service	liquid
Famphur	52-85-7	environmental contaminant	environmental contaminant	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	3.07E-05 - 3.07E-10	> 98%	Sigma	Powder
Fomesafen	72178-02-0	environmental contaminant	environmental contaminant	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	2.28E-04 - 2.28E-09	> 95%	Sigma	Powder
Heptachlor	76-44-8	Organochlorine	environmental contaminant	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	2.68E-04 - 2.68E-09	> 98%	Sigma	liquid
Hexachlobenzene	118-74-1	Organochlorine	environmental contaminant	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	3.51E-05 - 3.51E-10	99%	Sigma	Powder
2-Hydroxy Atrazin P	2163-68-0	environmental contaminant	environmental contaminant	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	5.07E-04 - 5.07E-09	99%	Protocol	liquid
Indeno(123,cd)pyrene	193-39-5	Natural Product	environmental contaminant	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	3.62E-05 - 3.62E-10	> 95%	Chem Service	liquid
Malathion	121-75-5	environmental contaminant	environmental contaminant	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	3.03E-04 - 3.03E-09	> 95%	Sigma	Powder
Methomyl	16752-77-5	environmental contaminant	environmental contaminant	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	5.20E-04 - 5.20E-09	> 95%	Sigma	Powder

Appendix B: Characterization of Substances Tested	CAS RN	Chemical Class	Product Class	Concentrations Tested			Purity	Supplier or Source of Substances	Physical and Chemical
				pg	mg/ml	Molar			
Methyl Parathion	298-00-0	environmental contaminant	environmental contaminant	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	3.80E-04 - 3.80E-09	> 95%	Sigma	Powder
2-Methylnaphthalene	91-57-6	environmental contaminant	environmental contaminant	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	7.03E-05 - 7.03E-10	> 95%	Sigma	Powder
Mirex	2385-85-5	pesticide	environmental contaminant	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	1.83E-04 - 1.83E-09	> 98%	Sigma	Powder
Napthalene	91-20-3	environmental contaminant	environmental contaminant	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	7.80E-05 - 7.80E-10	> 99%	Sigma	liquid
1,2 Naphthoquinone	524-42-5	environmental contaminant	environmental contaminant	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	6.32E-05 - 6.32E-10	97%	Sigma	Powder
Oxamyl	23135-22-0	environmental contaminant	environmental contaminant	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	4.56E-04 - 4.56E-09	> 95%	Sigma	Powder
Perylene	198-55-0	environmental contaminant	environmental contaminant	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	7.34E-05 - 7.34E-10	> 99%	Sigma	Powder
β-Pinene	127-91-3	environmental contaminant	environmental contaminant	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	3.96E-05 - 3.96E-10	> 98%	Sigma	Powder
Propoxur	114-26-1	environmental contaminant	environmental contaminant	4.00E+07 - 1.95E+04	1.00E+02 - 4.88E-02	4.78E-04 - 2.33E-07	> 95%	Sigma	Powder
Silvex	93-72-1	environmental contaminant	environmental contaminant	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	3.71E-05 - 3.71E-10	> 97%	Sigma	Powder
Taxifolin	480-18-2	environmental contaminant	environmental contaminant	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	3.29E-04 - 3.29E-09	> 97%	Sigma	Powder
Trichlorfon	52-68-6	environmental contaminant	environmental contaminant	4.00E+07 - 4.00E+02	1.00E+02 - 1.00E-03	3.88E-04 - 3.88E-09	> 95%	Sigma	Powder
p-Xylene	106-42-3	environmental contaminant	environmental contaminant	4.00E+06 - 4.00E+01	1.00E+01 - 1.00E-04	9.42E-05 - 9.42E-10	> 99%	Sigma	liquid

Appendix C - Betta Curve

Data for Appendix C is in "Appendix E - Raw Data for Plate-to-Plate Agonist Summary Data" - Betta Curve Tab



Appendix D: Agonist Plate-to-Plate Data Summary

Compounds Tested by XDS's LUMI CELL™ ER Recombinant Assay for Plate-to-Plate Variability

N/A - Not Applicable **ND** - Not Determined

P - Positive **PP**- Presumed Positive

PN - Presumed Negative **X** - No Data Available

? - Data not clear

Agonist Plate-to-Plate Data

ICCVAM Recommended Compounds for Validation of ER TA Assays	CAS RN	ICCVAM Historical Data					XDS's LUMI CELL™ ER Data for Validation of *In Vitro* ER TA Assay Plate-to-Plate Data				
		RBA Range	MCRG Studies ER Agonist	MCRG Studies ER Antagonist	MCRG Studies AR Agonist	MCRG Studies AR Antagonist	Statistically Significant Activity Above the Mean + 3 Times the Standard Dev. of the Negative Control	Cell Viability	EC 50 Molar ± Standard error	Relative Molar EC50 Induction (Relative to Estradiol EC50)	Plate to Plate Coefficient of Variation
ER POSITIVE:											
Apigenin	520-36-5	P	+	+	-	-	Active	Viable	5.30E-06 ± 1.15E-06	3.62E-06	57%
Bisphenol A	80-05-7	P	+	-	-	+	Active	Viable	7.91E-07 ± 1.71E-07	2.43E-05	37%
Butylbenzyl phthate	85-68-7	PP	+	-	-	-	Active	Viable	1.94E-06 ± 8.42E-07	9.91E-06	75%
Coumesterol	479-13-0	P	+	+	-	-	Active	Viable	4.30E-08 ± 4.01E-10	4.46E-04	2%
Daidzein	486-66-8	P	+	-	-	-	Active	Viable	2.64E-06 ± 3.59E-07	7.27E-06	24%
Dexamethasone	50-02-2	PN	+	-	+	-	Active	Viable	1.05E-05 ± 5.76E-06	1.83E-06	95%
p,p' -DDE	72-55-9	P	+	-	+	+	Active	Viable	5.26E-06 ± 2.05E-06	3.65E-06	78%
p,p'-DDT	50-29-3	P	+	+	-	+	Active	Viable	2.78E-06 ± 3.35E-07	6.91E-06	27%
Dibenzo(a,h)anthacene	53-70-3	PP	+	+	+	-	Weak - Active	Viable	ND	ND	ND
Di-n -butyl phthalate	84-74-2	PP	+	-	-	-	Active	Viable	8.29E-06 ± 1.64E-06	2.31E-06	44%
Diethylstilbestrol	56-53-1	P	+	-	-	+	Active	Viable	6.32E-11 ± 1.65E-11	3.04E-01	52%
17α-Estradiol	57-91-0	P	+	-	-	-	Active	Viable	3.31E-09 ± 8.87E-10	5.80E-03	46%
17β-Estradiol	50-28-2	P	+	-	+	+	Active	Viable	1.92E-11 ± 1.37E-12	1.00E+00	12%
17alpha-Ethynyl Estradiol	57-63-6	P	+	-	-	-	Active	Viable	1.44E-11 ± 5.81E-12	1.33E+00	81%
Estrone	53-16-7	P	+	-	+	-	Active	Viable	6.47E-10 ± 1.79E-10	2.97E-02	62%
Ethyl paraben	120-47-8	P	+	-	-	-	Active	Viable	1.26E-05 ± 7.05E-06	1.52E-06	97%
Fenarimol	60168-88-9	PP	+	-	-	+	Active	Viable	8.15E-06 ± 1.26E-06	2.36E-06	31%
Flavone	525-82-6	PN	+	+	-	-	Active	Viable	3.09E-06 ± 5.15E-07	6.22E-06	24%
Genistein	446-72-0	P	+	+	-	-	Active	Viable	5.46E-07 ± 1.06E-07	3.52E-05	27%

Appendix D: Agonist Plate-to-Plate Data	CAS RN	RAB Range	MCRG Studies ER		AR		Statistically Significant Activity Above the Mean + 3 Times the Standard Dev. of the Negative Control	Cell Viability	EC 50 mmol/ml ± Standard error	Relative EC50 Induction to Estradiol	Plate-to-Plate Coefficient of Variation
ER POSITIVE (cont.):			Agonist	Antagonist	Agonist	Antagonist					
Kaempferol	520-18-3	pp	+	-	-	-	Active	Viable	2.30E-06 ± 2.03E-07	8.33E-06	15%
Kepone	143-50-0	P	+	-	-	+	Active	Viable	2.82E-06 ± 9.89E-07	6.81E-06	61%
Methoxychlor	72-43-5	PP	+	-	-	+	Active	Viable	4.18E-06 ± 6.15E-07	4.59E-06	25%
n-Nonylphenol	104-40-5	P	+	?	-	?	Active	Viable	1.93E-07 ± 1.39E-08	9.95E-05	10%
Norethynodrel	68-23-5	P	+	-	-	-	Active	Viable	7.54E-10 ± 5.78E-11	2.54E-02	13%
4-tert-Octylphenol	140-66-9	P	+	-	-	+	Active	Viable	3.51E-07 ± 3.31E-08	5.48E-05	25%
Tamoxifen	10540-29-1	P	+	+	-	-	Non-Active	Viable	Non-Active	N/A	N/A
2,4,5-Trichlorophenoxyacetic acid	93-76-5	PN	+	-	-	-	Active	Viable	1.30E-05 ± 2.60E-06	1.48E-06	35%
Zearalenone	17924-92-4	P	+	+	-	-	Active	Viable	4.94E-10 ± 1.45E-10	3.88E-02	51%
ER NEGATIVE:											
Actinomycin D	50-76-0	PP	-	-	-	-	Non-Active	ND	Non-Active	N/A	N/A
Ammonium perchlorate	7790-98-9	PP	-	-	-	-	Non-Active	ND	Non-Active	N/A	N/A
4-Androstenedione	63-05-8	PP	-	-	+	-	Active	Viable	2.36E-05 ± 8.98E-07	8.13E-07	7%
Atrazine	1912-24-9	PP	-	-	-	-	Active ?	Viable	ND	ND	ND
2-sec-Butylphenol	89-72-5	PP	-	-	-	-	Active	Viable	5.04E-05 ± 1.33.E-05	3.81E-07	37%
Corticosterone	50-22-6	PN	-	-	-	-	Active	Viable	4.66E-06 ± 4.66E-07	4.12E-06	17%
Cycloheximide	66-81-9	PP	-	-	-	-	Non-Active	ND	Non-Active	N/A	N/A
Cyproterone acetate	427-51-0	PP	-	-	+	+	Non-Active	ND	Non-Active	N/A	N/A
Diethylhexyl phthalate	117-81-7	PN	-	-	-	-	Weak - Active	Viable	ND	ND	ND
Flutamide	13311-84-7	PP	-	-	-	+	Non-Active	ND	Non-Active	N/A	N/A
Haloperidol	52-86-8	PP	-	-	-	-	Non-Active	ND	Non-Active	N/A	N/A
4-Hydroxytamoxifen	68047-06-3	PP	+	+	-	-	Non-Active	ND	Non-Active - antagonist	N/A	N/A
Ketoconazole	65277-42-1	PP	-	-	+	-	Non-Active	ND	Non-Active	N/A	N/A
Linuron	330-55-2	PP	-	-	+	+	Weak - Active	Viable	1.26E-05 ± 5.28E-06	1.53E-06	73%
Medroxyprogesterone Acetate	71-58-9	PP	-	-	+	-	Active	Viable	7.54E-05 ± 9.86E-06	2.55E-07	29%
Mifepristone	8471-65-3	PP	-	-	+	+	Non-Active	ND	Non-Active	N/A	N/A

Appendix D: Agonist Plate-to-Plate Data	CAS RN	RBA Range	MCRG Studies ER Agonist	MCRG Studies ER Antagonist	MCRG Studies AR Agonist	MCRG Studies AR Antagonist	Statistically Significant Activity Above the Mean + 3 Times the Standard Dev. of the Negative Control	Cell Viability	EC 50 Molar ± Standard error	Relative EC50 Induction to Estradiol	Plate-to-Plate Coefficient of Variation
ER NEGATIVE (cont.):											
Morin	480-16-0	PP	-	-	-	-	Active	Viable	2.86E-05 ± 1.00E-06	6.72E-07	6%
Nilutamide	63612-50-0	PP	-	-	+	+	Non-Active	ND	Non-Active	N/A	N/A
Phenolphthlin	81-90-3	PP	-	-	-	-	Active	Viable	ND	ND	ND
Pimozide	2062-78-4	PP	-	-	-	-	Non-Active	ND	Non-Active	N/A	N/A
Procymidone	32809-16-8	PP	-	-	-	+	Non-Active	ND	Non-Active	N/A	N/A
Progesterone	57-83-0	PP	-	-	+	+	Non-Active	ND	Non-Active	N/A	N/A
Propylthiouracil	51-52-5	PP	-	-	-	-	Non-Active	ND	Non-Active	N/A	N/A
Reserpine	50-55-5	PP	-	-	-	-	Non-Active	ND	Non-Active	N/A	N/A
Spironolactone	52-01-7	PP	-	-	+	+	Weak - Active	Viable	ND	ND	ND
12-O -Tetradecanoylphorbol-13-acetate	16561-29-8	PP	-	-	-	-	Non-Active	ND	Non-Active	N/A	N/A
L-Thyroxine	51-48-9	PP	-	-	-	-	Weak - Active	Viable	ND	ND	ND
Vinclozolin	50471-44-8	PP	-	-	-	+	Non-Active	ND	Non-Active	N/A	N/A
Environmental Contaminants Not on ICCVAM List for Validation											
POSITIVE:											
Arochlor 1016	12674-11-2	X	X	X	X	X	Weak - Active	Viable	ND	ND	ND
Arochlor 1221	11104-28-2	X	X	X	X	X	Weak - Active	Viable	ND	ND	ND
Arochlor 1232	11141-16-5	X	X	X	X	X	Weak - Active	Viable	ND	ND	ND
Arochlor 1242	53469-21-9	X	X	X	X	X	Weak - Active	Viable	ND	ND	ND
Arochlor 1248	12672-29-6	X	X	X	X	X	Weak - Active	Viable	ND	ND	ND
Benzo(a)anthracene	56-55-3	X	X	X	X	X	Active	Viable	5.08E-06 ± 4.51E-07	3.78E-06	13%
Benzo(a)pyrene	50-32-8	X	X	X	X	X	Active	Viable	3.65E-06 ± 6.93E-07	5.25E-06	38%
Benzo(k)fluoranthene	207-08-9	X	X	X	X	X	Active	Viable	4.57E-06 ± 5.15E-07	4.20E-06	30%
Biochanin A	491-80-5	X	X	X	X	X	Active	Viable	7.76E-07 ± ND	2.47E-05	ND
Carbaryl	63-25-2	X	X	X	X	X	Active	Viable	2.74E-05 ± ND	7.01E-07	ND
α–Chlordane	57-74-9	X	X	X	X	X	Active	Viable	9.57E-07 ± 1.03E-07	2.00E-05	22%
?-Chlorodane	12789-03-6	X	X	X	X	X	Active	Viable	5.11E-06 ± 1.20E-06	3.75E-06	53%

Chrysene	218-01-9	X	X	X	X	X	Active	Viable	7.03E-06 ± 8.27E-07	2.73E-06	24%
Appendix D: Agonist Plate-to-Plate Data	**CAS RN**	**RBA Range**	**MCRG Studies**				**Statistically Significant Activity Above the Mean + 3 Times the Standard Dev. of the Negative Control**	**Cell Viability**	**EC 50 Molar ± Standard error**	**Relative EC50 Induction to Estradiol**	**Plate-to-Plate Coefficient of Variation**
			ER		**AR**						
			Agonist	Antagonist	Agonist	Antagonist					
POSITIVE (cont.):											
p-Cresol	106-44-5	X	X	X	X	X	Weak - Active	Viable	ND	ND	ND
DDD	72-54-8	X	X	X	X	X	Active	Viable	1.82E-06 ± 3.34E-07	1.05E-05	37%
Dieldrin	60-57-1	X	X	X	X	X	Active	Viable	8.36E-06 ± 1.14E-06	2.30E-06	43%
α–Endosulfan	959-98-8	X	X	X	X	X	Active	Viable	4.42E-06 ± 1.06E-06	4.34E-06	54%
β–Endosulfan	33213-65-9	X	X	X	X	X	Active	Viable	2.93E-06 ± 1.31E-06	6.55E-06	50%
Endrin	72-20-8	X	X	X	X	X	Active	Viable	9.12E-06 ± 1.76E-06	2.11E-06	43%
Fluorene	86-73-7	X	X	X	X	X	Active	Viable	1.13E-04 ± 1.21E-05	1.70E-07	26%
Indeno(123,cd)pyrene	193-39-5	X	X	X	X	X	Weak - Active	Viable	ND	ND	ND
Isodrin	465-73-6	X	X	X	X	X	Active	Viable	3.96E-05 ± 1.65E-05	4.85E-07	83%
Lindane	58-89-9	X	X	X	X	X	Active	Viable	2.17E-05 ± 7.25E-06	8.82E-07	35%
Naringenin	480-41-1	X	X	X	X	X	Active	Viable	5.34E-06 ± 5.03E-07	3.60E-06	13%
2-Phenylindole	948-65-2	X	X	X	X	X	Active	Viable	1.25E-06 ± 3.69E-08	1.53E-05	6%
4-Phenyl Toluene	644-08-6	X	X	X	X	X	Active	Viable	4.78E-05 ± 2.25E-06	4.02E-07	11%
Pyrene	129-00-0	X	X	X	X	X	Active	Viable	ND	ND	ND
2,4,5-Trichlorophenol	95-95-4	X	X	X	X	X	Active	Viable	2.28E-05 ± 5.10E-06	8.43E-07	39%
α-Zearalenol	36455-72-8	X	X	X	X	X	Active	Viable	4.41E-11 ± 1.31E-11	4.35E-01	59%
NEGATIVE:											
Acenaphthylene	208-96-8	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Aldicarb-sulfone	1646-88-4	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
5b-Androstane3A-OL-17-One	53-42-9	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Arochlor 1254	11097-69-1	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Arochlor 1260	11096-82-5	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Carbazole	86-74-8	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Carbofuran	1563-66-2	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Chlorpyrifos	2921-88-2	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Creosote	8001-58-9	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A

Appendix D: Agonist Plate-to-Plate Data	**CAS RN**	**RBA Range**	**MCRG Studies**				**Statistically Significant Activity Above the Mean + 3 Times the Standard Dev. of the Negative Control**	**Cell Viability**	**EC 50 Molar ± Standard error**	**Relative EC50 Induction to Estradiol**	**Plate-to-Plate Coefficient of Variation**
			ER		**AR**						
NEGATIVE (cont.):			Agonist	Antagonist	Agonist	Antagonist					
o-Cresol	95-48-7	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Cumene	98-82-8	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
p-Cymene	99-87-6	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Desethyl-Atrazin	6190-65-4	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Desisopropyl-Atrazin	1007-28-9	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Diazinon	333-41-5	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
1,4 Dioxane	123-91-1	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Disulfoton	298-04-4	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Endrin Aldehyde	7421-93-4	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Epichlorohydrin	106-89-8	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Famphur	52-85-7	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Fomesafen	72178-02-0	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Heptachlor	76-44-8	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Hexachlobenzene	118-74-1	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
2-Hydroxy Atrazin	2163-68-0	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Malathion	121-75-5	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Methomyl	16752-77-5	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Methyl Parathion	298-00-0	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
2-Methylnaphthalene	91-57-6	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Mirex	2385-85-5	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Napthalene	91-20-3	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
1,2 Naphthoquinone	524-42-5	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Oxamyl	23135-22-0	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Perylene	198-55-0	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
β-Pinene	127-91-3	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Propoxur	114-26-1	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Silvex	93-72-1	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A

Appendix D: Agonist Plate-to-Plate Data **NEGATIVE (cont.):**	CAS RN	RBA Range	MCRG Studies ER Agonist	MCRG Studies ER Antagonist	MCRG Studies AR Agonist	MCRG Studies AR Antagonist	Statistically Significant Activity Above the Mean + 3 Times the Standard Dev. of the Negative Control	Cell Viability	EC 50 Molar ± Standard error	Relative EC50 Induction to Estradiol	Plate-to-Plate Coefficient of Variation
Taxifolin	480-18-2	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
Trichlorfon	52-68-6	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A
p-Xylene	106-42-3	X	X	X	X	X	Non-Active	ND	Non-Active	N/A	N/A

Compounds on ICCVAM list not completed with explinations.

Anastrazole	120511-73-1	P	-	-	-	-	Not commercially available
Apomorphine	58-00-4	PP	-	-	-	-	Controlled Substance
Bisphenol B	77-40-7	P	+	-	-	-	Not completed due to cost considerations
Bicalutamide	90357-06-5	PP	-	-	+	+	Not commercially available
CGS 18320B	112808-99-8	PP	-	-	-	-	Not commercially available
Colomiphene citrate	50-41-9	P	+	+	-	-	Not completed due to cost considerations
5a-Dihydrotestosterone	521-18-6	P	+	-	+	-	Controlled Substance
Fadrozole	102676-47-1	PP	-	-	-	-	Not commercially available
Finasteride	98319-26-7	PP	-	-	-	-	Not commercially available
Fluoranthene	206-44-0	PN	-	-	-	+	Not completed due to cost considerations
Fluoxymestrone	76-43-7	PP	-	-	+	-	Not completed due to cost considerations
meso-Hexestrol	84-16-2	P	+	-	-	-	Not completed due to cost considerations
Hydroxyflutamide	52806-53-8	PP	-	-	+	+	Not commercially available
ICI 182,780	129453-61-8	P	-	+	-	-	Not commercially available
Methyl Testosterone	58-18-4	PN	+	-	+	-	Controlled Substance
Methyltrienolone	965-93-5	PP	-	-	+	-	Not commercially available
Oxazepam	604-75-1	PP	-	-	-	-	Not completed due to cost considerations
Testosterone	58-22-0	P	-	-	+	-	Controlled Substance
17b-Trenbolone	10161-33-8	PP	-	-	+	-	Not completed due to cost considerations
Phenobarbitol	57-30-7	PP	-	-	-	-	Controlled Substance

Appendix E

Appendix E of the

Submission of XDS's LUMI-CELL™ ER High-Throughput System for Screening Estrogen-Like Chemicals for Review by ICCVAM

is not available for web viewing. Please contact NICEATM for more information.

Appendix F: Antagonist Plate-to-Plate Data Summary

Compounds Tested by XDS's LUMI CELL™ ER Recombinant Assay for Plate-to-Plate Variability

Antagonist Plate-to-Plate Data

N/A - Not Applicable **ND** - Not Determined

P - Positive **PP**- Presumed Positive

PN - Presumed Negative **X** - No Data Available

ICCVAM Recommended Compounds for Validation of ER TA Assays	CAS RN	ICCVAM Historical Data					XDS's LUMI CELL™ ER Data for Validation of *In Vitro* ER TA Assay Antagonist Plate-to-Plate Data			
		RBA Range	MCRG Studies ER Agonist	MCRG Studies ER Antagonist	MCRG Studies AR Agonist	MCRG Studies AR Antagonist	Statistically Significant Activity Below the Mean + 3 Times the Standard Dev. of 10 pg B-Estradiol	IC 50 mmol/ml ± Standard error	Relative Molar EC50 Induction (Relative to Tamoxifen IC50)	Plate-to-Plate Coefficient of Variation
ER POSITIVE:										
Apigenin	520-36-5	P	+	+	-	-	Active	6.42E-05 ± 1.29E-05	7.33E-03	45%
Bisphenol A	80-05-7	P	+	-	-	+	Active	1.15E-04 ± 2.39E-05	4.08E-03	36%
Coumesterol	479-13-0	P	+	+	-	-	Non - Active	N/A	N/A	N/A
Daidzein	486-66-8	P	+	-	-	-	Weak - Active	ND	ND	ND
p,p'-DDT	50-29-3	P	+	+	-	+	Active	9.42E-05 ± 1.09E-06	4.99E-03	2%
Dibenzo(a,h)anthacene	53-70-3	PP	+	+	+	-	Active	3.60E-08 ± 8.82E-09	1.31E+01	49%
Diethylstilbestrol	56-53-1	P	+	-	-	+	Active	2.51E-05 ± 6.19E-06	1.88E-02	49%
17alpha-Ethynyl Estradiol	57-63-6	P	+	-	-	-	Active	1.05E-04 ± 1.59E-05	4.47E-03	26%
Flavone	525-82-6	PN	+	+	-	-	Active	ND	ND	ND
Genistein	446-72-0	P	+	+	-	-	Active	ND	ND	ND
Tamoxifen	10540-29-1	P	+	+	-	-	Active	4.70E-07 ± 3.18E-08	1.00E+00	31%
Zearalenone	17924-92-4	P	+	+	-	-	Active	3.13E-05 ± ND	1.50E-02	N/A
ER NEGATIVE:										
Corticosterone	50-22-6	PN	-	-	-	-	Weak - Active	ND	ND	ND
Medroxyprogesterone Acetate	71-58-9	PP	-	-	+	-	Non - Active	N/A	N/A	N/A
Spironolactone	52-01-7	PP	-	-	+	+	Active	ND	ND	ND
Vinclozolin	50471-44-8	PP	-	-	-	+	Active	ND	ND	ND

Appendix G

Appendix G of the

Submission of XDS's LUMI-CELL™ ER High-Throughput System for Screening Estrogen-Like Chemicals for Review by ICCVAM

is not available for web viewing. Please contact NICEATM for more information.

Appendix H: Agonist Well-to-Well Data Summary

Compounds Tested by XDS's LUMI CELL™ ER Recombinant Assay for Well-to-Well Variability

Agonist Well-to-Well Data

N/A - Not Applicable **ND** - Not Determined

P - Positive **PP**- Presumed Positive

PN - Presumed Negative **X** - No Data Available

ICCVAM Recommended Compounds for Validation of ER TA Assays	CAS RN	ICCVAM Historical Data					XDS's LUMI CELL™ ER Data for Validation of *In Vitro* ER TA Assay Well-to-Well Data			
			MCRG Studies							
			ER		AR					
ER POSITIVE:		RBA Range	Agonist	Antagonist	Agonist	Antagonist	Statistically Significant Activity Above the Mean + 3 Times the Standard Dev. of the Negative Control	EC 50 mmol/ml ± Standard error	Relative Molar EC50 Induction (Relative to Estradiol EC50)	Well-to-Well Coefficient of Variation
Apigenin	520-36-5	P	+	+	-	-	Active	1.30E-05 ± 4.63E-06	2.58E-06	62%
Bisphenol A	80-05-7	P	+	-	-	+	Active	ND	ND	ND
Coumesterol	479-13-0	P	+	+	-	-	Active	3.74E-07 ± 1.31E-07	8.99E-05	86%
Daidzein	486-66-8	P	+	-	-	-	Active	ND	ND	ND
17β-Estradiol	50-28-2	P	+	-	+	+	Active	3.36E-11 ± 2.64E-12	1.00E+00	16%
17alpha-Ethynyl Estradiol	57-63-6	P	+	-	-	-	Active	3.11E-11 ± 3.49E-12	1.08E+00	19%
Estrone	53-16-7	P	+	-	+	-	Active	6.38E-10 ± 1.40E-10	5.26E-02	38%
Ethyl paraben	120-47-8	P	+	-	-	-	Active	4.09E-05 ± 1.84E-06	8.21E-07	8%
Genistein	446-72-0	P	+	+	-	-	Active	ND	ND	ND
Kaempferol	520-18-3	P	+	-	-	-	Active	4.68E-06 ± 1.01E-07	7.17E-06	5%
Kepone	143-50-0	P	+	-	-	+	Active	6.91E-06 ± 5.47E-07	4.86E-06	14%
Norethynodrel	68-23-5	P	+	-	-	-	Active	ND	ND	ND

Appendix I

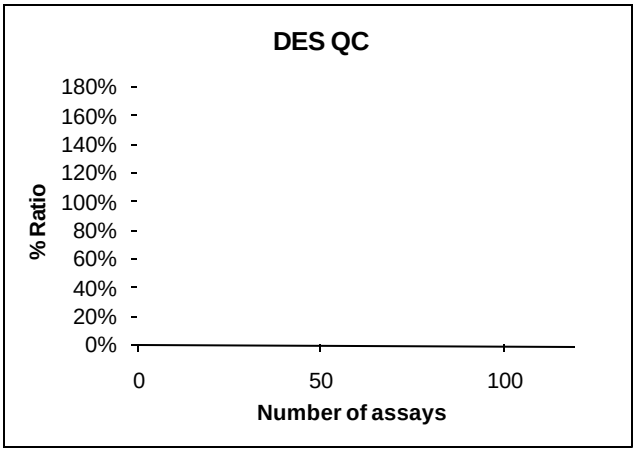
Appendix I of the

Submission of XDS's LUMI-CELL™ ER High-Throughput System for Screening Estrogen-Like Chemicals for Review by ICCVAM

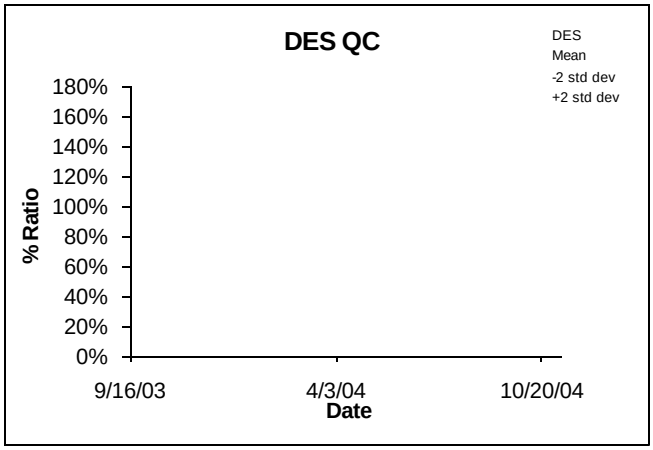
is not available for web viewing. Please contact NICEATM for more information.

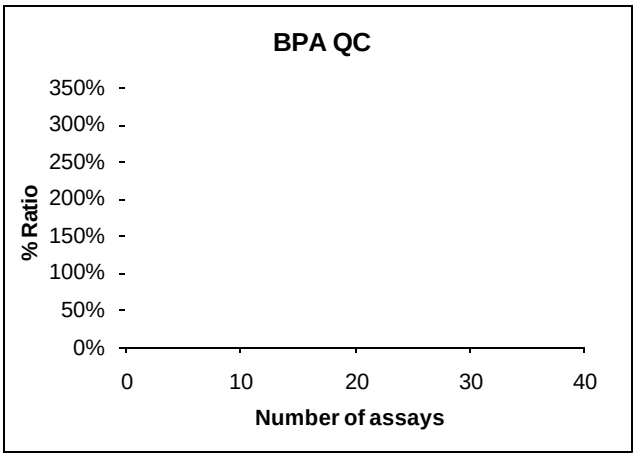
Appendix J - QC SCATTER CHARTS

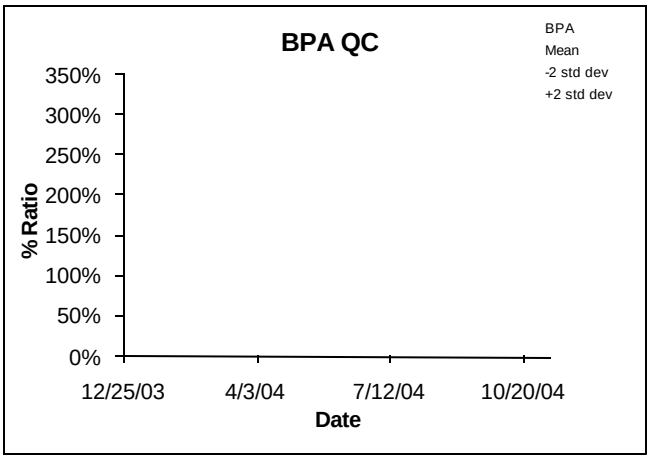
Number of Assays

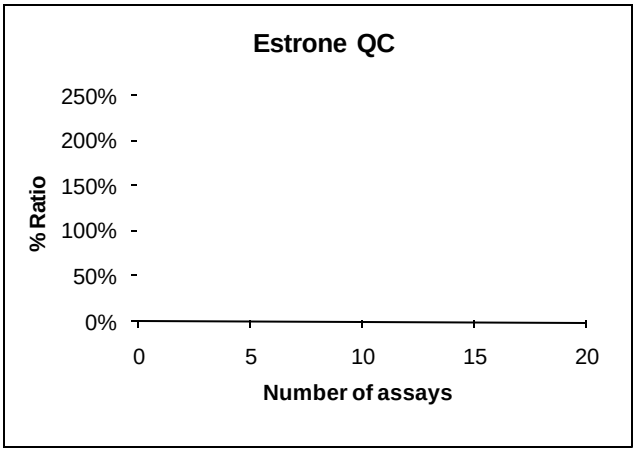


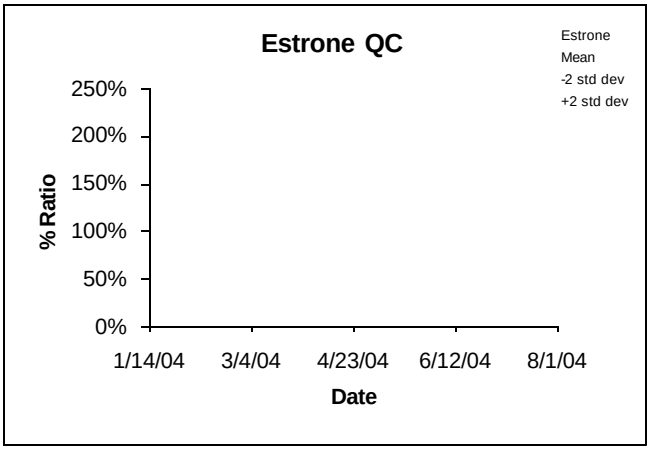
Date of Assay











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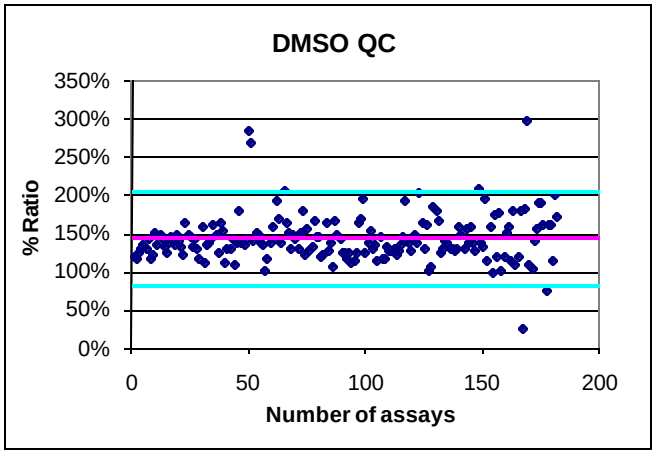

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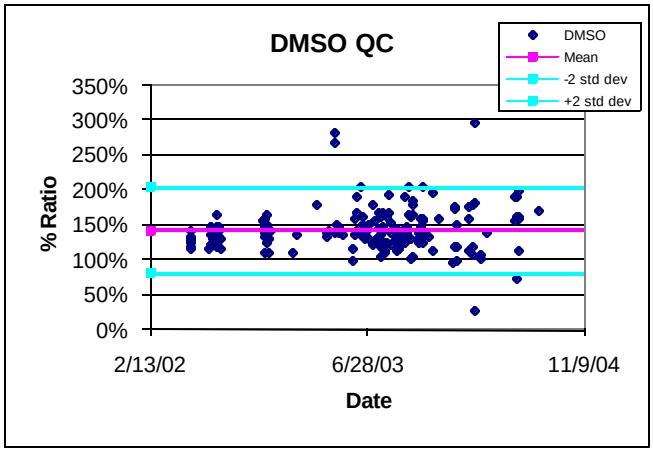

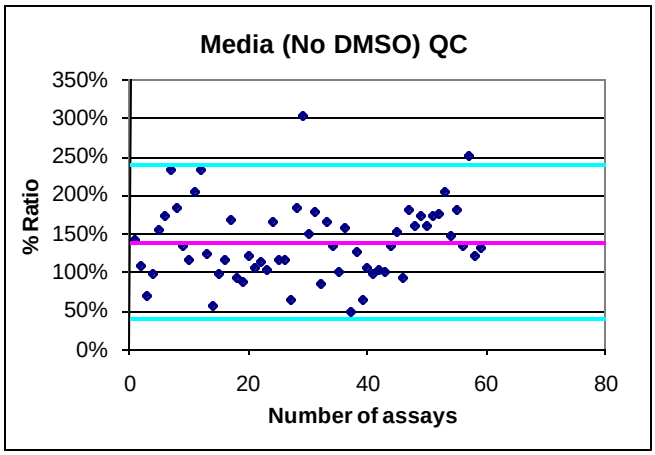

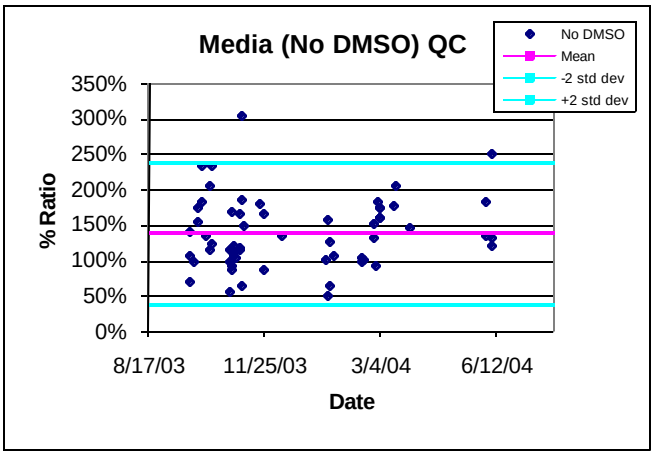

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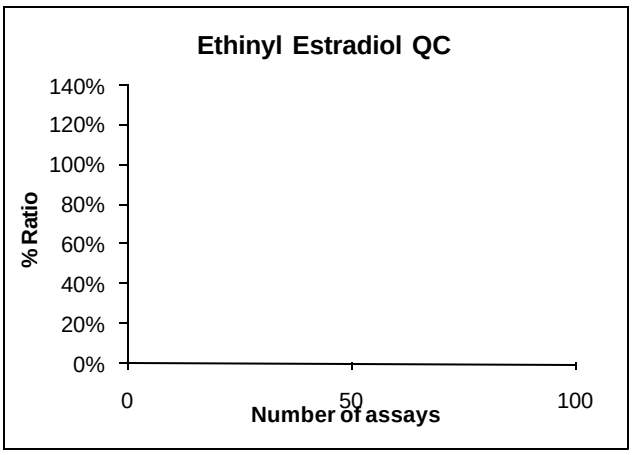

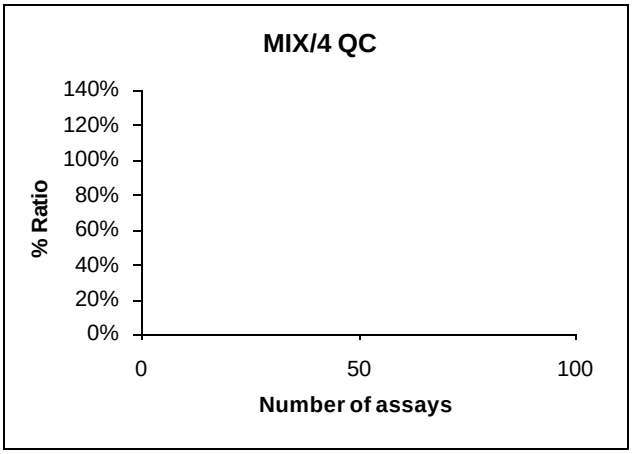

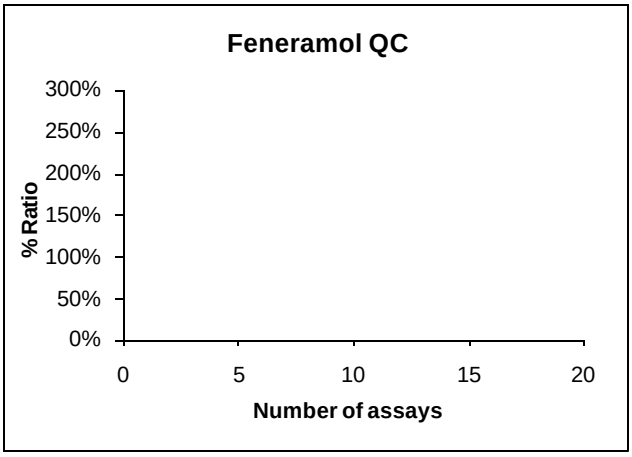

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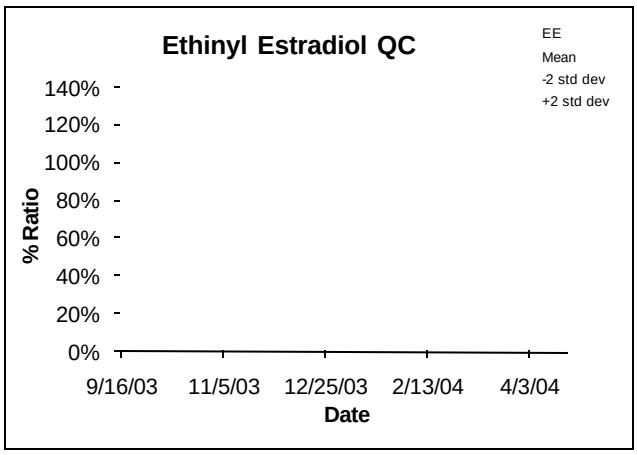

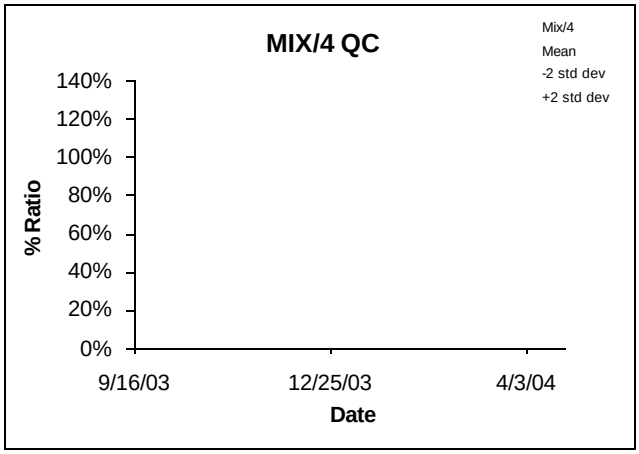

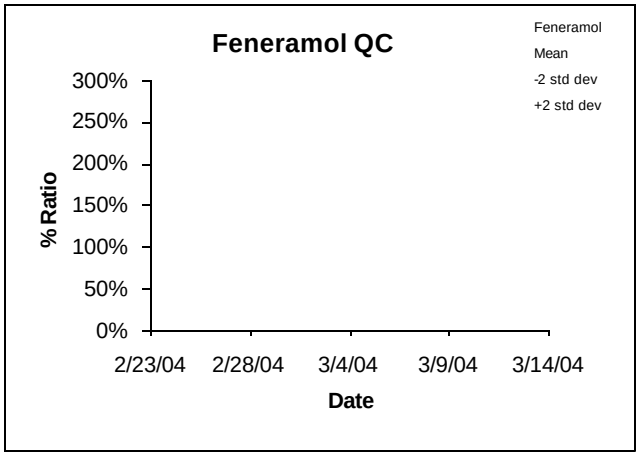

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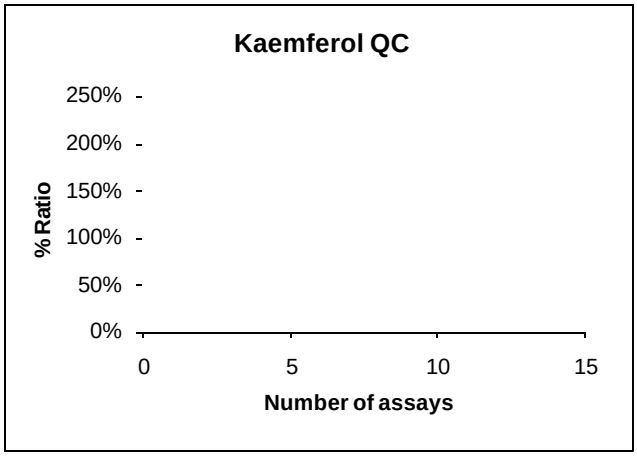

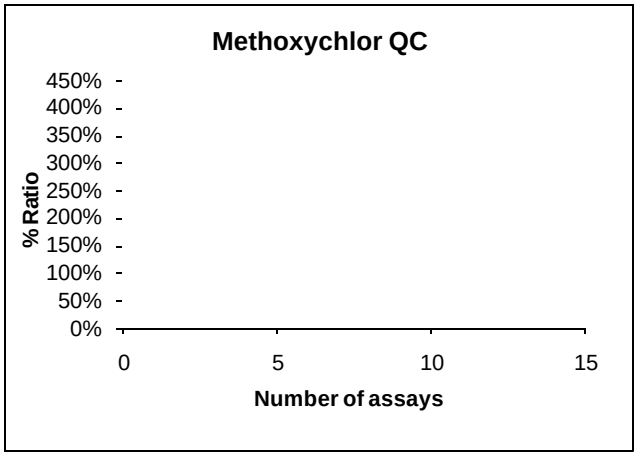

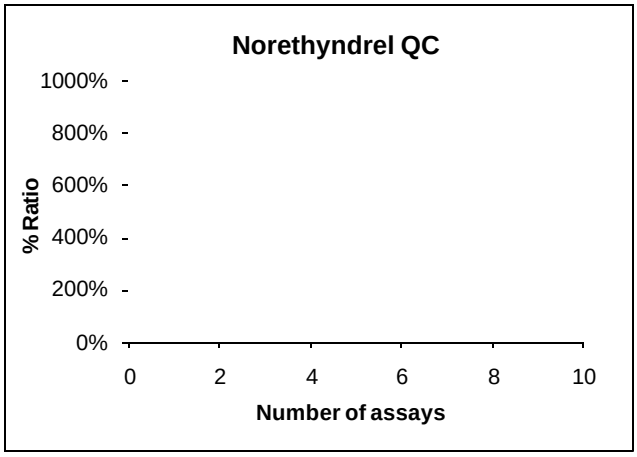

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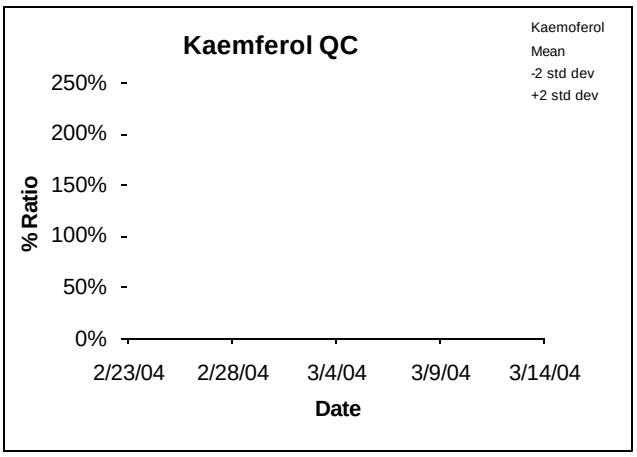

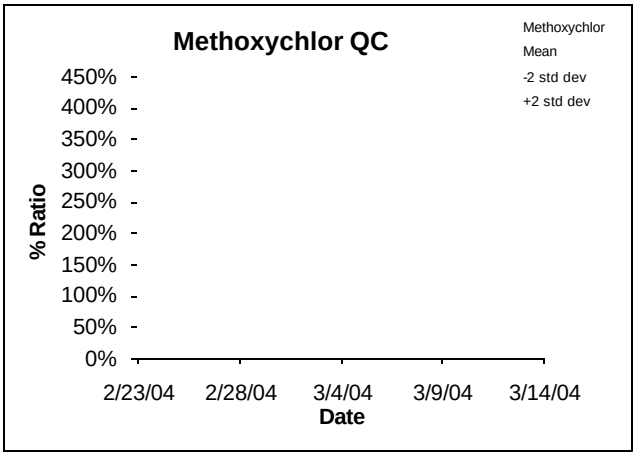

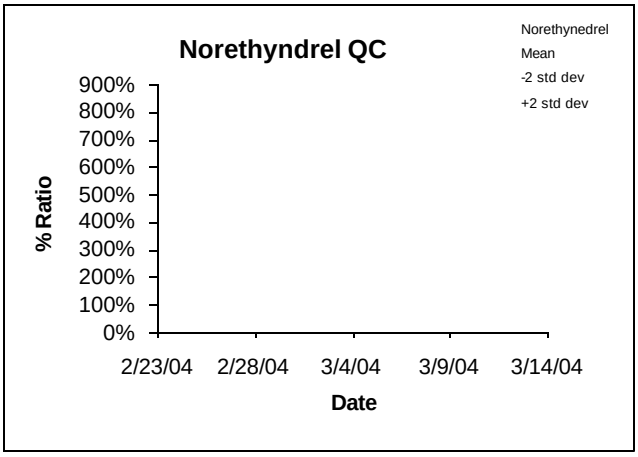

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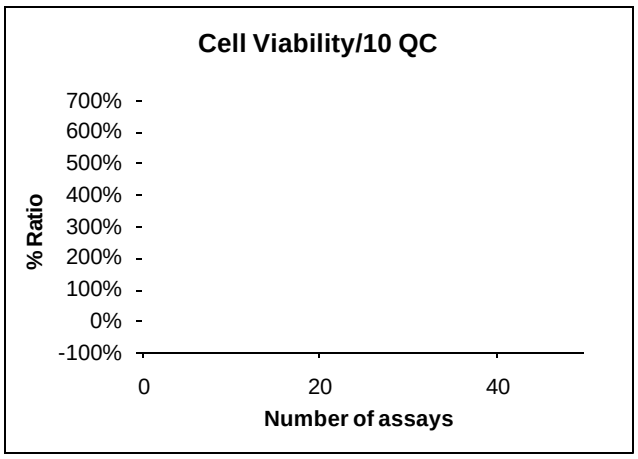

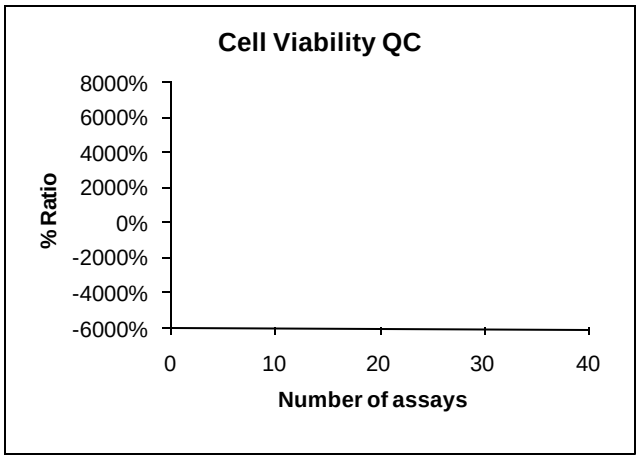

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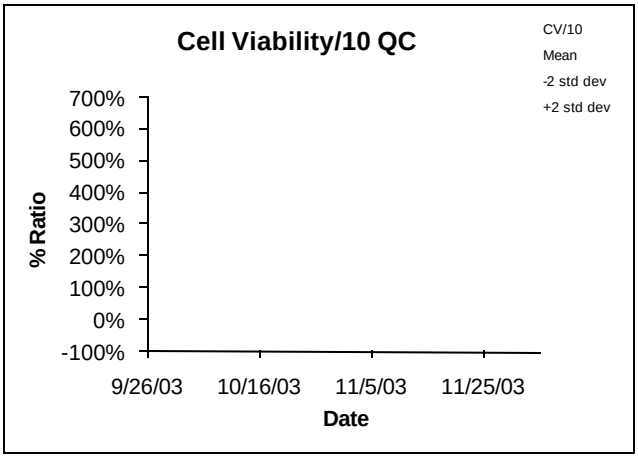

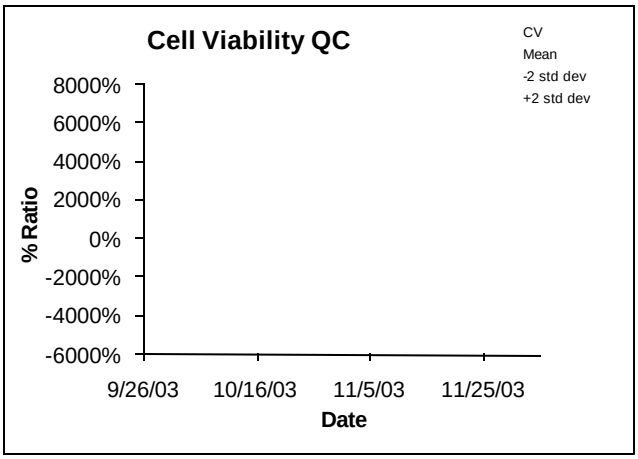

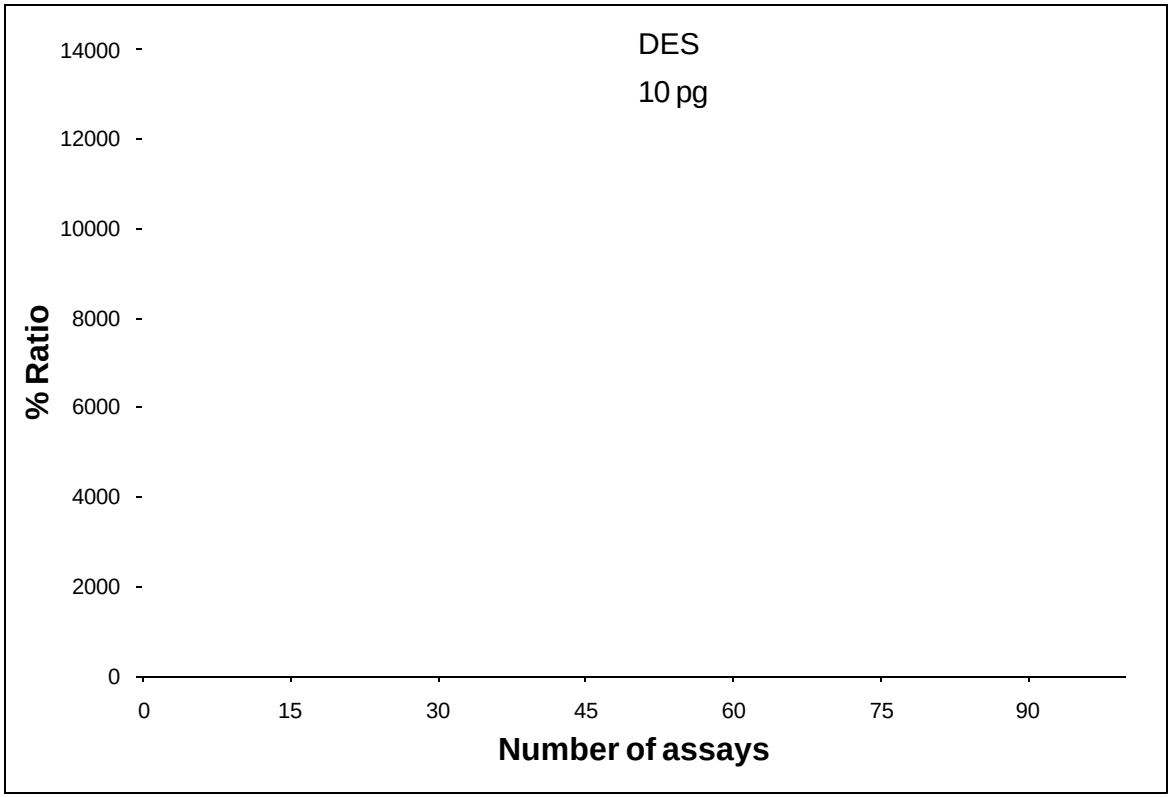
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