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**Annex C**

**THE XDS BG1LUC ER TA PROTOCOL STANDARDIZATION STUDY**

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**REPORT**  
**THE XDS BG1LUC ER TA PROTOCOL STANDARDIZATION STUDY**  
**AGONIST AND ANTAGONIST PROTOCOLS**

**National Toxicology Program (NTP) Interagency Center for the  
Evaluation of Alternative Toxicological Methods (NICEATM)**  
*06 April 2007*

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<b>LIST OF ACRONYMS AND ABBREVIATIONS</b>	
553	
554	
555	AR Androgen Receptor
556	ATP Adenosine Triphosphate
557	BBP Butylbenzyl Phthalate
558	BRD Background Review Document
559	CASRN Chemical Abstracts Service Registry Number
560	CV Coefficient of Variation
561	DBA Dibenzo[ <i>a,h</i> ]Anthracene
562	DMEM Dulbecco's Modification of Eagle's Medium
563	DMSO Dimethyl Sulfoxide
564	DMSO Control 1% v/v DMSO in Tissue Culture Medium
565	E2 17 $\beta$ -Estradiol
566	E2 control 2.5 x 10 <sup>-5</sup> $\mu$ g/mL 17 $\beta$ -Estradiol Control Used in the BG1LUC ER TA Antagonist Assay
567	E2 reference standard 10-point Serial Dilution of 17 $\beta$ -Estradiol Reference Standard for the BG1LUC ER
568	TA Agonist Assay
569	EC <sub>50</sub> Half-maximal effective concentration
570	EE 17 $\alpha$ -Ethinyl Estradiol
571	EDSP Endocrine Disruptor Screening Program
572	EDWG Endocrine Disruptor Working Group
573	EFM Dulbecco's Modification of Eagle's Medium (DMEM) Containing 4.5 g/L Glucose, with
574	Sodium Pyruvate, without Phenol, Containing 1% Penicillin/Streptomycin,
575	2% L-Glutamine, and 5% Charcoal/Dextran Treated FBS
576	EPA U.S. Environmental Protection Agency
577	ER Estrogen Receptor
578	EtOH Ethanol
579	Flavone control 25 $\mu$ g/mL Flavone with 2.5 x 10 <sup>-5</sup> $\mu$ g/mL 17 $\beta$ -Estradiol; Used as the Positive Control in
580	the BG1LUC ER TA Antagonist Assay
581	FBS Fetal Bovine Serum (Charcoal/Dextran Treated)

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582	FR	Federal Register
583	G418	Gentamycin
584	GLP	Good Laboratory Practices (OECD 1998)
585	IC <sub>50</sub>	Concentration of the test substance that inhibits the reference estrogen response by 50%
586	ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
587	MEM	Minimum Essential Medium
588	Methoxychlor	<i>p,p'</i> -Methoxychlor
589	Methoxychlor control	3.13 µg/mL Methoxychlor Positive Control for the BG1LUC ER TA Agonist Assay
590	MMTV	Mouse Mammary Tumor Virus
591	NICEATM	National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative
592		Toxicological Methods
593	Nonylphenol	<i>p</i> -n-nonylphenol
594	NTP	National Toxicology Program
595	NTPSI	National Toxicology Program Substances Inventory
596	OECD	Organisation for Economic Co-operation and Development
597	<i>o,p'</i> -DDT	1,1,1-Trichloro-2-( <i>o</i> -chlorophenyl)-2-( <i>p</i> -chlorophenyl)ethane
598	QC	Quality Control
599	Raloxifene	Raloxifene HCl
600	Ral/E2 reference standard	Nine-point Serial Dilution of Raloxifene with a Fixed Concentration of 2.5 x
601		10 <sup>-5</sup> µg/mL 17β-Estradiol Reference Standard for the BG1LUC ER TA Antagonist Assay
602	RLU	Relative Light Units
603	Std Dev	Standard Deviation
604	SOP	Standard Operating Procedure
605	TA	Transcriptional Activation
606	XDS	Xenobiotic Detection Systems
607		

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607 **PREFACE**

608  
609 In April of 2000, the U.S. Environmental Protection Agency (EPA) asked the Interagency Coordinating  
610 Committee on the Validation of Alternative Methods (ICCVAM) to evaluate the validation status of *in*  
611 *vitro* estrogen receptor (ER) and androgen receptor (AR) binding and transcriptional activation (TA) test  
612 methods, which were proposed as possible components of the EPA Endocrine Disruptor Screening  
613 Program (EDSP) (EPA 1998). Because a large number of *in vitro* ER- and AR-based test methods were  
614 known to exist, it was expected that at least some of these would have been adequately validated and  
615 could, following a review of existing data and verification of their validity, be included in the EDSP. The  
616 National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological  
617 Methods (NICEATM) subsequently compiled available data and information on the *in vitro* ER and AR  
618 binding and TA test methods. Four Background Review Documents (BRDs) were produced that provided  
619 comprehensive reviews of the available data for each of the four types of test methods (ICCVAM 2002d,  
620 2002b, 2002a, 2002c).

621  
622 On 20-21 May 2002, in collaboration with ICCVAM and the ICCVAM Endocrine Disruptor Working  
623 Group (EDWG), NICEATM organized an independent evaluation of these *in vitro* test methods for  
624 detecting substances with potential endocrine disrupting activity. This meeting was open to the public  
625 with time set aside for public comment. A 24-member scientific expert panel (Panel) reviewed the  
626 information and recommendations provided in the four draft BRDs and concluded that there were no  
627 adequately validated *in vitro* ER- or AR-based test methods. In addition, at the public meeting, the Panel  
628 provided recommendations on the following:

- 629
- 630 • Specific test methods that should undergo further evaluation in validation studies and their  
relative priority for evaluation
  - 631 • The adequacy of proposed minimum procedural standards
  - 632 • The adequacy of protocols for specific test methods recommended for validation
  - 633 • The adequacy and appropriateness of reference substances proposed for validation studies

634 In October, 2002, NICEATM published the Panel's report (ICCVAM 2002e) along with a *Federal*  
635 *Register (FR)* notice requesting public comment on this report (NIEHS 2002)

636  
637 ICCVAM considered the Panel's conclusions, recommendations, and public comments received in  
638 response to the *FR* notice. ICCVAM then developed test method recommendations that included  
639 minimum procedural standards and a list of 78 reference substances that should be used to standardize

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640 and validate *in vitro* ER and AR binding and TA test methods. In June 2003, ICCVAM issued an *FR*  
641 notice (NIEHS 2003) announcing the availability of a report defining these recommendations and  
642 minimum procedural standards entitled, “ICCVAM Evaluation of *In Vitro* Test Methods for Detecting  
643 Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional  
644 Activation Assays,” (ICCVAM 2003), as well as the final BRDs (ICCVAM 2002d, 2002b, 2002a,  
645 2002c). The *FR* notice also requested the nomination of *in vitro test* methods for use in the EDSP as part  
646 of the Tier I screening battery of *in vitro* and *in vivo* test assays that will be used to reach weight-of-  
647 evidence decisions on whether to conduct large multi-generational Tier 2 *in vivo* studies.

648  
649 In January 2004, NICEATM received a letter from Xenobiotic Detection Systems, Inc. (XDS) nominating  
650 the BG1Luc ER TA for validation. The development of the assay was supported by a Small Business  
651 Innovation Research grant from the U.S. National Institute of Environmental Health Sciences. NICEATM  
652 subsequently received a submission for the BG1Luc ER TA in April 2004 containing the historical  
653 development and rationale for the assay, assay protocols, and supporting materials. In accordance with the  
654 ICCVAM nomination process, NICEATM conducted a pre-screen evaluation of the submission to  
655 determine the extent that the proposed nomination addressed the ICCVAM prioritization criteria,  
656 submission guidelines, and recommendations for the standardization and validation of *in vitro* endocrine  
657 disruptor test methods (ICCVAM 2003). Based on the NICEATM pre-screen evaluation, ICCVAM  
658 recommended that:

- 659 • The BG1Luc ER TA should be considered as a high priority for validation studies as an *in*  
660 *vitro* test method for the detection of test substances with ER agonist and antagonist activity.
- 661 • To facilitate independent and timely standardization and validation studies, NICEATM  
662 should manage the needed studies by exercising a validation coordination option in its  
663 support contract.
- 664 • Validation studies should include coordination and collaboration with the European Centre  
665 for the Validation of Alternative Methods and the Japanese Center for the Validation of  
666 Alternative Methods to include one laboratory in each of the three respective geographic  
667 regions supported by the three Centers.
- 668 • In preparation for the interlaboratory validation study, XDS should conduct additional  
669 protocol standardization studies with an emphasis on conducting additional antagonist studies  
670 to more comprehensively demonstrate the suitability of the BG1Luc ER TA for the detection  
671 of substances with ER antagonist activity.

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672 NICEATM exercised a pre-validation coordination option in its support contract to conduct and manage a  
673 study to standardize BG1LUC ER TA protocols and to conduct additional antagonist testing. The study  
674 was initiated in October 2005 and was conducted at XDS.

675 The primary goal of the study was to develop standardized protocols for detecting ER agonists and  
676 antagonists that can be easily transferred to other laboratories and be used to obtain reproducible results.

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**EXECUTIVE SUMMARY**

692  
693  
694 The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative  
695 Toxicological Methods (NICEATM) has conducted a protocol standardization study of the BG1Luc4E2  
696 Estrogen Receptor (ER) Transcriptional Activation (TA) test method (hereafter referred to as BG1Luc ER  
697 TA) developed by Xenobiotic Detection Systems, Inc. (XDS). Protocol standardization procedures were  
698 based on recommendations made in the “Interagency Coordinating Committee on the Validation of  
699 Alternative Methods (ICCVAM) Evaluation of *In Vitro* Test Methods for Detecting Potential Endocrine  
700 Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays,”  
701 (ICCVAM 2003, 2006). The goal of the study was to develop and evaluate standardized protocols for the  
702 BG1Luc ER TA for detecting ER agonists and antagonists that can be transferred to other laboratories for  
703 use in validation studies. Reference standards, controls, and methods for assessing cell viability were  
704 selected and standardized for both BG1Luc ER TA agonist and antagonist protocols, and an historical  
705 database was established for quality control. The adequacy of the standardized agonist and antagonist  
706 protocols was evaluated using a subset of the substances recommended by ICCVAM for the development,  
707 optimization, and/or validation of ER binding and TA assays. Results from this pre-validation study were  
708 used to standardize protocols for the BG1LUC ER TA agonist and antagonist assays.

709

**710 Selection and Standardization of Reference Standards and Controls**

711 Reference standards and controls selected and standardized for the agonist assay were:

- 712 • A 10-point serial dilution of 17 $\beta$ -estradiol (E2) as the reference standard
- 713 • A 1% volume/volume (v/v) solution of dimethyl sulfoxide (DMSO) as the solvent control
- 714 • 3.13  $\mu\text{g/mL}$  methoxychlor as a weak acting positive control.

715 Reference standards and controls selected and standardized for the antagonist assay were:

- 716 • A nine-point serial dilution of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$   $\mu\text{g/mL}$  E2 as  
717 the reference standard
- 718 • A 1% volume/volume (v/v) solution of DMSO as the solvent control
- 719 •  $2.5 \times 10^{-5}$   $\mu\text{g/mL}$  E2 as the E2 control
- 720 • 25  $\mu\text{g/mL}$  flavone with  $2.5 \times 10^{-5}$   $\mu\text{g/mL}$  E2 as a weak acting positive control.

721 Historical data for the reference standards and controls were generated from 10 independent experiments.

722 These data were used to establish quality control measures for subsequent experiments.

723

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723 **Selection and Standardization of Assessment of Cell Viability Methods**

724 Two commercially available, quantitative cytotoxicity assays, CellTiter-Glo<sup>®</sup> and CellTiter-Blue<sup>®</sup>, were  
725 evaluated for incorporation into the BG1Luc ER TA. CellTiter-Glo<sup>®</sup> is a luminescence-based assay for  
726 measuring adenosine triphosphate (ATP) levels and requires the use of a separate plate from the one used  
727 to evaluate ER TA activity. CellTiter-Blue<sup>™</sup> is a fluorescence-based assay that measures cell viability by  
728 use of the indicator dye resazurin. Viable cells convert the dark blue resazurin to the fluorescent product  
729 resorufin. Nonviable cells cannot perform this conversion and do not fluoresce. The CellTiter-Blue<sup>™</sup> assay  
730 could theoretically be used on the same plate used to measure ER TA activity, but the timing for this  
731 assay was incompatible with BG1Luc ER TA. Therefore, CellTiter-Glo<sup>®</sup> was selected and standardized  
732 for use with BG1Luc ER TA protocols. Cytotoxicity data for the reference standards collected during an  
733 evaluation of this cytotoxicity assay indicated that a significant decrease in E2 agonist response occurred  
734 when the reduction in ATP level per well exceeded 20%. Therefore, concentrations of substance that  
735 caused a reduction in cell viability below 80% were classified as cytotoxic and were not used to assess ER  
736 TA activity. Assessment of cell viability was also conducted qualitatively using a method developed by  
737 XDS based on visual observations of cellular morphology and density.

738

739

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### 739 **Testing of Coded Substances in Agonist and Antagonist Protocols**

740 Eight coded substances (atrazine, bisphenol A, bisphenol B, corticosterone, *o,p'*-DDT, diethylstilbestrol,  
741 17 $\alpha$ -ethinyl estradiol, and flavone) covering a range of ER agonist activities and eight coded substances  
742 (butylbenzyl phthalate, dibenzo[*a,h*] anthracene, flavone, genistein, nonylphenol, progesterone,  
743 *o,p'*-DDT, and tamoxifen) covering a range of ER antagonist activities were each tested in three  
744 independent experiments to evaluate intralaboratory reproducibility and the ability of the test method to  
745 correctly identify substances having ER agonist or antagonist activity. Prior to comprehensive testing, a  
746 range finder experiment was conducted to establish the maximum concentration for testing based on the  
747 solubility of the test substance in 1% v/v DMSO/culture media and cytotoxicity, and/or, for agonist assay,  
748 the maximum ER TA response observed and for the antagonist assay, the minimum ER TA response  
749 observed when tested against  $2.5 \times 10^{-5}$   $\mu\text{g/mL}$  of E2. Due to precipitation of all coded substances in the  
750 culture media at 1 mg/mL, the standard limit concentration for this assay, the highest concentration tested  
751 in the range finder experiments and, in some cases in the definitive tests, was 100  $\mu\text{g/mL}$ . Following  
752 range finding, comprehensive testing of coded substances was conducted as an 11-point double serial  
753 dilution in triplicate for each of three independent experiments.

754  
755 Based on results obtained from agonist testing, 17 $\alpha$ -ethinyl estradiol ( $EC_{50}^1 = 3.87 \times 10^{-6}$   $\mu\text{g/mL}$ ),  
756 diethylstilbestrol ( $EC_{50} = 1.26 \times 10^{-5}$   $\mu\text{g/mL}$ ), bisphenol A ( $EC_{50} = 8.76 \times 10^{-2}$   $\mu\text{g/mL}$ ), bisphenol B  
757 ( $EC_{50} = 5.16 \times 10^{-2}$   $\mu\text{g/mL}$ ), *o,p'*-DDT ( $EC_{50} = 0.383$   $\mu\text{g/mL}$ ), and flavone ( $EC_{50} = 6.88$   $\mu\text{g/mL}$ ) were  
758 reproducibly classified as estrogenic agonists while atrazine and corticosterone did not induce a  
759 significant ER TA response. Based on results obtained from antagonist testing, tamoxifen  
760 ( $IC_{50}^2 = 0.158$   $\mu\text{g/mL}$ ), dibenzo[*a,h*]anthracene ( $IC_{50}$  could not be calculated), flavone ( $IC_{50}$  could not be  
761 calculated), and genistein ( $IC_{50}$  could not be calculated), were reproducibly classified as estrogenic  
762 antagonists, while butylbenzyl phthalate, progesterone, nonylphenol, and *o,p'*-DDT) did not significantly  
763 reduce ER TA activity induced by  $2.5 \times 10^{-5}$   $\mu\text{g/mL}$  of E2.

764

### 765 **Problems Encountered During Testing of Coded Substances**

766 Technical errors were made when making serial dilutions in individual experiments for atrazine,  
767 corticosterone, diethylstilbestrol, and 17 $\alpha$ -ethinyl estradiol, resulting in the exclusion of certain data

---

<sup>1</sup>EC<sub>50</sub> = half-maximal effective concentration

<sup>2</sup>IC<sub>50</sub> = Concentration of the test substance inhibiting the reference estrogen response by 50%

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768 points from single replicates of these individual experiments. Early in the study, cells that were being  
769 cultured for use in the assay did not perform to previously established historical norms, or exhibited  
770 decreased viability. A series of qualifying experiments indicated that the likely cause of these cell culture  
771 problems was a combination of factors including contaminated lots of gentamicin, L-glutamine, fetal  
772 bovine serum, and tissue culture flasks. Based on this information, protocols were specifically modified to  
773 test the performance of these components before use in cell culture.

774

#### 775 **Concordance of Testing Results with ICCVAM Published Data**

776 For each reference substance, there was agreement among the replicate experiments in terms of the  
777 classification of the substance as being positive or negative in the agonist or antagonist assays. Estrogenic  
778 activity for substances tested using the standardized agonist protocol exhibited 100% concordance with  
779 ICCVAM published data (ICCVAM 2003, 2006), classifying six substances (bisphenol A, bisphenol B,  
780 *o,p'*-DDT, diethylstilbestrol, 17 $\alpha$ -ethinyl estradiol, and flavone) as ER agonists and two (atrazine and  
781 corticosterone) as negative. The relative activities of the ER agonists, based on their calculated EC<sub>50</sub>  
782 concentrations, were in agreement with ICCVAM reported median activities. In terms of estrogenic  
783 antagonist activity, there was 75% concordance with ICCVAM published data. The classification of four  
784 substances (dibenzo[*a,h*]anthracene, flavone, genistein, and tamoxifen) as ER antagonists and two  
785 (butylbenzyl phthalate and progesterone) as negative for ER antagonism agreed with the ICCVAM  
786 published data. Two substances (*p,n*-nonylphenol and *o,p'*-DDT) classified as ER antagonists in the  
787 ICCVAM published data were classified as negative in the BG1Luc ER TA protocol standardization  
788 study. Although these substances caused a significant decrease in ER TA activity, they also caused a  
789 significant decrease in cell viability over the same concentration range. Thus, these two substances were  
790 classified as cytotoxic rather than as estrogenic antagonists. There was also a high degree of correlation  
791 between the visual observation and CellTiter-Glo<sup>®</sup> methods of assessing cell viability for all substances  
792 tested.

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## 793 **1.0 Introduction**

794 This document reports on the procedures and results of the agonist and antagonist protocol  
795 standardization study for the BG1Luc4E2 Estrogen Receptor (ER) Transcriptional Activation (TA) test  
796 method (hereafter referred to as BG1Luc ER TA) developed by Xenobiotic Detection Systems, Inc.  
797 (XDS). Protocol standardization procedures were based on recommendations made in the “ICCVAM  
798 Evaluation of *In Vitro* Test Methods for Detecting Potential Endocrine Disruptors: Estrogen Receptor and  
799 Androgen Receptor Binding and Transcriptional Activation Assays,” (ICCVAM Guidelines (ICCVAM  
800 2003, 2006)). Specific goals of the study were to:

- 801 • Standardize procedures for using the BG1Luc ER TA to identify ER agonists and antagonists
- 802 • Standardize procedures for a quantitative test of cell viability for use with the BG1LUC ER  
803 TA agonist and antagonist assays
- 804 • Develop two Good Laboratory Practice (GLP)-compliant (OECD 1998, 2004) protocols: one  
805 for identifying substances with ER agonist activity, and one for identifying substances with  
806 ER antagonist activity
- 807 • Develop a historical database for reference standards and controls for the agonist and  
808 antagonist versions of the BG1Luc ER TA
- 809 • Demonstrate the adequacy of the standardized protocols for detecting ER agonists or  
810 antagonists using eight substances covering a range of ER agonist and antagonist activities,  
811 respectively.

812 The study was sponsored and managed by the National Toxicology Program (NTP) Interagency Center  
813 for the Evaluation of Alternative Toxicological Methods (NICEATM) and conducted at the XDS facility  
814 in Durham, North Carolina.

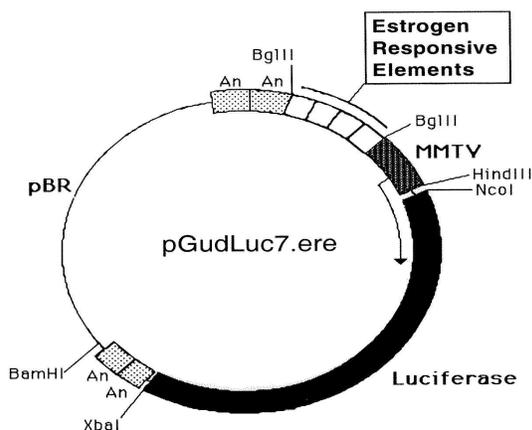
## 815 **2.0 Overview of the BG1Luc ER TA**

816 The BG1Luc ER TA measures whether and to what extent a substance induces or inhibits TA activity via  
817 ER mediated pathways in recombinant BG-1Luc4E2 cells (Rogers and Denison 2000; Rogers and  
818 Denison 2002). The BG-1Luc4E2 cell line was derived from BG-1 immortalized adenocarcinoma cells  
819 that endogenously express ER and have been have been stably transfected with the plasmid  
820 pGudLuc7.ERE. This plasmid contains four copies of a synthetic oligonucleotide containing the estrogen  
821 response element upstream of the mouse mammary tumor viral (MMTV) promoter and the firefly  
822 luciferase gene (**Figure 2-1**). BG1 cells that were transfected with the reporter gene construct and stable  
823 transfectants were selected by growth in minimum essential medium (MEM) containing gentamycin

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824 (G418) (Rogers and Denison 2000; Rogers and Denison 2002). Luciferase expression is driven by ligand  
825 binding of the estrogen receptor.

826 **Figure 2-1 pGudLuc7.ERE Plasmid**



827

828 To conduct the BG1Luc ER TA assay, BG-1Luc4E2 cells are cultured and selected with G418, and then  
829 conditioned in Dulbecco's Modification of Eagle's Medium (DMEM) containing 4.5 g/L Glucose, with  
830 Sodium Pyruvate, without Phenol Red, containing 1 % Penicillin/Streptomycin, 5% Charcoal/Dextran  
831 treated Fetal Bovine Serum (FBS), and 2% L-Glutamine (EFM). After conditioning, cells are seeded into  
832 96-well plates and incubated in EFM containing solvent and/or reference standard, control, or test  
833 substance. After 19 to 24 hours of exposure to test substance, cells are examined under a microscope for  
834 viability, lysed, and treated with luciferase enzyme reagent. Luminescence per well is measured in a  
835 luminometer as relative light units (RLU). RLUs are normalized for background and adjusted such that  
836 the maximal ER TA response induced by the E2 reference standard is 10,000 RLUs.

837

838 The BG1LUC ER TA assay has been proposed by XDS for use in the U.S. Environmental Protection  
839 Agency (EPA) Endocrine Disruptor Screening Program (EDSP) as part of the Tier I screening battery of  
840 *in vitro* and *in vivo* test assays that will be used to reach weight-of-evidence decisions on whether to  
841 conduct large multi-generational Tier 2 *in vivo* studies.

### 842 **3.0 Overview of the Protocol Standardization Study Design**

843 The purpose of the study was to test eight coded substances for agonism and eight coded substances for  
844 antagonism to determine whether the results were reproducible. Also integral to the study design was the

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845 standardization of reference standards and controls, and the development of a quantitative method to  
846 assess cell viability.

847 The criteria for selection of substances for the prevalidation study was based on the following:

848 • Their inclusion on the subset of minimum substances recommended by ICCVAM for  
849 validating *in vitro* ER assays

850 • Their ER agonist activity classification, including those that are negative for agonism:

851 —Strongly active = half maximal effective concentration [EC<sub>50</sub>] value was <0.001 μM

852 —Moderately active = EC<sub>50</sub> value was between 0.001 and 0.1 μM

853 —Weakly active = EC<sub>50</sub> value was >0.1 μM

854 • Their ER antagonist classification:

855 —Uniformly active in multiple assays

856 —Active in the majority of assays in which it was tested

857 —Active in the single assay in which it was tested

858 —Uniformly negative in all assays

859 • Substances were also included that would likely be cytotoxic in the assay or that might pose  
860 solubility problems

861 The selected substances for protocol standardization, eight for agonism and eight for antagonism, are  
862 detailed in **Table 3-1**.  
863

864

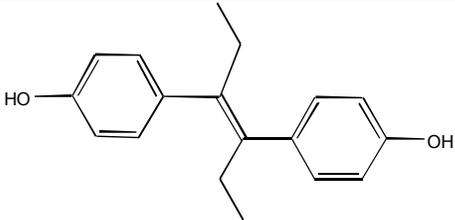
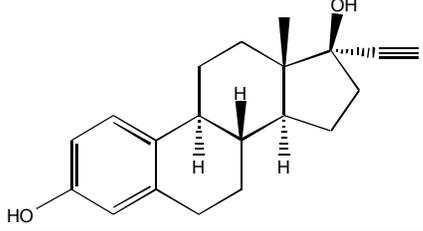
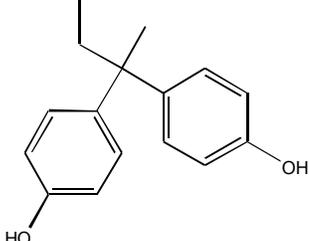
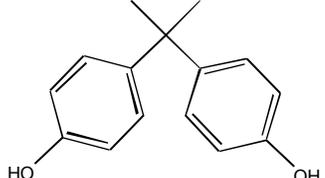
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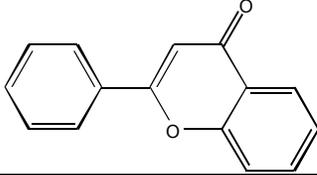
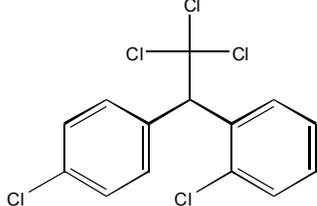
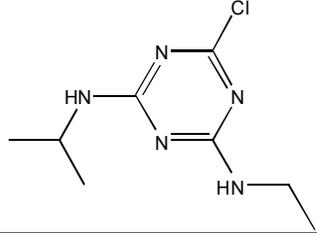
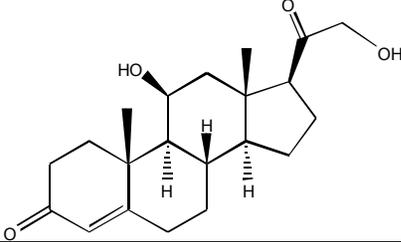
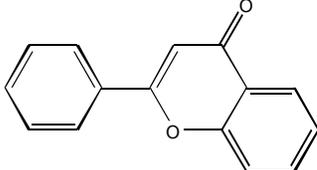
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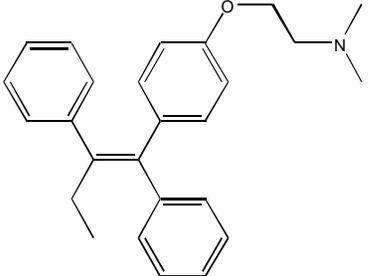
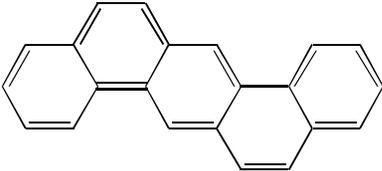
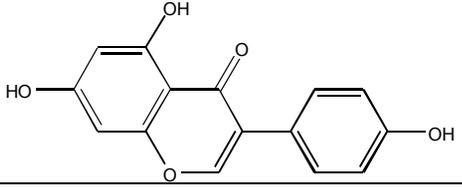
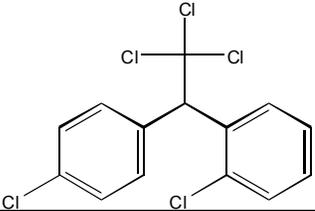
878 Table 3-1 Substances Selected for BG1Luc ER TA Protocol Standardization

Selected for Agonism (A) or Antagonism (Z)	Substance	CASRN	Structure	ER Agonist Activity <sup>1,2</sup>	ER Antago Activit
A	Diethylstilbestrol	56-53-1		+++	-
A	17 $\alpha$ -Ethinyl estradiol	57-63-6		+++	-
A	Bisphenol B	77-40-7		++	
A	Bisphenol A	80-5-7		+	-

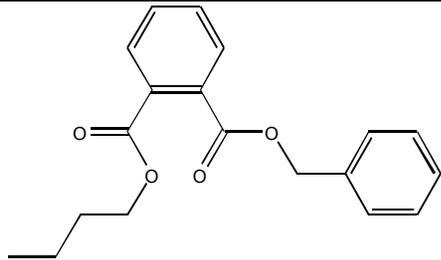
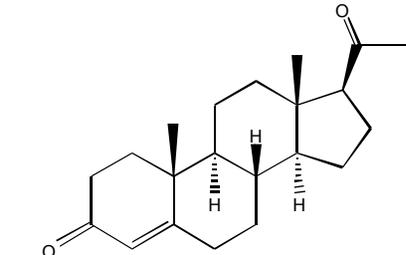
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Selected for Agonism (A) or Antagonism (Z)	Substance	CASRN	Structure	ER Agonist Activity <sup>1,2</sup>	ER Antagonist Activity <sup>1,3</sup>	Comments
A	Flavone	525-82-6		+	###	
A	<i>o,p'</i> -DDT <sup>4,5</sup>	789-2-6		+	#	Cytotoxic
A	Atrazine	1912-24-9		-	-	Cytotoxic
A	Corticosterone	50-22-6		-	-	Negative for Agonism
Z	Flavone <sup>4</sup>	525-82-6		+/-	###	

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Selected for Agonism (A) or Antagonism (Z)	Substance	CASRN	Structure	ER Agonist Activity <sup>1,2</sup>	ER Antagonist Activity <sup>1,3</sup>	Comments
Z	Tamoxifen	10540-29-1		-	###	Cytotoxic
Z	Dibenzo[ <i>a,h</i> ]anthracene	53-70-3		-	##	
Z	Genistein	446-72-0		+	#	Insoluble
Z	<i>p</i> -n - Nonylphenol	104-40-5		++	#	
Z	<i>o,p'</i> -DDT <sup>4,5</sup>	789-2-6		+	#	Cytotoxic

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Selected for Agonism (A) or Antagonism (Z)	Substance	CASRN	Structure	ER Agonist Activity <sup>1,2</sup>	ER Antagonist Activity <sup>1,3</sup>	Comments
Z	Butylbenzyl phthalate	85-68-7		++	-	Negative for Antagonism
Z	Progesterone	57-83-0		+	-	Negative for Antagonism

879 <sup>1</sup>Data on agonist and antagonist activities were derived from (ICCVAM 2003, 2006)

880 <sup>2</sup>+++ Indicates that the substance was relatively active (half maximal effective concentration [EC50] value was <0.001 μM); ++ indicates that the substance was  
 881 moderately active (EC50 value was between 0.001 and 0.1 μM); + indicates that the substance was weakly active (EC50 value was >0.1 μM); +- indicates that  
 882 the substance was positive for agonism in the one assay in which it was tested; - indicates that the substance was uniformly negative in multiple assays.

883 <sup>3</sup>### indicates that the substance was uniformly positive in multiple assays; ## indicates that the substance was positive in the majority of assays in which it was  
 884 tested; # indicates that the substance was positive in the single assay in which it was tested; - indicates that the substance was uniformly negative in all assays.

885 <sup>4</sup>Please note that two substances are being used in both the agonist and antagonist assay with *o,p'*-DDT acting as a potential cytotoxin in both assays and flavone  
 886 acting as a positive in both the agonist and antagonist assay.

887 <sup>5</sup>*o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane

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888 The study was conducted in the following sequence:

- 889 • Selection of a positive control for agonist assays
- 890 • Selection of an antagonist reference standard and controls
- 891 • Development of historical databases
- 892 • Standardization of procedures for evaluation of cell viability
- 893 • Range finder testing of eight coded substances for agonism
- 894 • Range finder testing of eight coded substances for antagonism
- 895 • Comprehensive testing of eight coded substances for agonism
- 896 • Comprehensive testing of eight coded substances for antagonism

#### 897 **4.0 Initial Protocol Development**

898 During initial protocol development and prior to the initiation of the protocol standardization study, XDS  
899 conducted experiments to determine cell doubling times and appropriate seeding densities for the BG-  
900 1Luc4E2 cell line, along with the appropriate concentration for solvents, optimal test substance exposure  
901 duration, and selection of reference standards. Important elements of this initial protocol development  
902 were:

- 903 • Cell doubling times and seeding densities. BG-1Luc4E2 cells have a doubling time of 48 to  
904 72 hours. XDS performed experiments with several different seeding densities to determine  
905 which would provide adequate growth over the incubation and substance exposure periods  
906 without reaching 100% monolayer confluence. A seeding density of  $4 \times 10^4$  cells/well was  
907 found to be optimal.
- 908 • Appropriate concentration of solvent. The BG1Luc ER TA was developed to use dimethyl  
909 sulfoxide (DMSO) as the solvent, at a concentration of 1% volume/volume (v/v) (ICCVAM  
910 Guidelines (ICCVAM 2003, 2006) recommend that the solvent used in transcriptional  
911 activation assays be water, ethanol or DMSO). Testing determined that a concentration of 1%  
912 DMSO did not cause a reduction of activity in the BG1Luc ER TA and was not cytotoxic to  
913 the cell line.
- 914 • Optimal exposure duration. Testing indicated that the optimal substance exposure duration  
915 was between 19 and 24 hours.
- 916 • Reference Standards. ICCVAM Guidelines (ICCVAM 2003, 2006) recommend the use of  
917  $17\beta$ -estradiol (E2) for ER TA agonist assays and ICI 182,780 for ER TA antagonist assays.  
918 The BG1LUC ER TA agonist protocol was developed using E2 as the reference standard and

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919 the BG1LUC ER TA antagonist protocol was developed using tamoxifen as the reference  
920 standard.

921

## 922 **5.0 Selection and Standardization of Reference standards and controls**

### 923 **5.1 Standardization of Agonist Reference Standard**

924 ICCVAM Guidelines (ICCVAM 2003, 2006) recommend the use of E2 as the reference standard for ER  
925 TA agonist assays; therefore, this substance was retained as the reference standard for the BG1LUC ER  
926 TA agonist protocol. In order to maximize the number of concentrations and replicates of coded substance  
927 that could be tested on a single plate, experiments were conducted to determine the optimal number of E2  
928 reference standard concentrations and replicates per plate.

929 Two E2 reference standard configurations were compared, an eight point, half-log serial with samples run  
930 in triplicate wells, and a nine-point, double serial dilution with samples run in duplicate wells (**Table 5-1**).

931 **Table 5-1 Concentrations of E2 Tested in Eight-Point**  
932 **Half-Log vs. Nine-Point Serial Dilution Design**

Eight-Point Half-Log Dilution <sup>1</sup>	Nine-Point Serial Dilution <sup>1</sup>
1.00 x 10 <sup>-4</sup>	1.00 x 10 <sup>-4</sup>
3.33 x 10 <sup>-5</sup>	5.00 x 10 <sup>-5</sup>
1.11 x 10 <sup>-5</sup>	1.25 x 10 <sup>-5</sup>
3.70 x 10 <sup>-6</sup>	6.25 x 10 <sup>-6</sup>
1.23 x 10 <sup>-6</sup>	3.13 x 10 <sup>-6</sup>
4.12 x 10 <sup>-7</sup>	1.56 x 10 <sup>-6</sup>
1.37 x 10 <sup>-7</sup>	7.83 x 10 <sup>-7</sup>
4.57 x 10 <sup>-8</sup>	1.95 x 10 <sup>-7</sup>
-	9.77 x 10 <sup>-8</sup>

933 Abbreviations: E2 = 17β-estradiol

934 <sup>1</sup>Concentrations are presented as µg/mL.

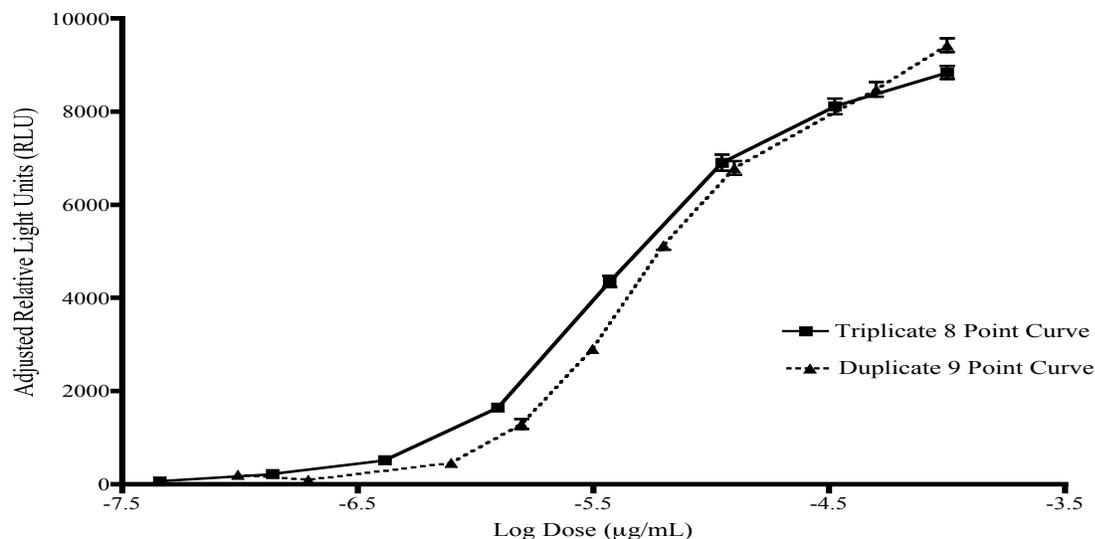
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936 Results were compared after performing 10 independent experiments with both configurations run on the  
937 same 96-well plate (**Figure 5-1**).

938

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938 **Figure 5-1 Comparison of Eight-Point Triplicate and Nine-Point**  
939 **Duplicate E2 Configurations<sup>1</sup>**



940

941 Abbreviations: E2 = 17β-estradiol

942 <sup>1</sup>Each line represents the mean and standard deviation of 10 separate experiments.

943

944 Results indicated that there was no significant difference between the two reference standard  
945 configurations. Therefore, the duplicate E2 configuration was selected for use in the agonist assay in  
946 order to maximize the testing of coded substances as 11-point serial dilutions in triplicate on a 96-well  
947 plate. This also allowed for the addition of the  $2.5 \times 10^{-5}$  µg/mL concentration to better define the top of  
948 the E2 reference standard curve.

## 949 5.2 Selection and Standardization of Agonist Controls

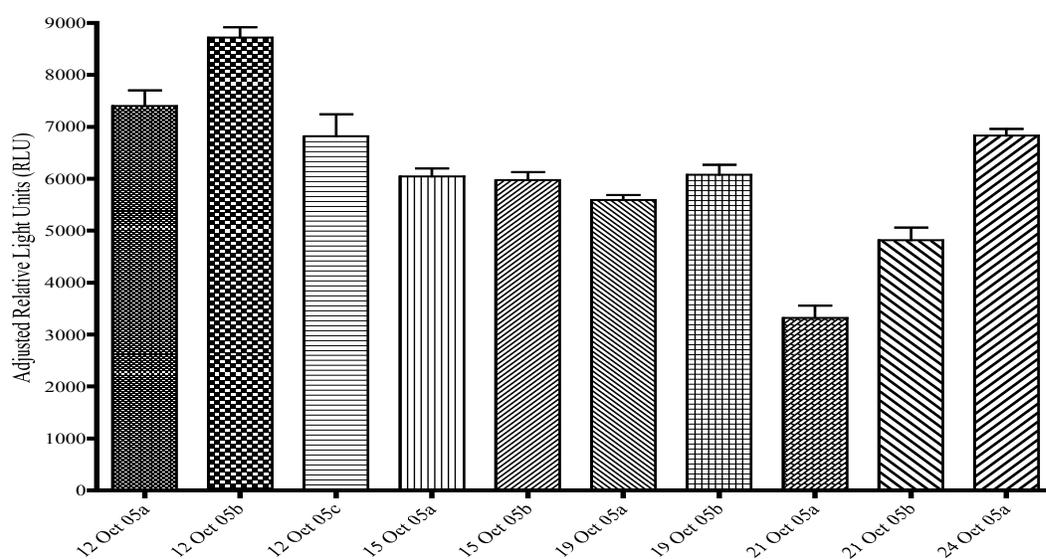
950 ICCVAM Guidelines (ICCVAM 2003, 2006) recommend the inclusion of a weak agonist having a  
951 maximal ER TA response two to three orders of magnitude lower than the E2 reference standard as a  
952 weak positive control to demonstrate the sensitivity and reproducibility of the assay. Prior to the initiation  
953 of the protocol standardization study, XDS used several different substances as quality control standards  
954 in the development of the BG1Luc ER TA agonist protocol. These substances were diethylstilbestrol,  
955 bisphenol A, estrone, 17α-ethinyl estradiol (EE), fenarimol, kaempferol, *p,p'*-methoxychlor  
956 (methoxychlor), and norethynodrel. An objective of the study was the selection and standardization of a  
957 weak positive control for the agonist protocol.

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958 Three substances, kaempferol, methoxychlor, and zearalenone, were selected from the list of  
959 recommended substances for ER TA test methods found in the ICCVAM Guidelines (ICCVAM 2003,  
960 2006) and evaluated as potential weak agonist positive controls.

961 The three substances were evaluated in 10 independent experiments over a two-week period at  
962 concentrations that had previously been determined to have similar ER TA activities in terms of  
963 magnitude of response as E2, but at a significantly higher concentration than E2. The resulting data was  
964 evaluated for consistency of response (**Figures 5-2, 5-3, and 5-4**).

965 **Figure 5-2 Evaluation of Kaempferol as an Agonist Positive Control<sup>1,2</sup>**



966

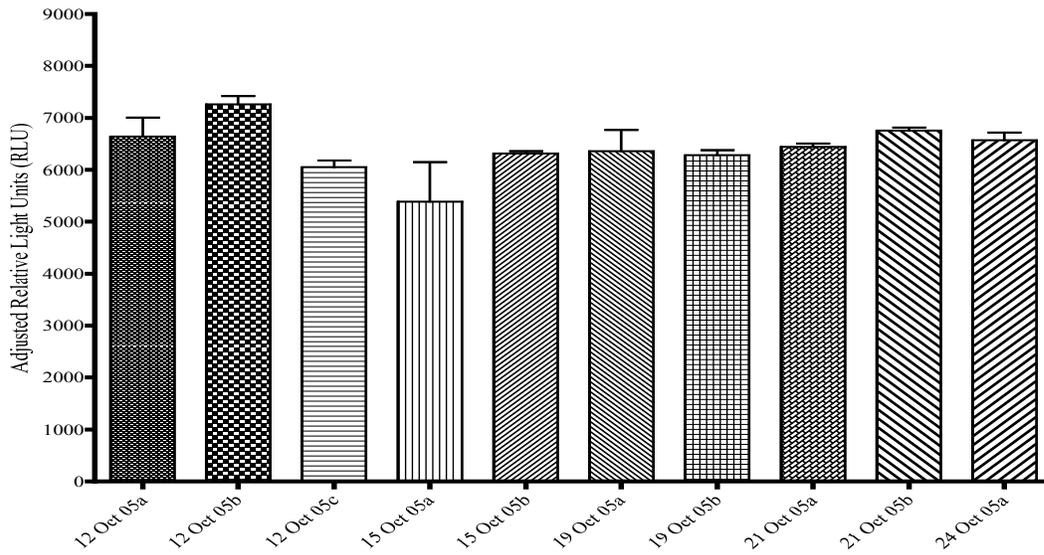
967 <sup>1</sup>Each bar represents the mean and standard deviation of triplicate wells.

968 <sup>2</sup>Letters after the date on bar labels indicate that the experiment was performed multiple times on the same day.

969

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970 **Figure 5-3 Evaluation of Methoxychlor as an Agonist Positive Control<sup>1,2</sup>**



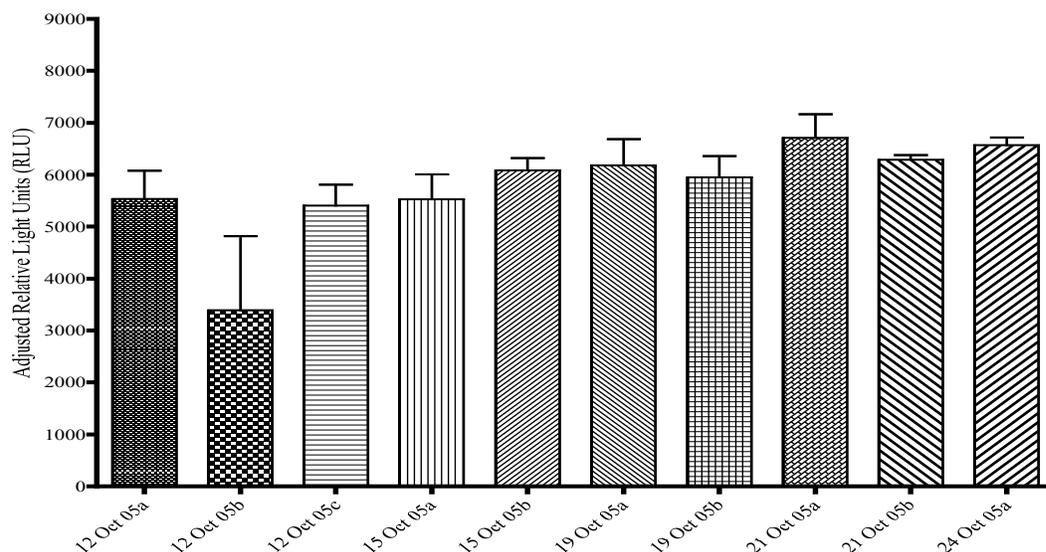
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972 <sup>1</sup>Each bar represents the mean and standard deviation of triplicate wells.

973 <sup>2</sup>Letters after the date on bar labels indicate that the experiment was performed multiple times on the same day.

974

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974 **Figure 5-4 Evaluation of Zearalenone as an Agonist Positive Control<sup>1,2</sup>**

975

976 <sup>1</sup>Each bar represents the mean and standard deviation of triplicate wells.977 <sup>2</sup>Letters after the date on bar labels indicate that the experiment was performed multiple times on the same day.

978

979 Consistency of response across time led to the selection of 3.13 µg/mL methoxychlor as the positive  
 980 control for agonism.

### 981 5.3 Selection and Standardization of Antagonist Reference Standard

982 During the initial development of the BG1Luc ER TA antagonist protocol, XDS used tamoxifen as a  
 983 reference standard. However, tamoxifen requires metabolic activation to 4-hydroxytamoxifen and was  
 984 cytotoxic at the higher concentrations of the reference standard needed to establish saturation of response.  
 985 Therefore, an objective of the study was to select and standardize the use of an alternative reference  
 986 standard. Although ICCVAM Guidelines (*ICCVAM 2003, 2006*) recommend the use of ICI 182,780 as a  
 987 reference standard in ER TA antagonist assays, this substance has limited commercial availability  
 988 (ICCVAM 2006). As an alternative, raloxifene HCl (raloxifene), a strong estrogen antagonist that is listed  
 989 in the ICCVAM Guidelines (ICCVAM 2003, 2006) for validation testing, was evaluated for use as the  
 990 reference standard in the BG1LUC ER TA antagonist assay. In order to maximize the number of  
 991 concentrations and replicates of coded substance that could be examined on a single plate, experiments  
 992 were conducted to determine the optimal number of raloxifene reference standard concentrations and  
 993 replicates per plate. Two raloxifene reference standard configurations were compared, an eight-point,  
 994 half-log dilution with samples run in triplicate, and a nine-point, serial dilution with samples run in

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995 duplicate (**Table 5-2**). These concentrations of raloxifene were combined with a fixed concentration of  
 996  $2.5 \times 10^{-5}$   $\mu\text{g/mL}$  E2 (Ral/E2) to establish the concentration-response curve for antagonism.

997 **Table 5-2 Concentrations of Raloxifene Tested in Eight-point**  
 998 **Half-Log vs. Nine-point Serial Dilution Ral/E2 Design**

Eight-point Half-Log Dilution ( $\mu\text{g/mL}$ )	Nine-point Serial Dilution ( $\mu\text{g/mL}$ )
$2.50 \times 10^{-2}$	$2.50 \times 10^{-2}$
$8.33 \times 10^{-3}$	$1.25 \times 10^{-2}$
$2.78 \times 10^{-3}$	$6.25 \times 10^{-3}$
$9.26 \times 10^{-4}$	$3.13 \times 10^{-3}$
$3.09 \times 10^{-4}$	$1.56 \times 10^{-3}$
$1.03 \times 10^{-4}$	$7.81 \times 10^{-4}$
$3.43 \times 10^{-5}$	$3.91 \times 10^{-4}$
$1.14 \times 10^{-5}$	$1.95 \times 10^{-4}$
-	$9.77 \times 10^{-5}$

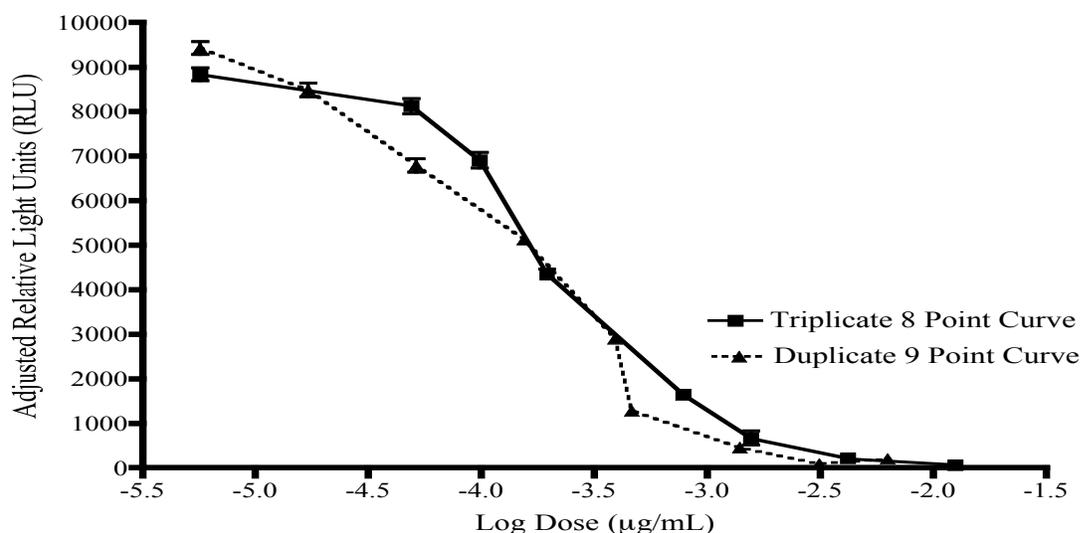
999 Abbreviations: Ral/E2 = concentrations of raloxifene with a fixed concentration of  
 1000  $2.5 \times 10^{-5}$   $\mu\text{g/mL}$  17 $\beta$ -estradiol

1001 Results were compared after performing 10 independent experiments with both configurations run on the  
 1002 same 96-well plate (**Figure 5-5**).

1003

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1003 **Figure 5-5 Comparison of Eight-point Triplicate and Nine-point**  
1004 **Duplicate Ral/E2 Configurations<sup>1</sup>**



1005

1006 Abbreviations: Ral/E2 = concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$  µg/mL  $17\beta$ -estradiol

1007 <sup>1</sup>Each line represents the mean and standard deviation of 10 separate experiments.

1008

1009 Results indicated that the duplicate nine-point curve had more data points that fell within the linear  
1010 portion of the concentration-response curve. In order to maximize the testing of coded substances as  
1011 11 point, double serial dilutions in triplicate on a 96-well plate, the duplicate Ral/E2 reference standard  
1012 configuration was selected for use in the antagonist assay.

1013 The concentration-response curve for Ral/E2 was then tested using different concentrations of raloxifene  
1014 in order to establish a concentration curve that completely reduces the ability of the E2 reference estrogen  
1015 to induce estrogenic activity at the highest concentrations of raloxifene used and that had no ability to  
1016 reduce the estrogenic activity of the E2 reference estrogen at the lowest concentrations of raloxifene  
1017 tested. Concentrations tested are presented in **Table 5-3** and results are presented in **Figure 5-6** as the  
1018 aggregate data from 10 replicate experiments. The Ral/E2 reference standard was examined as a nine-  
1019 point serial dilution, with each concentration run in duplicate.

1020

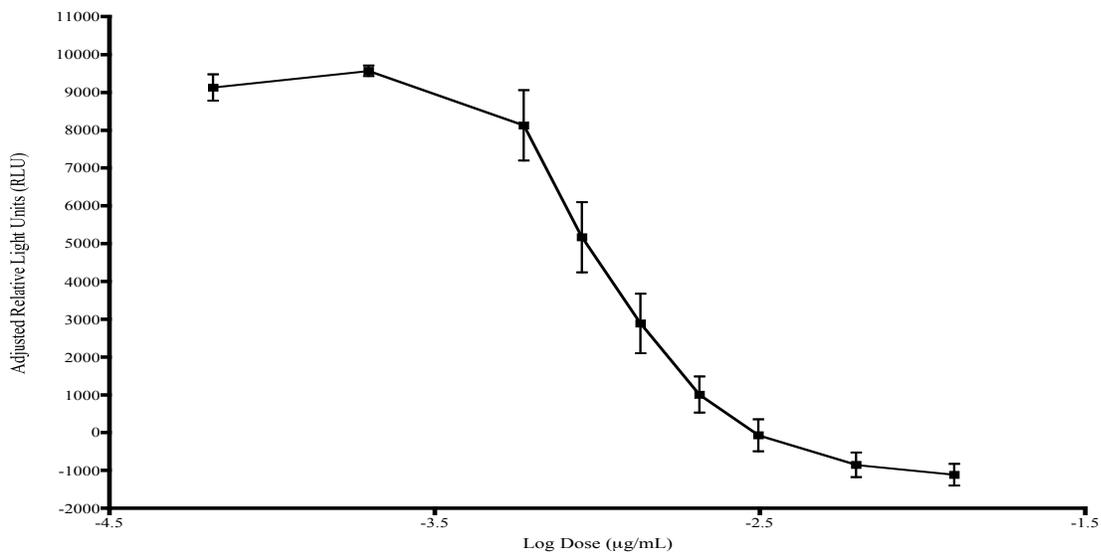
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1020 **Table 5-3 Concentrations of Raloxifene in**  
 1021 **Reference Standard**

Raloxifene Concentration ( $\mu\text{g/mL}$ )		
$1.25 \times 10^{-2}$	$1.56 \times 10^{-3}$	$1.95 \times 10^{-4}$
$6.25 \times 10^{-3}$	$7.81 \times 10^{-4}$	$9.77 \times 10^{-5}$
$3.13 \times 10^{-3}$	$3.91 \times 10^{-4}$	$4.88 \times 10^{-5}$

1022

1023 **Figure 5-6 Evaluation of Ral/E2 as an Antagonist Reference Standard<sup>1</sup>**



1024

1025 Abbreviations: Ral/E2 = concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5} \mu\text{g/mL}$   
 1026  $17\beta$ -estradiol

1027 <sup>1</sup>The line represents the mean and standard deviation of 10 separate experiments.

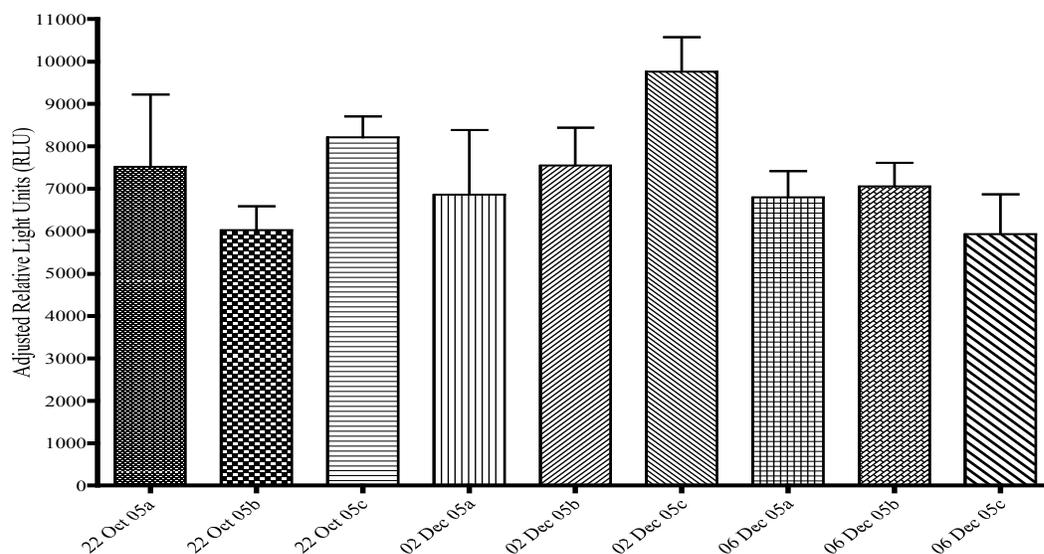
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## 1028 5.4 Selection and Standardization of Antagonist Controls

1029 ICCVAM Guidelines (ICCVAM 2003, 2006) also recommend the inclusion of a weak positive control  
1030 that would reduce the ability of the reference estrogen to induce maximum ER TA in the test system by  
1031 70 to 90% in an antagonist assay. The purpose of a weak positive control is to facilitate the demonstration  
1032 of the sensitivity and reproducibility of the assay. Three substances, dibenzo[*a,h*]anthracene (DBA),  
1033 flavone, and tamoxifen, were selected from recommended substances for ER TA test methods listed in the  
1034 ICCVAM Guidelines (ICCVAM 2003, 2006) and evaluated as potential antagonist positive controls.  
1035 These three candidates were evaluated for their potential to reduce the induction of ER TA caused by  
1036  $2.5 \times 10^{-5}$   $\mu\text{g/mL}$  of E2 in multiple independent experiments over a two week period at concentrations  
1037 that had been determined by XDS in previous experiments to cause a decrease in ER TA by  
1038 approximately 70%. Results (Figures 5-7, 5-8, and 5-9) were evaluated for consistency of response.

1039 **Figure 5-7 Evaluation of DBA as an Antagonist Positive Control<sup>1,2</sup>**



1040

1041 Abbreviations: DBA = dibenzo[*a,h*]anthracene

1042 <sup>1</sup>Each bar represents the mean and standard deviation of triplicate wells.

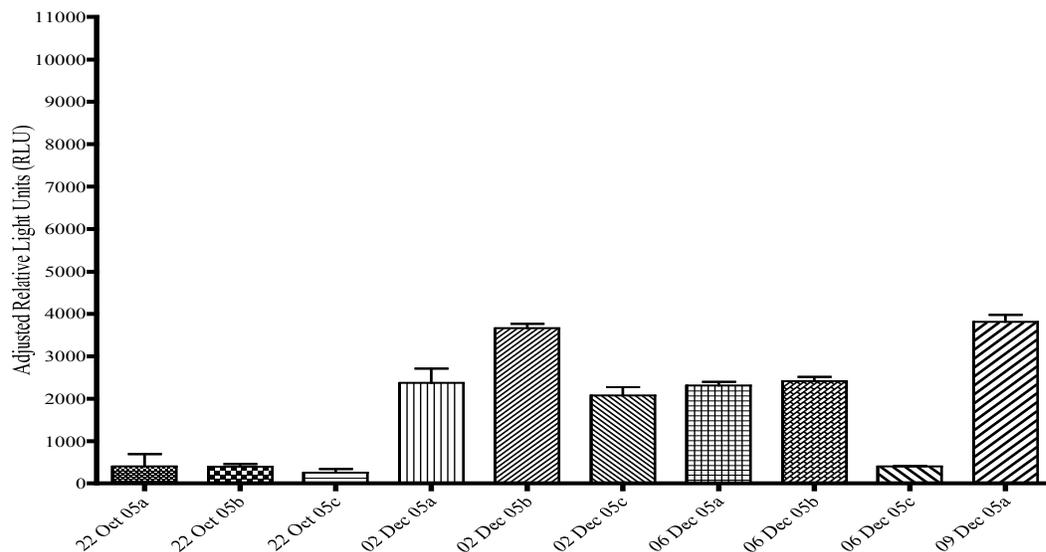
1043 <sup>2</sup>Letters after the date on bar labels indicate that the experiment was performed multiple times on the same day.

1044

1045

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1045 **Figure 5-8 Evaluation of Flavone as an Antagonist Positive Control<sup>1,2,3</sup>**



1046

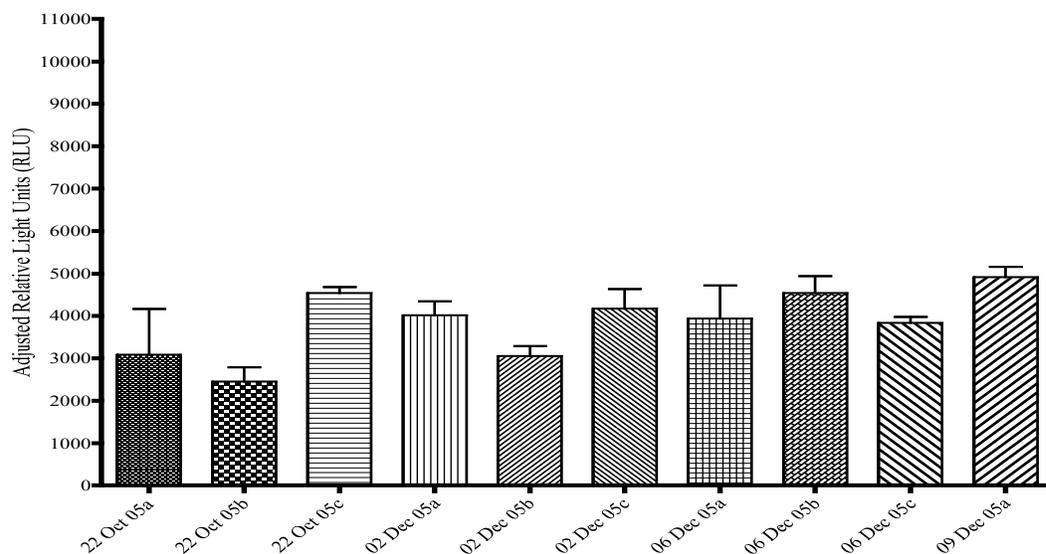
1047 <sup>1</sup>Each bar represents the mean and standard deviation of triplicate wells.

1048 <sup>2</sup>Letters after the date on bar labels indicate that the experiment was performed multiple times on the same day.

1049 <sup>3</sup>Replicates run on 22 Oct 05 were run at 50 µg/mL. All further flavone replicates were run at 25 µg/mL in order  
 1050 to provide a less robust inhibition of E2 than that observed at 50 µg/mL.

1051

1052 **Figure 5-9 Evaluation of Tamoxifen as an Antagonist Positive Control<sup>1,2</sup>**



1053

1054 <sup>1</sup>Each bar represents the mean and standard deviation of triplicate wells.

1055 <sup>2</sup>Letters after the date on bar labels indicate that the experiment was performed multiple times on the same day.

1056

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1057 DBA was not selected because previous experiments by XDS indicated that this substance had the  
1058 potential to produce a biphasic concentration-response curve, which could potentially introduce errors if  
1059 used for quality control. Tamoxifen was not selected because of concerns about potential cytotoxicity at  
1060 concentrations required for a 70% reduction of E2 induction. Flavone, at a concentration of 25 µg/mL,  
1061 was selected as the weak positive control in the antagonist assay as it was neither biphasic, nor were there  
1062 concerns about it being cytotoxic.

1063

#### 1064 **5.5 Summary of Selected Reference Standards and Controls**

1065 The selected reference standards and controls, listed in **Table 5-4**, were used during the testing of the  
1066 coded substances phase of the protocol standardization study. The agonist assay reference standard was a  
1067 10-point serial dilution of E2 (E2 reference standard), the solvent control was a 1% v/v solution of DMSO  
1068 (DMSO control), and the weak positive control was 3.13 µg/mL methoxychlor (methoxychlor control).  
1069 The antagonist assay reference standard was a nine-point serial dilution of raloxifene with a fixed  
1070 concentration of  $2.5 \times 10^{-5}$  µg/mL E2 (Ral/E2 reference standard), the solvent control was DMSO control,  
1071 the E2 control was  $2.5 \times 10^{-5}$  µg/mL E2 (E2 control), and the weak positive control was 25 µg/mL flavone  
1072 with  $2.5 \times 10^{-5}$  µg/mL E2 (flavone control).

1073

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1073 **Table 5-4 Solvent, Reference Estrogen, Agonist, and Antagonist Controls**

Use	Substance Name	CASRN	Supplier	Catalog Number	Purity	ER TA Agonist Activity <sup>1,2</sup>	ER TA Antagonist Activity <sup>1,3</sup>
Solvent	Dimethyl sulfoxide	67-68-5	Sigma-Aldrich Corp	D8418	99.9%	-	-
Agonist Reference Standard	17 $\beta$ -estradiol	50-28-2	Sigma-Aldrich Corp	E8875	98%	+++	-
Agonist Positive Control	p,p'-methoxychlor	72-43-5	Supelco	49054	99.9%	+	-
Antagonist Reference Standard	Raloxifene HCl	82640-04-8	Sigma-Aldrich Corp	R1402	99.5%	-	###
Antagonist Positive Control	Flavone	525-82-6	Sigma-Aldrich Corp	F2003	99%	+	###
Antagonist E2 Control	17 $\beta$ -estradiol	50-28-2	Sigma-Aldrich Corp	E8875	98%	+++	-

1074 Abbreviations: CASRN = Chemical Abstracts Service Registry Number; Corp = Corporation; ER = estrogen receptor; TA = transcriptional  
1075 activation

1076 <sup>1</sup>Data on agonist and antagonist activities were derived from (ICCVAM 2006)

1077 <sup>2</sup>+++ Indicates that the substance was strongly active (EC<sub>50</sub> value was <0.001  $\mu$ M); + indicates that the substance was weakly active (EC<sub>50</sub> value  
1078 was >0.1  $\mu$ M), or a positive response was reported without an EC<sub>50</sub> value;  
1079 - indicates that the substance was uniformly negative in multiple assays.

1080 <sup>3</sup>### Indicates that the substance was uniformly positive in multiple assays; - indicates that the substance was uniformly negative in multiple  
1081 assays.

1082

## 1083 6.0 Historical Databases

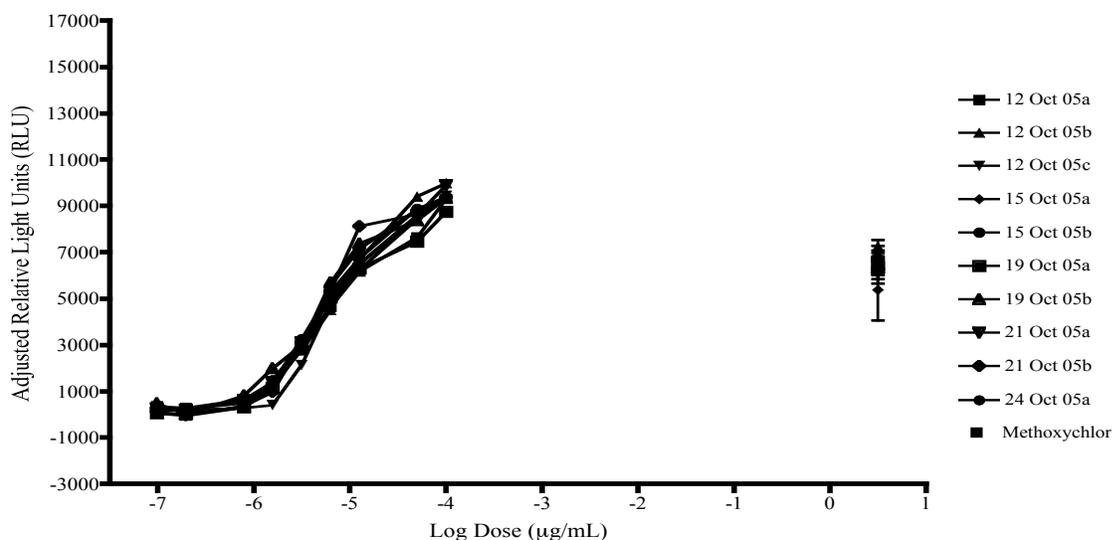
1084 Historical databases were established for both agonist and antagonist assays after selection of reference  
1085 standards and controls to provide reference values to be used as acceptance criteria and to provide an  
1086 ongoing measure of intralaboratory reproducibility. These databases were established by conducting 10  
1087 independent experiments using each protocol.

1088

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1089 **6.1 Agonist Historical Database**

1090 The agonist historical database was established by conducting 10 independent experiments using the 10-  
 1091 point E2 reference standard run in duplicate, DMSO control run in quadruplicate, and the methoxychlor  
 1092 control run in triplicate in each 96-well plate (**Figure 6-1**).

1093 **Figure 6-1 Agonist Historical Database**<sup>1,2,3</sup>

1094

1095 <sup>1</sup>Each line represents the 17 $\beta$ -estradiol reference standard for a single experiment. Each point on the line  
 1096 represents the mean and standard deviation of duplicate wells.

1097 <sup>2</sup>Each point at "0" on the abscissa represents the methoxychlor control for a single experiment  
 1098 (mean and standard deviation of triplicate wells).

1099 <sup>3</sup>Letters after the date on line labels indicate that the experiment was performed multiple times  
 1100 on the same day.

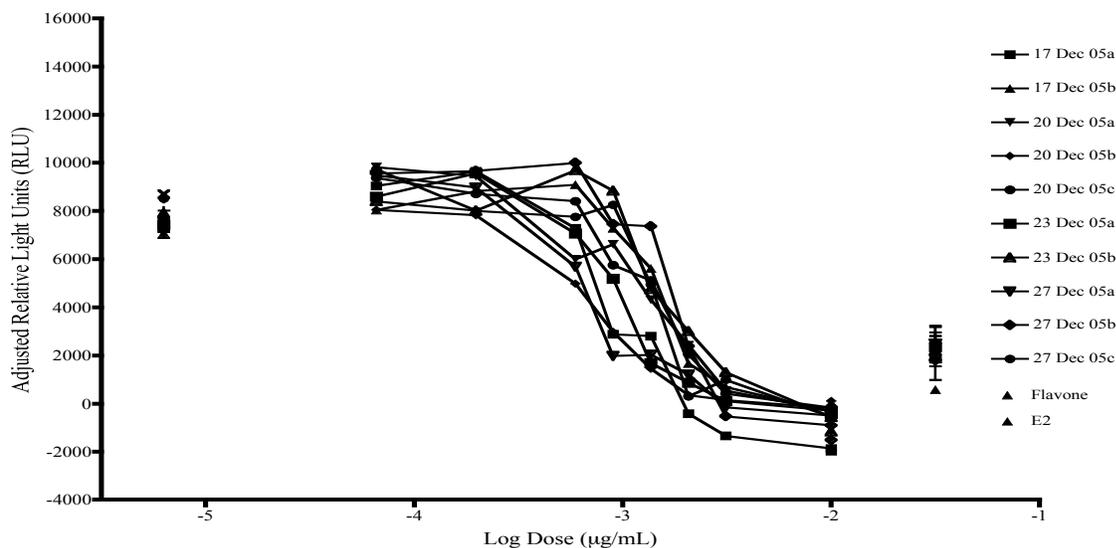
1101

1102 **6.2 Antagonist Historical Database**

1103 The antagonist historical database was established by conducting 10 independent experiments using the  
 1104 nine-point Ral/E2 reference standard run in duplicate, DMSO solvent control run in triplicate, and the E2  
 1105 control and flavone control run in triplicate in each 96-well plate (**Figure 6-2**).

1106

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1106 **Figure 6-2 Antagonist Historical Database<sup>1,2,3,4</sup>**

1107

1108 <sup>1</sup>Each line represents the Ral/E2 (concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$  µg/mL  
 1109 17β-estradiol) reference standard for a single experiment. Each point on the line  
 1110 represents the mean and standard deviation of duplicate wells.

1111 <sup>2</sup>Each point at “0” on the abscissa represents the flavone control for a single experiment (mean and standard  
 1112 deviation of triplicate wells).

1113 <sup>3</sup>Each point at “-7” on the abscissa represents the 17β-estradiol control for a single experiment (mean and standard  
 1114 deviation of triplicate wells).

1115 <sup>4</sup>Letters after the date on line labels indicate that the experiment was performed multiple times  
 1116 on the same day.

1117

## 1118 7.0 Assessment of Cell Viability

### 1119 7.1 Qualitative Evaluation of Cell Viability

1120 Prior to the initiation of the protocol standardization study, XDS developed a method of assessing cell  
 1121 viability based on visual observations of cellular morphology using an inverted microscope. **Table 7-1**  
 1122 provides the scoring system used to qualify cell viability by visual inspection during the testing.

1123

1124

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1124 **Table 7-1 Scoring System for Visual Inspection for Cell Viability.**

Score	Observation
1	Normal Cell Morphology and Density
2	Altered Cell Morphology, and/or Small Gaps between Cells
3	Altered Cell Morphology, and/or Large Gaps between Cells
4	Few (or no) Visible Cells
1P	Score of 1 with Precipitate
2P	Score of 2 with Precipitate
3P	Score of 3 with Precipitate
4P	Score of 4 with Precipitate
5P	Unable to View Cells Due to Precipitate

1125

1126 **7.2 Quantitative Evaluation of Cell Viability**

1127 ICCVAM Guidelines (ICCVAM 2003, 2006) recommend the use of quantitative tests for the  
 1128 measurement of cell viability. Therefore, two commercially available quantitative cell viability assays,  
 1129 CellTiter-Blue™ and CellTiter-Glo® (Promega, Inc.) were evaluated in the standardization study.

1130

1131 **7.3 CellTiter-Blue™**

1132 CellTiter-Blue™ measures cell viability by use of the indicator dye resazurin. Viable cells convert the  
 1133 dark blue resazurin to the fluorescent product resorufin, while nonviable cells cannot perform this  
 1134 conversion and do not fluoresce. The CellTiter-Blue™ assay had the potential to be conducted in the same  
 1135 plate as the BG1Luc ER TA. Testing of CellTiter-Blue™ in BG-1Luc4E2 cells failed to produce a  
 1136 fluorescent signal, even when cells were exposed to CellTiter-Blue™ reagent for up to six times the  
 1137 recommended incubation period. At six times the recommended incubation period, visual observation of  
 1138 cells indicated a significant decrease in cell viability. Therefore, CellTiter-Blue™ was not considered to  
 1139 be appropriate for use with the BG1Luc ER TA.

1140

1141 **7.4 CellTiter-Glo®**

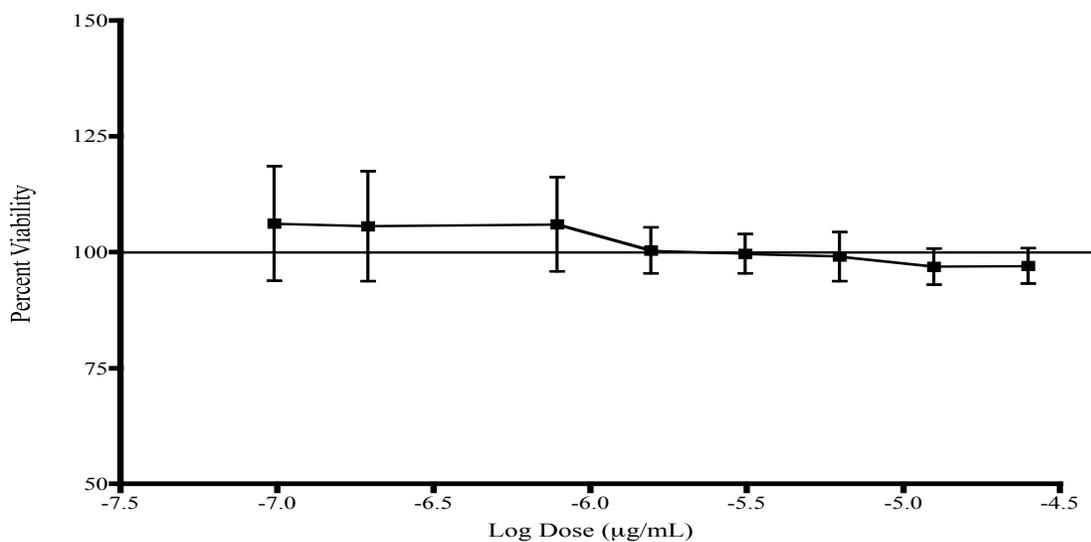
1142 CellTiter-Glo® is a method of determining the number of viable cells in culture based on quantitation of  
 1143 adenosine triphosphate (ATP) in viable cells. This method requires the use of concurrent parallel  
 1144 experimental plates because the assay format results in cell lysis and generation of a luminescent signal

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1145 proportional to the amount of ATP present. Results for CellTiter-Glo<sup>®</sup> testing using the E2 reference  
1146 standard and the Ral/E2 reference standard are presented in **Figures 7-1** and **7-2**. Based on these results,  
1147 the CellTiter-Glo<sup>®</sup> method was selected to quantitatively measure cytotoxicity for the protocol  
1148 standardization study.

1149

1150 **Figure 7-1 CellTiter-Glo<sup>®</sup> Agonist Viability Testing Trials<sup>1,2</sup>**



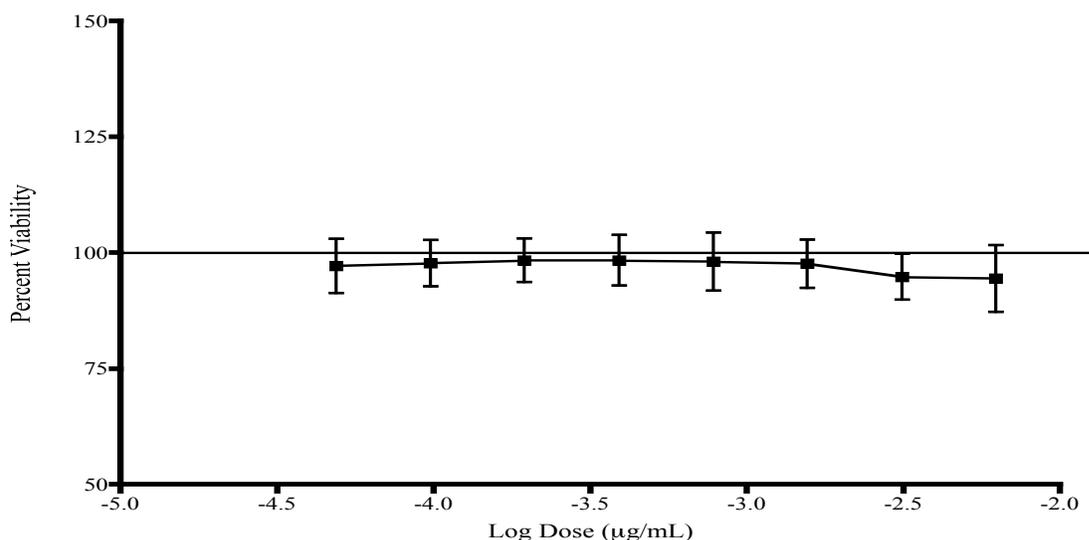
1151

1152 <sup>1</sup>Graph represents the mean and standard deviation of 10 replicate experiments.

1153 <sup>2</sup>Horizontal line represents 100% Viability as measured in dimethyl sulfoxide control.

1154

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1155 **Figure 7-2 CellTiter-Glo® Antagonist Viability Testing Trials<sup>1,2</sup>**

1156

1157 <sup>1</sup>Graph represents the mean and standard deviation of 10 replicate experiments.1158 <sup>2</sup>Horizontal line represents 100% Viability as measured in dimethyl sulfoxide control.

1159

1160 **7.3 Cell Viability Limit**

1161 Examination of CellTiter-Glo® cell viability data for the E2 and Ral/E2 reference standards demonstrated  
1162 that viability for these reference standards did not fall below 80% (**Figures 7-1 and 7-2**). No decrease in  
1163 response in the BG1Luc ER TA resulted from this level of reduction in cell viability (**Figures 6-1 and 6-**  
1164 **2**), and therefore, the limit for cell viability was set at 80%. Test substance concentrations that reduced the  
1165 percentage of viable cells below 80% were classified as cytotoxic and were not used to assess ER TA  
1166 activity.

1167

1168 **8.0 Procedures for Testing of Coded Substances**

1169 A summary of procedures and results for agonist and antagonist testing are presented in **Sections 9.0**  
1170 through **12.0**. Raw data files were provided to NICEATM, and included all data collected during protocol  
1171 standardization, including outlier values that were not used to perform data analyses. A list of the  
1172 experiments performed during the course of the protocol standardization effort is provided in **Appendix**  
1173 **A**. The detailed agonist and antagonist protocols for the BG1Luc ER TA are provided in **Appendices B**  
1174 and **C**, respectively.

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1175 **8.1 Coded Test Substances**

1176 NICEATM, through the National Toxicology Program Substances Inventory (NTPSI), acquired 14  
 1177 substances (flavone and 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane [*o,p'*-DDT] were  
 1178 used for both agonist and antagonist testing) from commercial sources (**Table 8-1**).

1179

1180 **Table 8-1 Coded Test Substances Used for Protocol Standardization**

NICEATM Substance Code	Laboratory Substance Code	Substance Name	CASRN	Supplier	Catalog Number	Purity
N0001	R00115B	Atrazine	1912-24-9	ChemService, Inc	PS-380	98%
N0002	R00107B	Bisphenol A	80-5-7	Sigma-Aldrich Corp	133027	100 %
N0003	R00116B	Bisphenol B	77-40-7	City Chemical, LLC	B2427	97.4 %
N0004	R00117B	Corticosterone	50-22-6	Sigma-Aldrich Corp	C2505	99%
N0005	R00118	<i>o,p'</i> -DDT	789-02-6	ChemService, Inc	PS-698	98%
N0006	R00108	Diethylstilbestrol	56-53-1	Sigma-Aldrich Corp	D4628	99%
N0007	R00109	17 $\alpha$ -ethinyl estradiol	57-63-6	Sigma-Aldrich Corp	E4876	99%
N0008	R00110	Flavone <sup>1</sup>	525-82-6	Sigma-Aldrich Corp	F2003	99%
N0009	R00111A	Butylbenzyl phthalate	85-68-7	Sigma-Aldrich Corp	308501	98%
N0010	R00119A	Dibenzo[ <i>a,h</i> ] anthracene	53-70-3	Sigma-Aldrich Corp	48574	99%
N0011	R00122A	Genistein	446-72-0	Sigma-Aldrich Corp	G6649	99%
N0012	R00112A	Flavone <sup>1</sup>	525-82-6	Sigma-Aldrich Corp	F2003	99%
N0013	R00120A	<i>p</i> -n-nonylphenol	104-40-5	Alfa Aesar, Corp	A15609	100 %
N0014	R0013A	Progesterone	57-83-0	Sigma-Aldrich Corp	P8783	100 %

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NICEATM Substance Code	Laboratory Substance Code	Substance Name	CASRN	Supplier	Catalog Number	Purity
N0015	R00121A	<i>o,p'</i> -DDT <sup>1</sup>	789-02-6	ChemService, Inc	PS-698	98%
N0016	R00114A	Tamoxifen	10540-29-1	Sigma-Aldrich Corp	T5648	99%

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; Corp = Corporation; Inc = Incorporated; LLC = Limited Liability Corporation; *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane

<sup>1</sup>Flavone and *o,p'*-DDT were obtained as a single lot, apportioned out, and assigned a separate code for agonist and antagonist testing.

1181  
1182  
1183  
1184

1185 All but four of the test substances (atrazine, bisphenol B, butylbenzyl phthalate [BBP], and *o,p'*-DDT)  
1186 were 99% pure or greater. NICEATM coded each substance with a unique identifier, and NTPSI  
1187 repackaged the test substances and distributed them to the laboratory. The coded test substances were  
1188 packaged and shipped such that their identities were concealed; however, a sealed envelope containing  
1189 the identity of each test substance as well as its material safety data sheet (MSDS) was provided to the  
1190 laboratory to be opened in the case of an accident (e.g., chemical spill).

1191 Upon receipt, the laboratory assigned each test substance a unique, laboratory-specific coded  
1192 identification, which was used in laboratory notebooks to refer to the test substance (**Table 8-1**).

1193 The laboratory reported all data using the NICEATM substance codes. NICEATM revealed the identity  
1194 of the test substances on completion of the protocol standardization study.

1195

## 1196 **8.2 Lot-to-Lot Consistency of Test Substances**

1197 Each substance was purchased as a single lot, and the laboratory received aliquots from this same lot  
1198 throughout the protocol standardization study. The substance suppliers provided certificates of analysis  
1199 for each lot, along with MSDS documents containing physical/chemical, safety, and handling  
1200 information.

## 1201 **9.0 General Procedure for Agonist Testing**

1202 Agonist range finder experiments were conducted by testing substances at serial log concentrations.  
1203 Results from range finder testing were then used to select starting concentrations for comprehensive  
1204 testing of test substances. Agonist range finder and comprehensive testing were conducted on 96-well  
1205 plates using 10 concentrations of E2 in duplicate as the reference standard (**Table 9-1**). Four replicate  
1206 wells of the DMSO control and three replicate wells of the methoxychlor control were included on each

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1207 plate. In order to avoid edging effects<sup>3</sup>, wells on the perimeter of the plate were not used for experiments.  
 1208 These wells did not contain cells but did contain cell culture media to prevent drying out of experimental  
 1209 wells.

1210 **Table 9-1 Concentrations of E2 Reference Standard Used in Range**  
 1211 **Finder and Comprehensive Testing**

E2 Concentrations (µg/mL)		
1.00 x 10 <sup>-4</sup>	6.25 x 10 <sup>-6</sup>	1.95 x 10 <sup>-7</sup>
5.00 x 10 <sup>-5</sup>	3.13 x 10 <sup>-6</sup>	9.78 x 10 <sup>-8</sup>
2.50 x 10 <sup>-5</sup>	1.56 x 10 <sup>-6</sup>	
1.25 x 10 <sup>-5</sup>	7.83 x 10 <sup>-7</sup>	

1212 Abbreviations: E2 = 17β-estradiol

1213 Luminescence of treated, reference standard, and control wells was corrected by subtracting the averaged  
 1214 luminescence of the DMSO controls from the RLU measured in each well. Data was transferred into  
 1215 GraphPad PRISM<sup>®</sup> 4.0 statistical software (PRISM<sup>®</sup>), graphed, and evaluated for positive or negative  
 1216 response. For substances that were positive, the concentration of test substance that caused a half-  
 1217 maximal response (EC<sub>50</sub>) was calculated using the Hill function analysis. The Hill function is a four-  
 1218 parameter logistic mathematical model relating the substance concentration to the RLU values in a  
 1219 sigmoidal shape:

$$1220 \quad Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log \text{EC}_{50} - X) \text{HillSlope}}}$$

1221 where Y=response (i.e., relative light units), X is the logarithm of the test substance concentration,  
 1222 Bottom is the minimum response, Top is the maximum response, log EC<sub>50</sub> is the logarithm of X as the  
 1223 response midway between Top and Bottom, and HillSlope describes the steepness of the curve. The  
 1224 model calculates the best fit for the Top, Bottom, HillSlope, and EC<sub>50</sub> parameters.

1225 Acceptance or rejection of a test was based on evaluation of reference standard and control results from  
 1226 each experiment conducted on a 96-well plate. Results were compared to quality controls for these  
 1227 parameters derived from the historical database established during development and standardization of the  
 1228 BG1Luc ER TA agonist protocol. The quality control parameters are as follows:

---

<sup>3</sup>Edging effects are variations in response seen in the outermost wells in a tissue culture plate. These variations are believed to be due to variations in temperature, evaporation, etc., that may occur in these wells that would

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- 1229 • Induction – Plate induction (i.e., the highest E2 reference standard RLU value divided by the  
1230 averaged DMSO solvent RLU value) must be greater than three fold.
- 1231 • Reference standard results – Calculated E2 reference standard EC<sub>50</sub> values must be within 2.5  
1232 times the standard deviation of the historical database EC<sub>50</sub> mean values.
- 1233 • DMSO control results - DMSO control RLU values must be within 2.5 times the standard  
1234 deviation of the historical database solvent control mean RLU values.
- 1235 • Positive control results – Methoxychlor control RLU values must be within 2.5 times the  
1236 standard deviation of the historical database methoxychlor control mean RLU values.

1237

## 1238 10.0 Agonist Testing

1239 The substances selected for agonist testing were atrazine, bisphenol A, bisphenol B, corticosterone, *o,p'*-  
1240 DDT, diethylstilbestrol, EE, and flavone (**Table 10-1**). These substances were selected from the subset of  
1241 minimum substances recommended for validation of *in vitro* ER assays in the ICCVAM Guidelines  
1242 (ICCVAM 2003, 2006). They were selected to represent a range of ER agonist activity classification  
1243 (including those that are negative for agonism) and to evaluate substances that are potentially problematic  
1244 (e.g., limited solubility, cytotoxicity). .

1245

1246 Because they were insoluble in cell culture media containing 1% DMSO, none of the selected substances  
1247 could be tested at the recommended limit concentration (1 mg/mL). Therefore, the limit concentration for  
1248 protocol standardization was set at 100 µg/mL.

1249

1250 **Table 10-1 Test Substances for Agonist Testing**

Code	Substance Name	CASRN	ER TA Agonist Activity <sup>1,2</sup>	Additional Basis for Selection <sup>3</sup>
N0001	Atrazine	1912-24-9	-	Cytotoxic
N0002	Bisphenol A	80-5-7	+	
N0003	Bisphenol B	77-40-7	++	
N0004	Corticosterone	50-22-6	-	
N0005	<i>o,p'</i> -DDT	789-2-6	+	Cytotoxic
N0006	Diethylstilbestrol	56-53-1	+++	
N0007	17α-ethinyl estradiol	57-63-6	+++	
N0008	Flavone	525-82-6	+	

---

ultimately affect cellular growth and health.

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1251 Abbreviations: CASRN = Chemical Abstracts Service Registry Number; ER = estrogen receptor;  
1252 TA = transcriptional activation *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane

1253 <sup>1</sup>Data on agonist activities were derived from ICCVAM (ICCVAM 2006)

1254 <sup>2</sup>+++ Indicates that the substance was strongly active (EC<sub>50</sub> value was <0.001 μM); ++ indicates that  
1255 the substance was moderately active (EC<sub>50</sub> value was between 0.001 and 0.1 μM); + indicates that  
1256 the substance was weakly active (EC<sub>50</sub> value was >0.1 μM), or a positive response was reported  
1257 without an EC<sub>50</sub> value; - indicates that the substance was uniformly negative in multiple assays.

1258 <sup>3</sup>Information on solubility and cytotoxicity were derived from the scientific literature.

1259  
1260 All data presented for agonist range finding and comprehensive testing have met acceptance criteria. Data  
1261 and tests that did not meet acceptance criteria are discussed in **Section 14**.

1262

### 1263 **10.1 Agonist Range Finding**

1264 Agonist range finding for coded substances consisted of eight-point, logarithmic serial dilutions, with  
1265 each concentration tested in a single well of the 96-well plate. All agonist range finder experiments used  
1266 the same concentrations of test substance (**Table 10-2**). Concentrations for comprehensive testing were  
1267 selected based on the response observed in range finder testing.

1268

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1268 **Table 10-2 Agonist Range Finder Concentrations**  
 1269 **for Coded Substances**

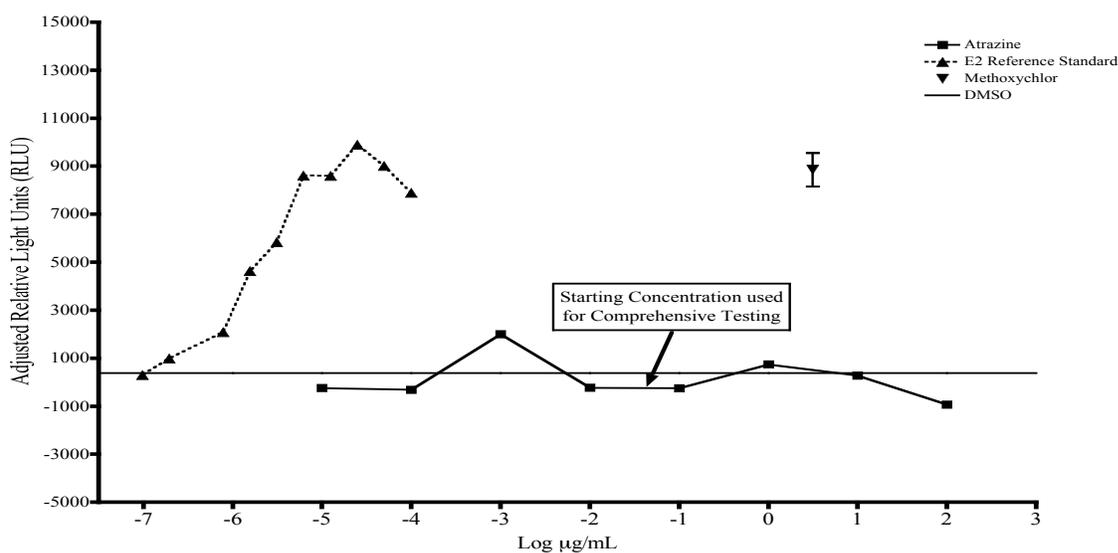
Range Finder Concentrations (µg/mL)		
100	0.1	1.00 x 10 <sup>-4</sup>
10	1.00 x 10 <sup>-2</sup>	1.00 x 10 <sup>-5</sup>
1	1.00 x 10 <sup>-3</sup>	

1270

1271 Results for agonist range finder experiments are presented in **Figures 10-1 through 10-8**.

1272

1273 **Figure 10-1 Agonist Range Finder for N0001 – Atrazine<sup>1</sup>**



1274

1275 Abbreviations: E2 = 17β-estradiol; Methoxychlor = 3.13 µg/mL methoxychlor control; DMSO = dimethyl sulfoxide;

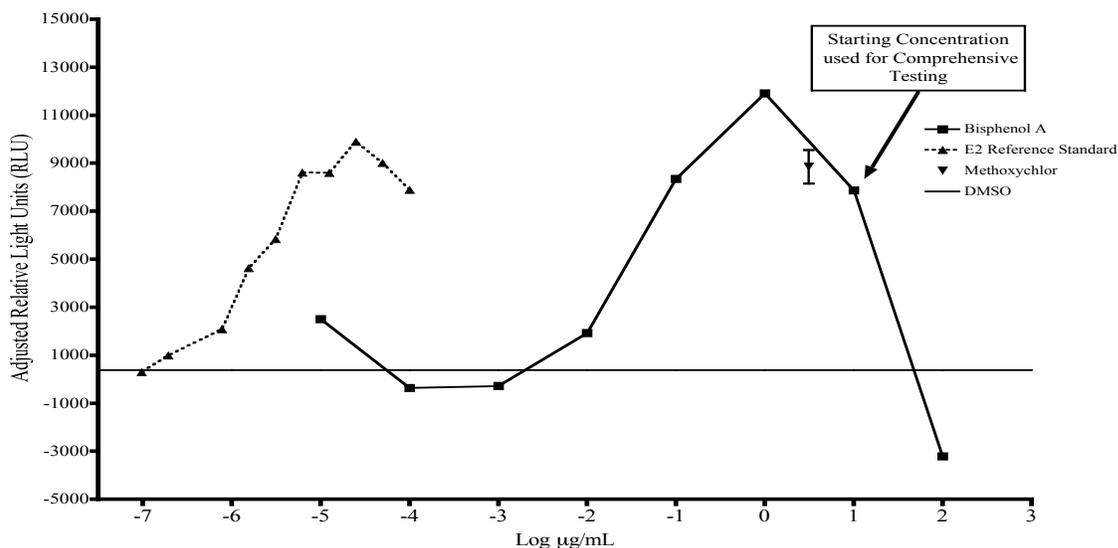
1276 <sup>1</sup>Horizontal line represents the mean of four DMSO control replicates plus three times the standard deviation of the  
 1277 DMSO control mean.

1278

1279

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1279 **Figure 10-2 Agonist Range Finder for N0002 - Bisphenol A<sup>1</sup>**



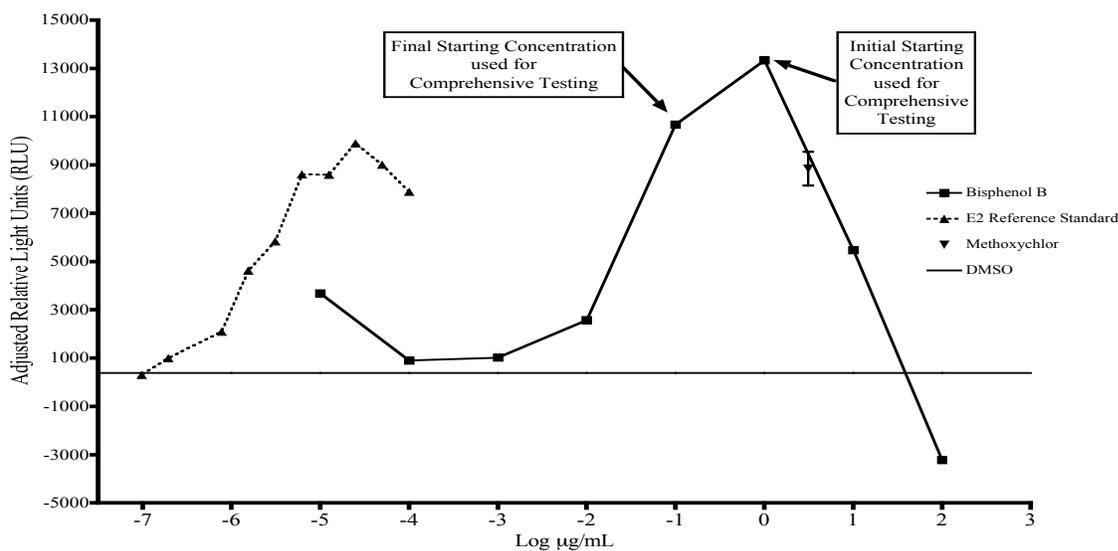
1280

1281 Abbreviations: E2 = 17β-estradiol; Methoxychlor = 3.13 µg/mL methoxychlor control; DMSO = dimethyl sulfoxide;

1282 <sup>1</sup>Horizontal line represents the mean of four DMSO control replicates plus three times the standard deviation of the  
1283 DMSO control mean.

1284

1285 **Figure 10-3 Agonist Range Finder for N0003 - Bisphenol B<sup>1</sup>**



1286

1287 Abbreviations: E2 = 17β-estradiol; Methoxychlor = 3.13 µg/mL methoxychlor control; DMSO = dimethyl sulfoxide;

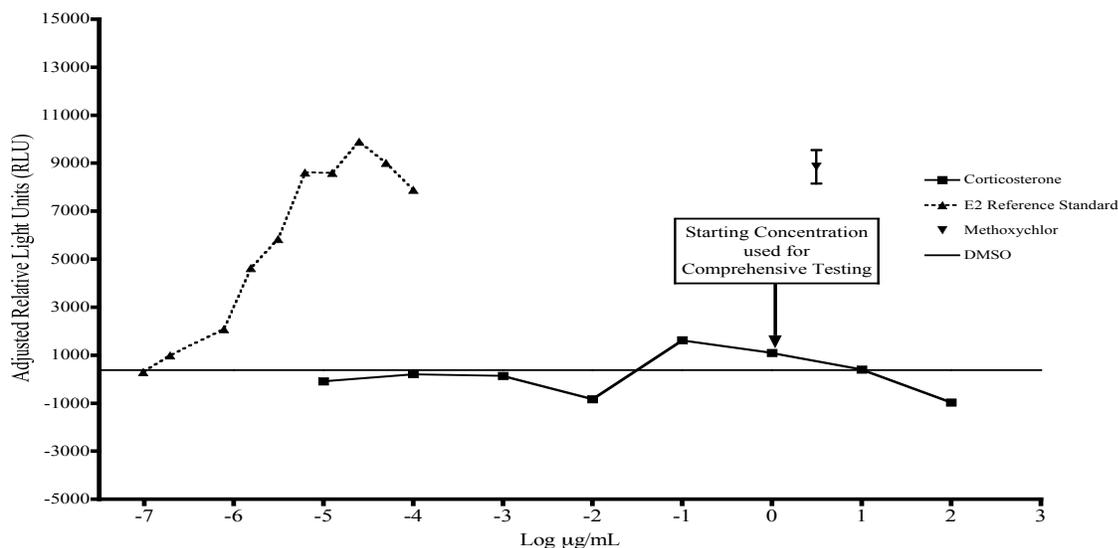
1288 <sup>1</sup>Horizontal line represents the mean of four DMSO control replicates plus three times the standard deviation of the  
1289 DMSO control mean.

1290

1291

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1291 **Figure 10-4 Agonist Range Finder for N0004 – Corticosterone<sup>1</sup>**



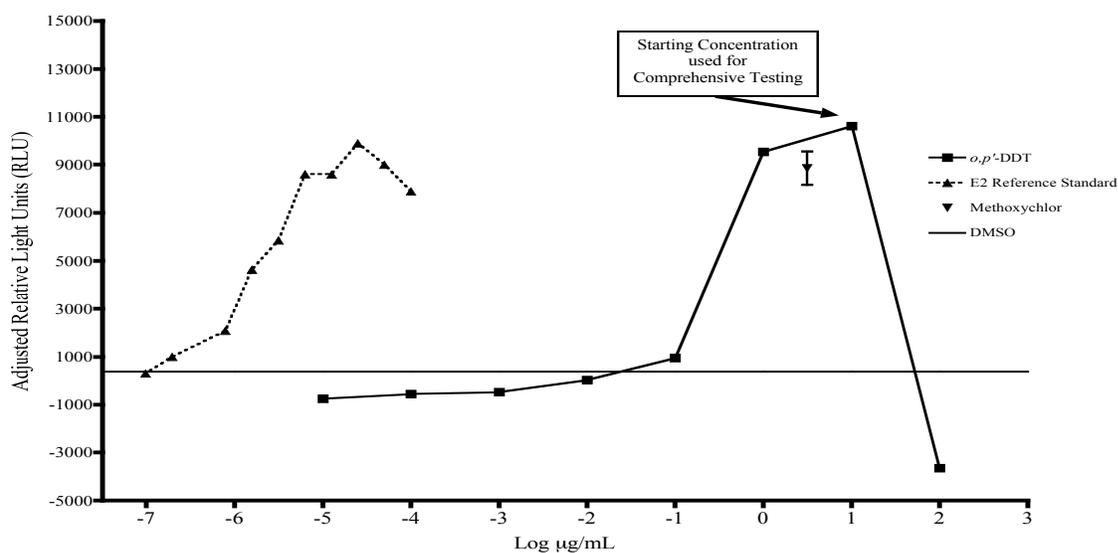
1292

1293 Abbreviations: E2 = 17β-estradiol; Methoxychlor = 3.13 µg/mL methoxychlor control; DMSO = dimethyl sulfoxide;

1294 <sup>1</sup>Horizontal line represents the mean of four DMSO control replicates plus three times the standard deviation of the  
1295 DMSO control mean.

1296

1297 **Figure 10-5 Agonist Range Finder for N0005 - *o,p'*-DDT<sup>1</sup>**



1298

1299 Abbreviations: *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane; E2 = 17β-estradiol; Methoxychlor = 3.13  
1300 µg/mL methoxychlor control; DMSO = dimethyl sulfoxide;

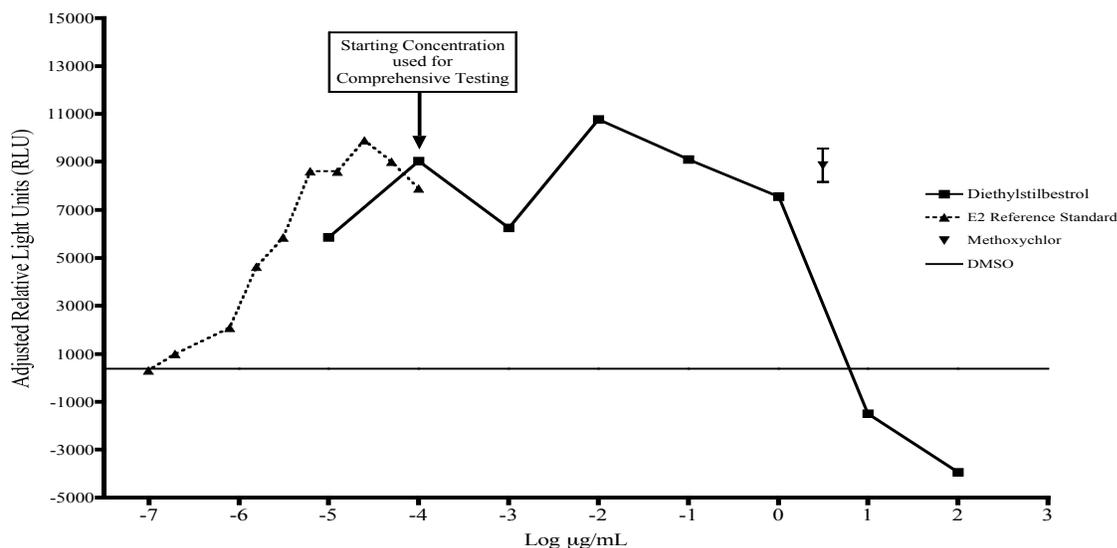
1301 <sup>1</sup>Horizontal line represents the mean of four DMSO control replicates plus three times the standard deviation of the  
1302 DMSO control mean.

1303

1304

PREDECISIONAL MATERIAL: DO NOT CITE, QUOTE OR DISTRIBUTE

1304 **Figure 10-6 Agonist Range Finder for N0006 – Diethylstilbestrol<sup>1</sup>**



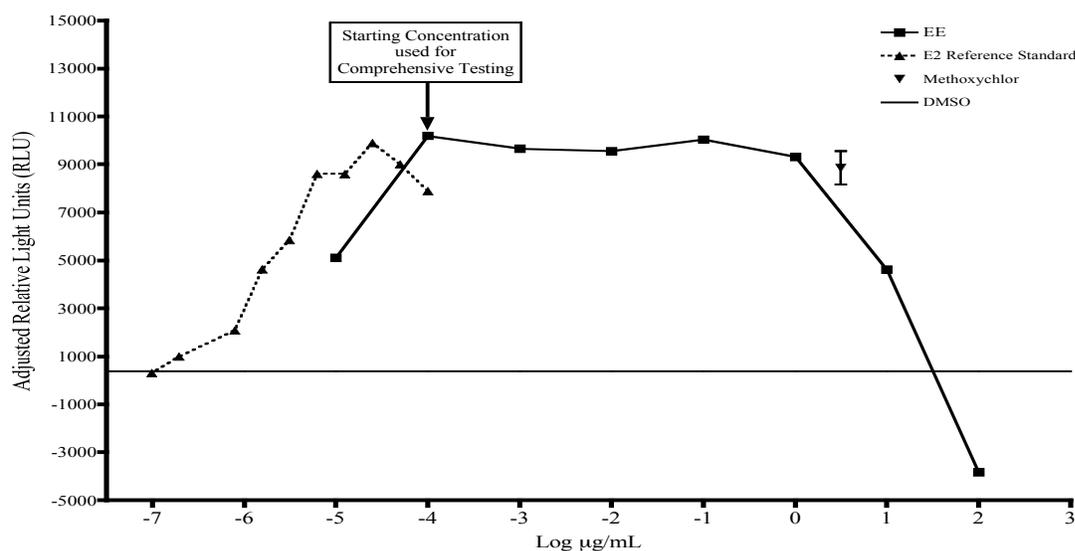
1305

1306 Abbreviations: E2 = 17β-estradiol; Methoxychlor = 3.13 µg/mL methoxychlor control; DMSO = dimethyl sulfoxide;

1307 <sup>1</sup>Horizontal line represents the mean of four DMSO control replicates plus three times the standard deviation of the  
 1308 DMSO control mean.

1309

1310 **Figure 10-7 Agonist Range Finder for N0007 – EE<sup>1</sup>**



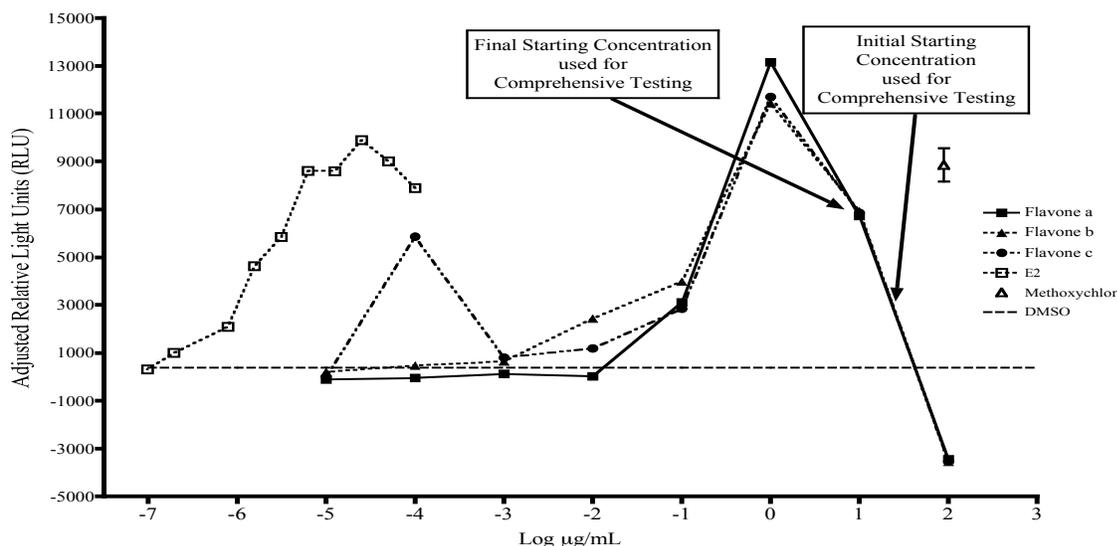
1311

1312 Abbreviations: EE = 17α-ethinyl estradiol; E2 = 17β-estradiol; Methoxychlor = 3.13 µg/mL methoxychlor control; DMSO = dimethyl  
 1313 sulfoxide;

1314 <sup>1</sup>Horizontal line represents the mean of four DMSO control replicates plus three times the standard deviation of the  
 1315 DMSO control mean.

1316

PREDECISIONAL MATERIAL: DO NOT CITE, QUOTE OR DISTRIBUTE

1316 **Figure 10-8 Agonist Range Finder for N0008 – Flavone<sup>1,2,3</sup>**

1317

1318 Abbreviations: E2 = 17 $\beta$ -estradiol; Methoxychlor = 3.13  $\mu\text{g/mL}$  methoxychlor control; DMSO = dimethyl sulfoxide;1319 <sup>1</sup>Horizontal line represents the mean of four DMSO control replicates plus three times the standard deviation of the  
1320 DMSO control mean.1321 <sup>2</sup>Each line represents a single flavone experiment replicate. Flavone range finding was repeated in triplicate  
1322 after an abnormal initial range finder experiment.1323 <sup>3</sup>The 3.13  $\mu\text{g/mL}$  methoxychlor controls are not shown at the concentration at which they were tested. They have been  
1324 placed on the graph in such a way as to maximize visibility.1325  
1326 Due to concerns about possible experimental error, flavone range finding was repeated in triplicate (see  
1327 also **Section 15.0**).1328  
1329 Visual observations for cell viability were conducted for all experimental plates just prior to BG1LUC ER  
1330 TA evaluation. Cell viability testing (i.e., CellTiter-Glo<sup>®</sup>) was conducted in parallel plates on the same  
1331 day. Comparisons of cell viability data from CellTiter-Glo<sup>®</sup> assays and visual observations are shown in  
1332 **Table 10-3**.

1333

1334

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1334 **Table 10-3 CellTiter-Glo® and Visual Observation Data for Agonist Range Finder Experiments**

Substance	Concentration (µg/mL)	CellTiter-Glo®	Visual Observation Score <sup>1</sup>
N0001 - Atrazine	100	93%	1
	10	104%	1
	1	99%	1
	0.1	99%	1
	1.00 x 10 <sup>-2</sup>	107%	1
	1.00 x 10 <sup>-3</sup>	90%	1
	1.00 x 10 <sup>-4</sup>	98%	1
	1.00 x 10 <sup>-5</sup>	107%	1
N0002 - Bisphenol A	<b>100<sup>2</sup></b>	<b>6%</b>	<b>4</b>
	10	105%	1
	1	99%	1
	0.1	108%	1
	1.00 x 10 <sup>-2</sup>	105%	1
	1.00 x 10 <sup>-3</sup>	95%	1
	1.00 x 10 <sup>-4</sup>	109%	1
	1.00 x 10 <sup>-5</sup>	96%	1
N0003 - Bisphenol B	<b>100</b>	<b>6%</b>	<b>4</b>
	10	102%	1
	1	100%	1
	0.1	105%	1
	1.00 x 10 <sup>-2</sup>	108%	1
	1.00 x 10 <sup>-3</sup>	106%	1
	1.00 x 10 <sup>-4</sup>	102%	1
	1.00 x 10 <sup>-5</sup>	102%	1
N0004 - Corticosterone	100	80%	2
	10	94%	1
	1	97%	1
	0.1	102%	1
	1.00 x 10 <sup>-2</sup>	104%	1
	1.00 x 10 <sup>-3</sup>	103%	1
	1.00 x 10 <sup>-4</sup>	107%	1
	1.00 x 10 <sup>-5</sup>	103%	1
N0005 - <i>o,p'</i> -DDT	<b>100</b>	<b>12%</b>	<b>4</b>
	10	94%	1
	1	103%	1
	0.1	93%	1
	1.00 x 10 <sup>-2</sup>	97%	1
	1.00 x 10 <sup>-3</sup>	101%	1
	1.00 x 10 <sup>-4</sup>	101%	1
	1.00 x 10 <sup>-5</sup>	108%	1

1335 Abbreviations: *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane; EE = 17α-ethinyl estradiol1336 <sup>1</sup>Visual observations are scored using the scale provide in **Table 7.1**1337 <sup>2</sup>Bolded text indicates substances and concentrations that caused a decrease in cell viability below 80%

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1338 **Table 10-3 (Continued) CellTiter-Glo® and Visual Observation Data for Agonist Range Finder**1339 **Experiments**

Substance	Concentration (µg/mL)	CellTiter-Glo®	Visual Observation Score <sup>1</sup>
N0006 - Diethylstilbestrol	<b>100</b>	<b>6%</b>	<b>4</b>
	10	111%	1
	1	111%	1
	0.1	107%	1
	1.00 x 10 <sup>-2</sup>	99%	1
	1.00 x 10 <sup>-3</sup>	92%	1
	1.00 x 10 <sup>-4</sup>	96%	1
	1.00 x 10 <sup>-5</sup>	101%	1
N0007 - EE	<b>100</b>	<b>30%</b>	<b>3</b>
	10	96%	1
	1	104%	1
	0.1	107%	1
	1.00 x 10 <sup>-2</sup>	112%	1
	1.00 x 10 <sup>-3</sup>	104%	1
	1.00 x 10 <sup>-4</sup>	102%	1
	1.00 x 10 <sup>-5</sup>	93%	1
N0008 - Flavone	<b>100</b>	<b>12%</b>	<b>4</b>
	10	92%	1
	1	102%	1
	0.1	103%	1
	1.00 x 10 <sup>-2</sup>	101%	1
	1.00 x 10 <sup>-3</sup>	92%	1
	1.00 x 10 <sup>-4</sup>	102%	1
	1.00 x 10 <sup>-5</sup>	100%	1

1340 Abbreviations: *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane; EE = 17 $\alpha$ -ethinyl estradiol1341 <sup>1</sup>Visual observations are scored using the scale provide in **Table 7.1**1342 <sup>2</sup>Bolded text indicates substances and concentrations that caused a decrease in cell viability below 80%

1343

1344 Six of the eight substances caused a decrease in cell viability at the highest concentration used for range

1345 finder testing. The decrease in cell viability was observed with both visual observations and CellTiter-

1346 Glo®.

1347

1348 **10.2 Agonist Comprehensive Testing**1349 **10.2.1 N0001 – Atrazine**

1350 Atrazine was selected for agonist testing because it was listed as negative for ER agonist activity in the

1351 ICCVAM Guidelines (ICCVAM 2003, 2006) and was indicated as potentially cytotoxic (Freyberg 2005).

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1352 The highest concentration of atrazine used for comprehensive testing was  $1.00 \times 10^{-2}$   $\mu\text{g/mL}$ . This  
1353 concentration was selected as the starting point for a double serial dilution because it was a single log  
1354 dilution higher than the concentration giving the highest adjusted RLU value during range finder testing.  
1355 The concentrations of atrazine tested are listed in **Table 10-4**.

1356

1357 **Table 10-4**      **Concentrations of N0001 - Atrazine Used in**  
1358                      **Comprehensive Testing**

N0001 – Atrazine ( $\mu\text{g/mL}$ )		
$1.00 \times 10^{-2}$	$6.25 \times 10^{-4}$	$3.91 \times 10^{-5}$
$5.00 \times 10^{-3}$	$3.13 \times 10^{-4}$	$1.95 \times 10^{-5}$
$2.50 \times 10^{-3}$	$1.56 \times 10^{-4}$	$9.77 \times 10^{-5}$
$1.25 \times 10^{-3}$	$7.81 \times 10^{-5}$	

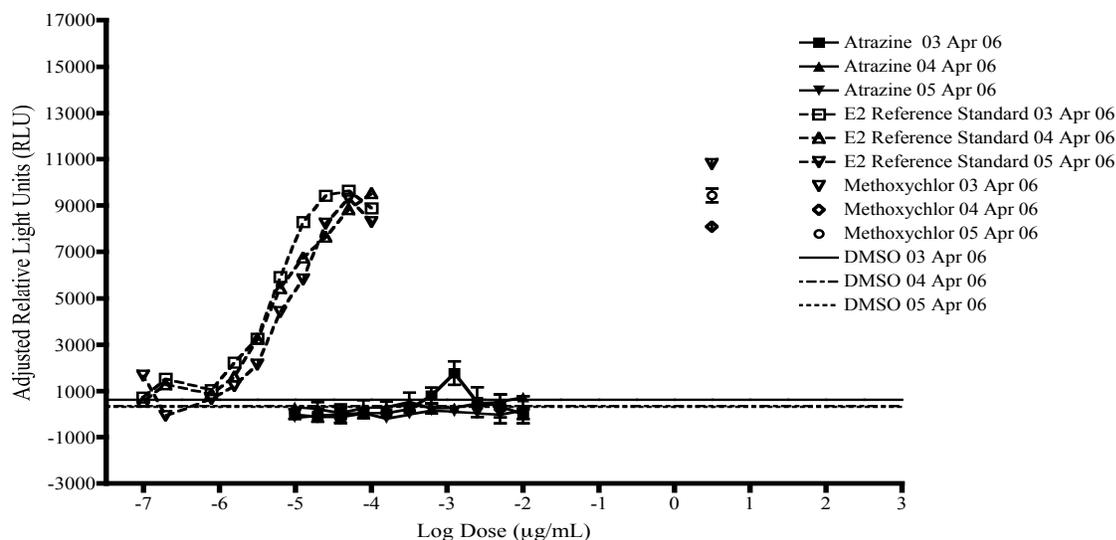
1359

1360 Results of individual agonist experiments for atrazine are shown in **Figure 10-9**.

1361

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1361 **Figure 10-9 Agonist Comprehensive Testing for N0001 – Atrazine:**  
 1362 **Individual Experiments<sup>1</sup>**



1363

1364 Abbreviations: E2 = 17 $\beta$ -estradiol; Methoxychlor = 3.13  $\mu$ g/mL methoxychlor control; DMSO = dimethyl sulfoxide;

1365 <sup>1</sup>Horizontal lines represent the mean of four DMSO control replicates plus three times the standard deviation of the DMSO  
 1366 control mean. Values must be above this line in order to be considered positive for agonism.

1367

1368 Atrazine was negative for agonism at all concentrations tested on 4 April 06 and 5 April 06. On 3 April  
 1369 06, one concentration of atrazine ( $1.25 \times 10^{-3}$   $\mu$ g/mL) yielded a positive response. However, because this  
 1370 response was only observed for a single concentration in a single experiment, atrazine was classified as a  
 1371 negative for agonism.

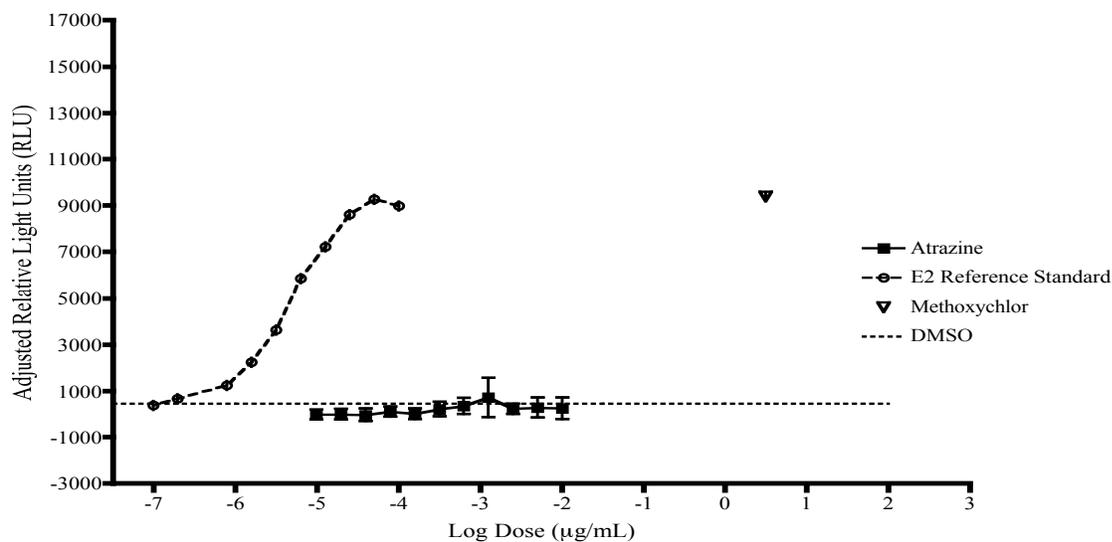
1372

1373 Results of averaged agonist experiments for atrazine are shown in **Figure 10-10**.

1374

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1374 **Figure 10-10 Agonist Comprehensive Testing for N0001 – Atrazine:**  
 1375 **Averaged Experiments 1,2,3**



1376  
 1377

Abbreviations: E2 = 17β-estradiol; Methoxychlor = 3.13 µg/mL methoxychlor control; DMSO = dimethyl sulfoxide.

1378 <sup>1</sup>Historical mean and standard deviation of the E2 reference standard.

1379 <sup>2</sup>Historical mean and standard deviation of the methoxychlor control.

1380 <sup>3</sup>Horizontal line represents the historical mean of DMSO vehicle control plus three times the standard

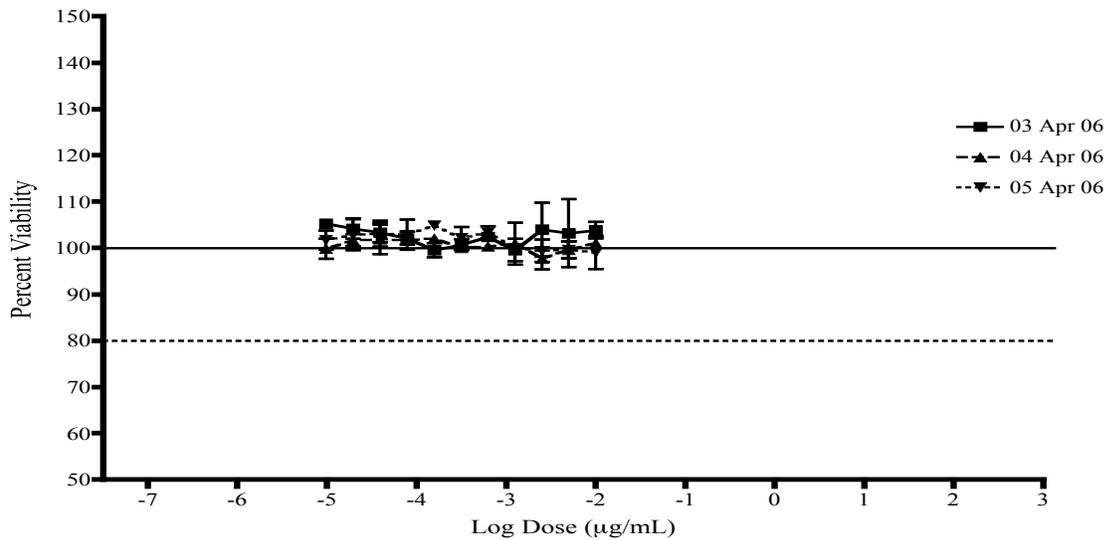
1381 deviation of the DMSO control mean. Values must be above this line in order to be considered positive for agonism.

1382  
 1383 Atrazine did not decrease cell viability in range finder or comprehensive testing at any concentration  
 1384 tested (**Figures 10-11, 10-12 and 10-13**).

1385  
 1386

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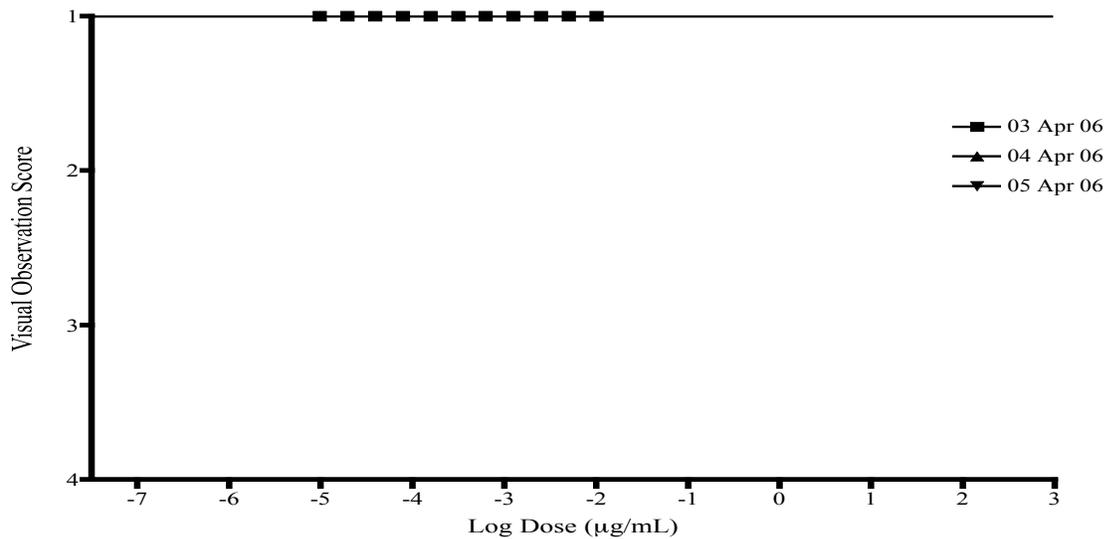
1386 **Figure 10-11 CellTiter-Glo® Viability Assessment for N0001 – Atrazine1,2**



1387  
1388 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

1389  
1390 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability below this line are considered cytotoxic and are not included in the evaluation of agonist activity.

1391  
1392 **Figure 10-12 Visual Observation Viability Assessment for N0001 –**  
1393 **Atrazine1**



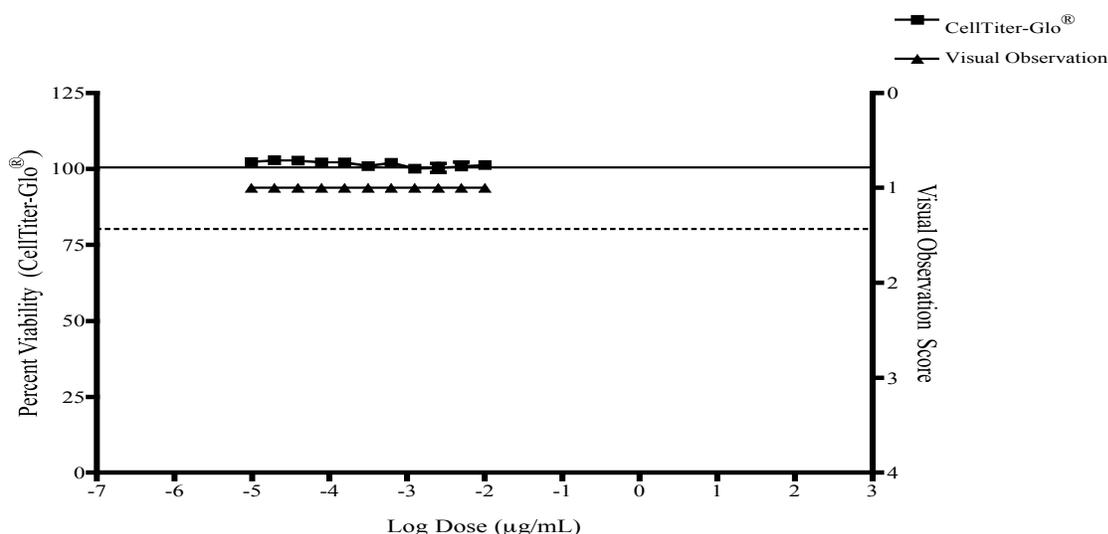
1394  
1395  
1396 <sup>1</sup>Wells containing cells that exhibit normal morphology and density are given a visual observation score of 1. Wells  
1397 that are exposed to concentrations resulting in visual observation scores  $\geq 2$  are considered cytotoxic and are not  
included in the evaluation of antagonist activity.

1398

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1399 **Figure 10-13 Combined Qualitative and Quantitative Viability**

1400 **Assessments for N0001 – Atrazine<sup>1,2</sup>**



1401 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

1402 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability below this line are considered cytotoxic and are not included in the evaluation of agonist activity.

1406 **10.2.2N0002 – Bisphenol A**

1407 Bisphenol A was selected for agonist testing because it was listed as weakly positive for ER agonist  
1408 activity in the ICCVAM Guidelines (ICCVAM 2003, 2006).

1409 The highest concentration of bisphenol A used in comprehensive testing was 10 µg/mL. This  
1410 concentration was selected as the starting point because it was a single log dilution higher than the  
1411 concentration giving the highest adjusted RLU value during range finder testing. The concentrations of  
1412 bisphenol A tested are listed in **Table 10-5**.

1414 **Table 10-5 Concentrations of N0002 - Bisphenol A**  
1415 **used in Comprehensive Testing**

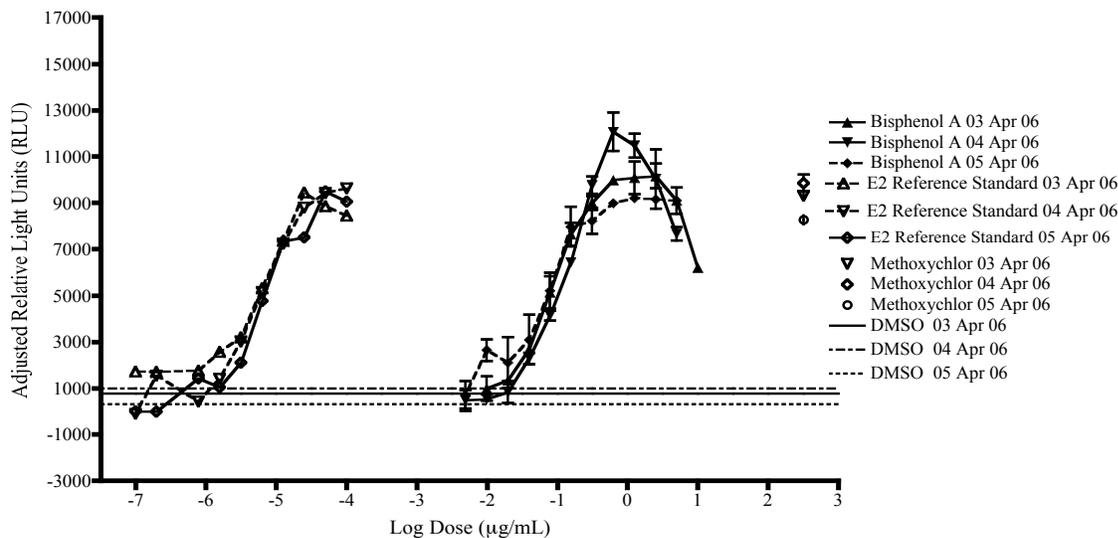
N0002 – Bisphenol A (µg/mL)		
10	0.63	$3.91 \times 10^{-2}$
5	0.31	$1.95 \times 10^{-2}$
2.5	0.16	$9.77 \times 10^{-3}$
1.25	$7.81 \times 10^{-2}$	

1416

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1417 Results of individual agonist experiments for bisphenol A are shown in **Figure 10-14**.

1418 **Figure 10-14 Agonist Comprehensive Testing for N0002 – Bisphenol A: Individual**  
 1419 **Experiments 1,2**



1420

1421 Abbreviations: E2 = 17 $\beta$ -estradiol; Methoxychlor = 3.13  $\mu$ g/mL methoxychlor control; DMSO = dimethyl sulfoxide.

1422 <sup>1</sup>Horizontal lines represent the mean of four DMSO control replicates plus three times the standard deviation of the DMSO  
 1423 control mean. Values must be above this line in order to be considered positive for agonism.

1424 <sup>2</sup>The 3.13  $\mu$ g/mL methoxychlor controls are not shown at the concentration at which they were tested. They have been placed  
 1425 on the graph in such a way as to maximize visibility.

1426

1427 Bisphenol A showed agonist activity in the three experiments that were conducted. EC<sub>50</sub> values for  
 1428 individual experiments are shown in **Table 10-6**.

1429

1430 **Table 10-6 Individual EC<sub>50</sub> Values for N0002 – Bisphenol A**

Experiment Date	EC <sub>50</sub> ( $\mu$ g/mL)
3 April 06	$7.55 \times 10^{-2}$
4 April 06	0.11
5 April 06	$8.00 \times 10^{-2}$

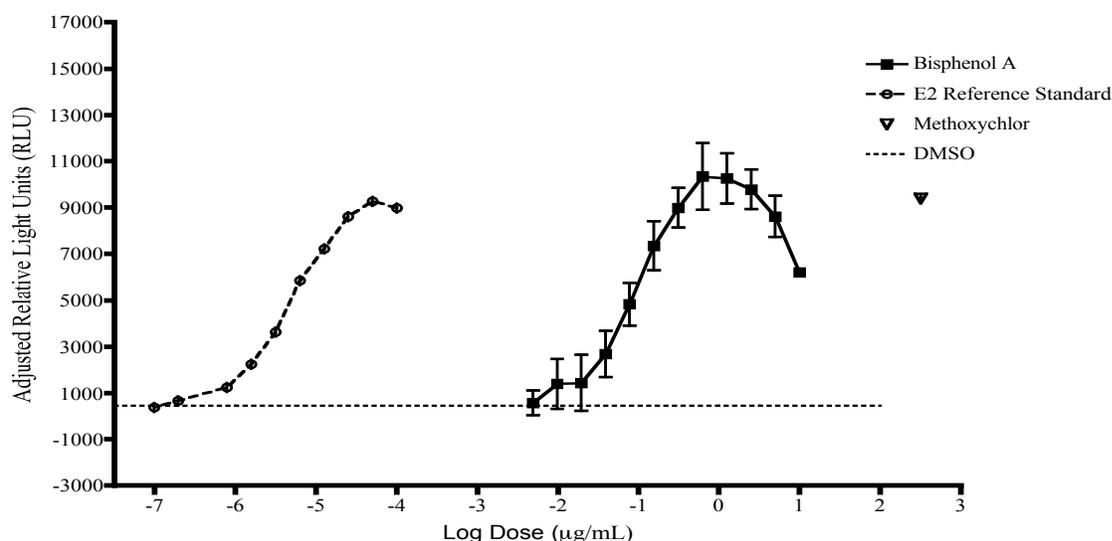
1431 Abbreviations: EC<sub>50</sub> = half-maximal effect concentration

1432 Results of averaged agonist experiments for bisphenol A are shown in **Figure 10-15**.

1433

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1434 **Figure 10-15 Agonist Comprehensive Testing for N0002 –**  
 1435 **Bisphenol A: Averaged Experiments<sup>1,2,3,4</sup>**



1436

1437 Abbreviations: E2 = 17 $\beta$ -estradiol; Methoxychlor = 3.13  $\mu$ g/mL methoxychlor control; DMSO = dimethyl sulfoxide.

1438 <sup>1</sup>Historical mean and standard deviation of the E2 reference standard.

1439 <sup>2</sup>Historical mean and standard deviation of the methoxychlor control).

1440 <sup>3</sup>Horizontal line represents the historical mean of DMSO vehicle control plus three times the standard  
 1441 deviation of the DMSO control mean. Values must be above this line in order to be considered positive for agonism.

1442 <sup>4</sup>The 3.13  $\mu$ g/mL methoxychlor controls are not shown at the concentration at which they were tested. They have been placed on the graph in  
 1443 such a way as to maximize visibility.

1444

1445 Bisphenol A was positive for agonism at all but the lowest concentration tested ( $7.96 \times 10^{-2}$   $\mu$ g/mL). The  
 1446 averaged EC<sub>50</sub> value (**Table 10-7**) was calculated as the mean of three experiments.

1447

1448 **Table 10-7 Averaged EC<sub>50</sub> Value for N0002 – Bisphenol A**

EC <sub>50</sub> ( $\mu$ g/mL)	STD DEV	CV
$8.76 \times 10^{-2}$	$1.75 \times 10^{-2}$	20%

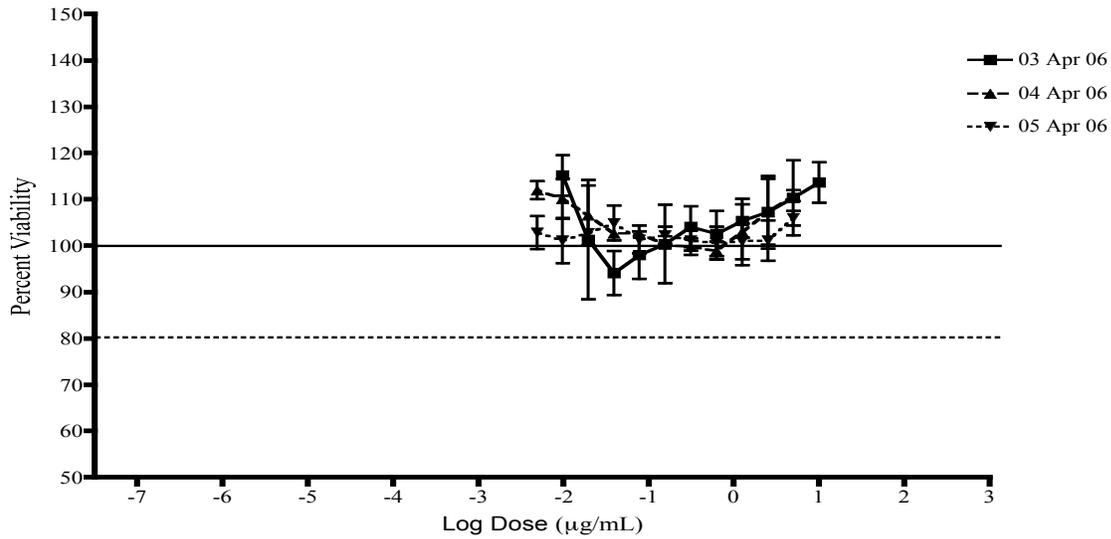
1449 Abbreviations: EC<sub>50</sub> = half-maximal effect concentration; STD DEV = Standard  
 1450 Deviation of the Mean; CV = Coefficient of Variation

1451

1452 Bisphenol decreased cell viability at the highest concentration tested in the range finder (100  $\mu$ g/mL), but  
 1453 did not decrease cell viability at any concentration tested in comprehensive testing, (**Figures 10-16, 10-  
 1454 17, and 10-18**).

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1455 **Figure 10-16 CellTiter-Glo® Viability Assessment for N0002 – Bisphenol A<sup>1,2</sup>**



1456

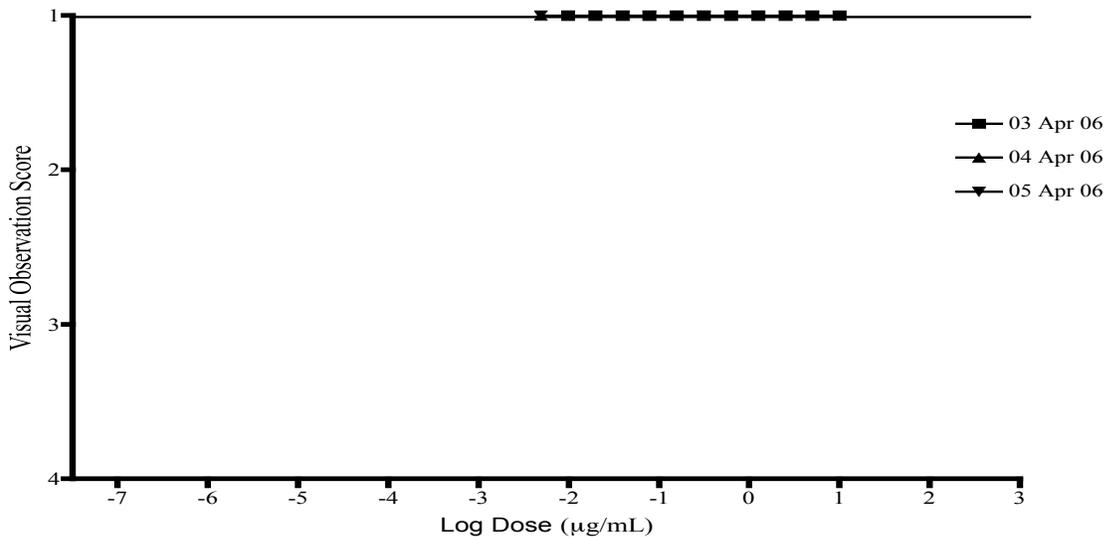
1457 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

1458 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability

1459 below this line are considered cytotoxic and are not included in the evaluation of agonist activity.

1460

1461 **Figure 10-17 Visual Observation Viability Assessment for N0002 – Bisphenol A<sup>1</sup>**



1462 <sup>1</sup>Wells containing cells that exhibit normal morphology and density are given a visual observation score of 1. Wells

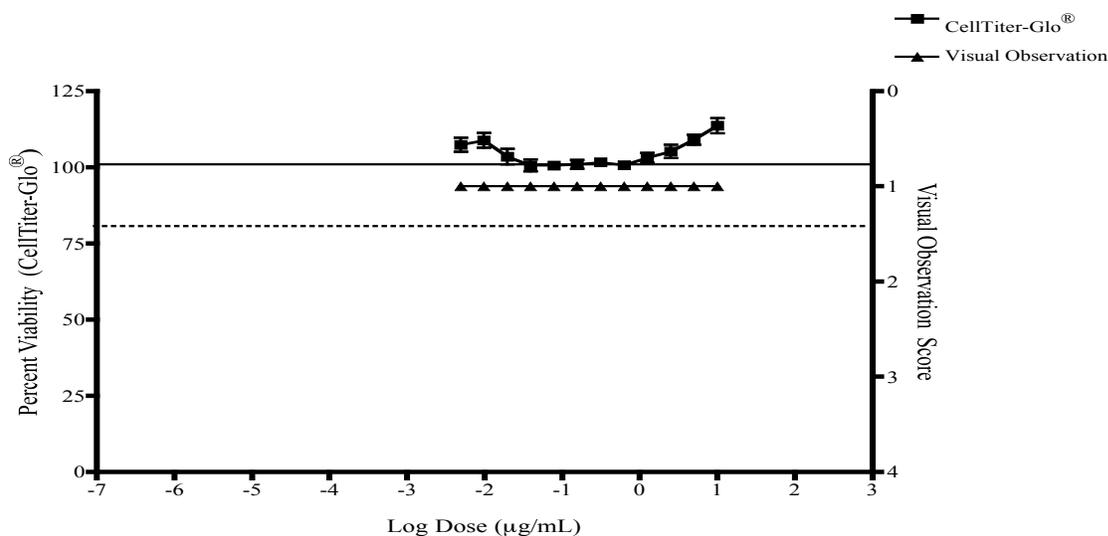
1463 that are exposed to concentrations resulting in visual observation scores  $\geq 2$  are considered cytotoxic and are not

1464 included in the evaluation of antagonist activity.

1465

1466

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1466 **Figure 10-18 Combined Qualitative and Quantitative Viability**1467 **Assessment for N0002 – Bisphenol A<sup>1,2</sup>**

1468

1469 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.1470 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability  
1471 below this line are considered cytotoxic and are not included in the evaluation of agonist activity.

1472

1473 **10.2.3N0003 – Bisphenol B**1474 Bisphenol B was selected for agonist testing because it was listed as moderately positive for ER agonist  
1475 activity in the ICCVAM Guidelines (ICCVAM 2003, 2006).

1476 The highest concentration of bisphenol B used in comprehensive testing was 1.25 µg/mL. This  
 1477 concentration was selected as the starting point for a double serial dilution because it was within one log  
 1478 dilution of the concentration giving the highest adjusted RLU value during range finder testing. The  
 1479 concentrations of bisphenol B tested are listed in **Table 10-8**. Initial comprehensive testing indicated that  
 1480 there were an insufficient number of concentrations to demonstrate baseline activity at the lower end of  
 1481 the concentration-response curve. The new starting concentration for bisphenol B was 0.63 µg/mL, and an  
 1482 additional concentration of  $6.10 \times 10^{-4}$  µg/mL was added.

1483

1484

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1484 **Table 10-8 Concentrations of N0003 - Bisphenol B**  
 1485 **Used in Comprehensive Testing**

N0003 – Bisphenol B (µg/mL)		
1.25 <sup>*</sup>	7.81 x 10 <sup>-2</sup>	4.88 x 10 <sup>-3</sup>
0.63 <sup>#</sup>	3.91 x 10 <sup>-2</sup>	2.44 x 10 <sup>-3</sup>
0.31	1.95 x 10 <sup>-2</sup>	1.22 x 10 <sup>-3</sup>
0.16	9.77 x 10 <sup>-3</sup>	6.10 x 10 <sup>-4</sup>

1486 <sup>\*</sup>Final starting concentration for bisphenol B testing

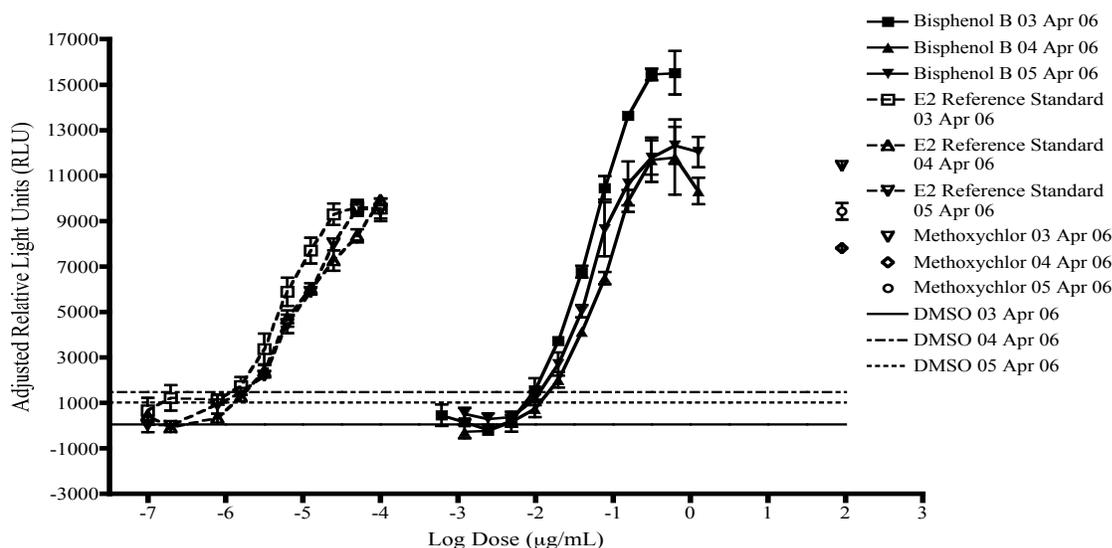
1487 <sup>#</sup>Initial starting concentration for bisphenol B testing

1488

1489 Results of individual agonist experiments for bisphenol B are shown in **Figure 10-19**.

1490

1491 **Figure 10-19 Agonist Comprehensive Testing for N0003 –**  
 1492 **Bisphenol B: Individual Experiments<sup>1,2</sup>**



1493

1494 Abbreviations: Abbreviations: E2 = 17β-estradiol; Methoxychlor = 3.13 µg/mL methoxychlor control; DMSO = dimethyl sulfoxide.

1495 <sup>1</sup>Horizontal lines represent the mean of four DMSO control replicates plus three times the standard deviation of the DMSO  
 1496 control mean. Values must be above this line in order to be considered positive for agonism.

1497 <sup>2</sup>The 3.13 µg/mL methoxychlor controls are not shown at the concentration at which they were tested. They have been placed on  
 1498 the graph in such a way as to maximize visibility of these controls.

1499

1500 Bisphenol B showed agonist activity at the majority of concentrations tested. EC<sub>50</sub> values for individual  
 1501 experiments are shown in **Table 10-9**.

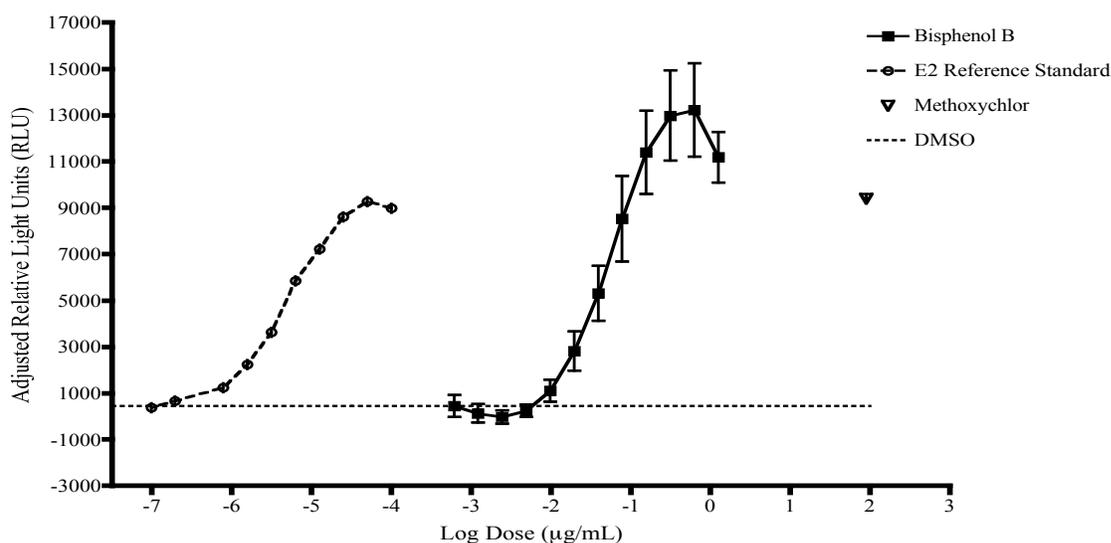
PREDECISIONAL MATERIAL: DO NOT CITE, QUOTE OR DISTRIBUTE

1502 **Table 10-9 Individual EC<sub>50</sub> Values for N0003 – Bisphenol B**

Experiment Date	EC <sub>50</sub> (µg/mL)
3 April 06	4.90 x 10 <sup>-2</sup>
4 April 06	5.70 x 10 <sup>-2</sup>
5 April 06	4.90 x 10 <sup>-2</sup>

1503 EC<sub>50</sub> = half-maximal effect concentration1504 Results of averaged agonist experiments for bisphenol B are shown in **Figure 10-20**.

1505

1506 **Figure 10-20 Agonist Comprehensive Testing for N0003 –**1507 **Bisphenol B: Averaged Experiments<sup>1,2,3,4</sup>**

1508

1509 Abbreviations: E2 = 17β-estradiol; Methoxychlor = 3.13 µg/mL methoxychlor control; DMSO = dimethyl sulfoxide.

1510 <sup>1</sup>Historical mean and standard deviation of the E2 reference standard.1511 <sup>2</sup>Historical mean and standard deviation of the positive methoxychlor control.1512 <sup>3</sup>Horizontal line represents the historical mean of DMSO vehicle control plus three times the standard deviation of the DMSO control mean.  
1513 Values must be above this line in order to be considered positive for agonism.1514 <sup>4</sup>The 3.13 µg/mL methoxychlor controls are not shown at the concentration at which they were tested. They have been placed  
1515 on the graph in such a way as to maximize visibility.

1516

1517 Bisphenol B was positive for agonism at the majority of concentrations tested. The averaged EC<sub>50</sub> value1518 (**Table 10-10**) was calculated as the mean of three experiments.

1519

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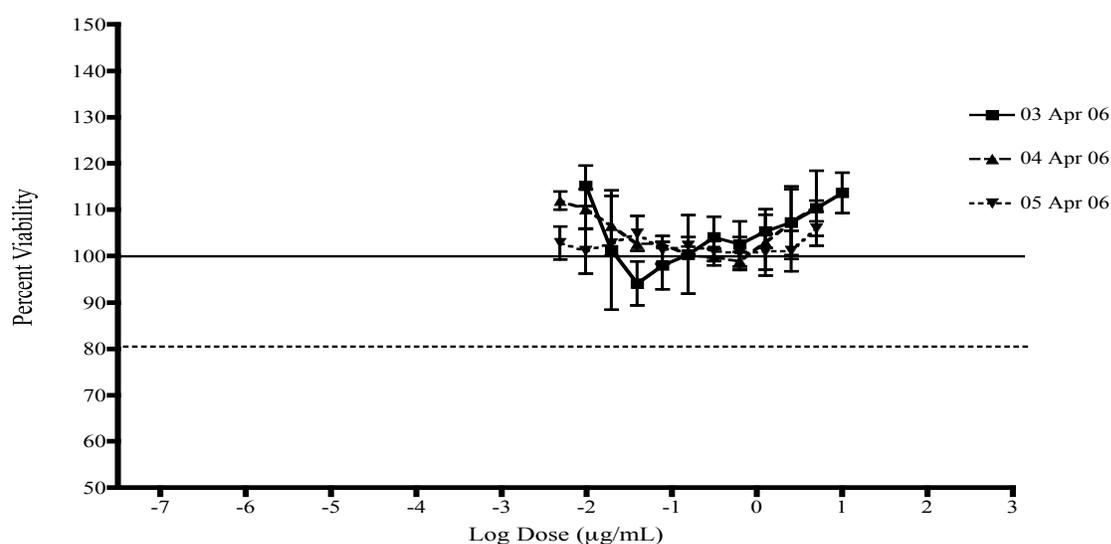
1520 **Table 10-10 Averaged EC<sub>50</sub> Value for N0003 – Bisphenol B**

EC <sub>50</sub> (µg/mL)	STD DEV	CV
5.16 x 10 <sup>-2</sup>	4.63 x 10 <sup>-3</sup>	9%

1521 Abbreviations: EC<sub>50</sub> = half-maximal effect concentration; STD DEV = Standard  
 1522 Deviation of the Mean; CV = Coefficient of Variation

1523  
 1524 Bisphenol B was cytotoxic at the highest concentration tested (100 µg/mL) in the range finder assay, but  
 1525 did not decrease cell viability at any concentration tested in comprehensive testing (**Figures 10-21, 10-22,**  
 1526 **and 10-23**).

1527

1528 **Figure 10-21 CellTiter-Glo<sup>®</sup> Viability Assessment for N0003 – Bisphenol B<sup>1,2</sup>**

1529  
 1530 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

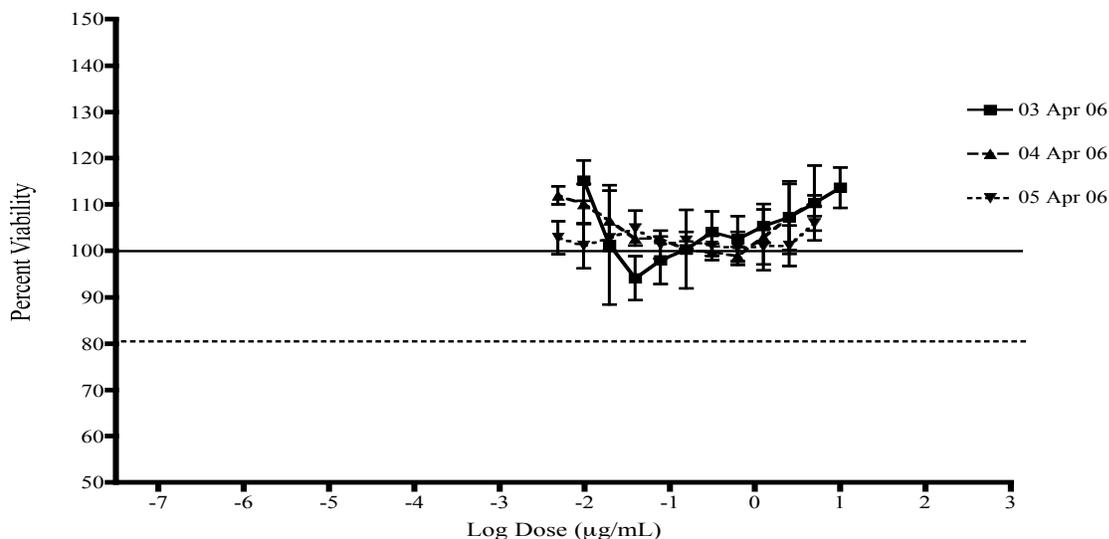
1531  
 1532 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability below this line are considered cytotoxic and are not included in the evaluation of agonist activity.

1533

1534

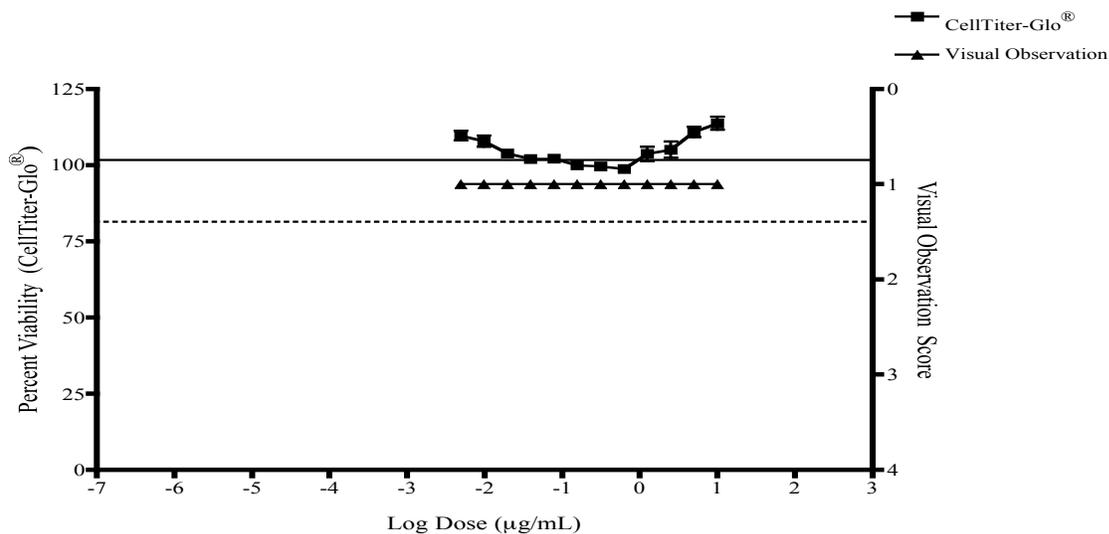
PREDECISIONAL MATERIAL: DO NOT CITE, QUOTE OR DISTRIBUTE

1534 **Figure 10-22 Visual Observation Viability Assessment for**  
 1535 **N0003 – Bisphenol B<sup>1</sup>**



1536  
 1537 <sup>1</sup>Wells containing cells that exhibit normal morphology and density are given a visual observation score of 1. Wells  
 1538 that are exposed to concentrations resulting in visual observation scores  $\geq 2$  are considered cytotoxic and are not  
 1539 included in the evaluation of antagonist activity.

1540  
 1541 **Figure 10-23 Combined Qualitative and Quantitative**  
 1542 **Viability Assessment for N0003 – Bisphenol B<sup>1,2</sup>**



1543  
 1544 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in DMSO control.

1545  
 1546 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability  
 below this line are considered cytotoxic and are not included in the evaluation of agonist activity.

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1547 **10.2.4N0004 – Corticosterone**

1548 Corticosterone was selected for agonist testing because it was listed as negative for ER agonist activity in  
 1549 the ICCVAM Guidelines (ICCVAM 2003, 2006). The highest concentration of corticosterone used in  
 1550 comprehensive testing was 1 µg/mL. This concentration was selected as the starting point for a double  
 1551 serial dilution because it was a single log dilution higher than the concentration giving the highest  
 1552 adjusted RLU value during range finder testing. The concentrations of corticosterone tested are listed in  
 1553 **Table 10-11**.

1554

1555 **Table 10-11 Concentrations of N0004 - Corticosterone**  
 1556 **used in Comprehensive Testing**

N0004 – Corticosterone (µg/mL)		
1	$6.25 \times 10^{-2}$	$3.91 \times 10^{-3}$
0.5	$3.13 \times 10^{-2}$	$1.95 \times 10^{-3}$
0.25	$1.56 \times 10^{-2}$	$9.77 \times 10^{-4}$
0.13	$7.81 \times 10^{-3}$	

1557

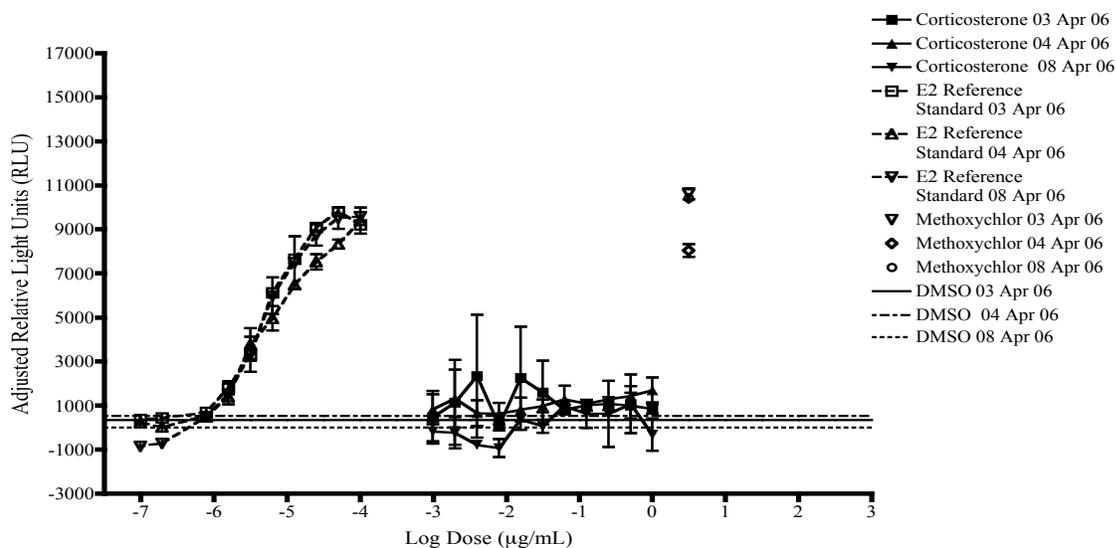
1558 Results of individual agonist experiments for corticosterone are shown in **Figure 10-24**.

1559

1560

PREDECISIONAL MATERIAL: DO NOT CITE, QUOTE OR DISTRIBUTE

1560 **Figure 10-24 Agonist Comprehensive Testing for N0004 –**  
 1561 **Corticosterone: Individual Experiments<sup>1</sup>**



1562  
 1563

Abbreviations: E2 = 17 $\beta$ -estradiol; Methoxychlor = 3.13  $\mu$ g/mL methoxychlor control; DMSO = dimethyl sulfoxide.

1564  
 1565

<sup>1</sup>Horizontal lines represent the mean of four DMSO control replicates plus three times the standard deviation of the DMSO control mean. Values must be above this line in order to be considered positive for agonism

1566

1567 On 3 April 06, there were three concentrations of corticosterone ( $3.91 \times 10^{-3}$ ,  $1.56 \times 10^{-2}$ ,  $3.13 \times 10^{-2}$   
 1568  $\mu$ g/mL) that yielded a positive response. However, this response was only observed in a single  
 1569 experiment, so corticosterone was classified as negative for agonism.

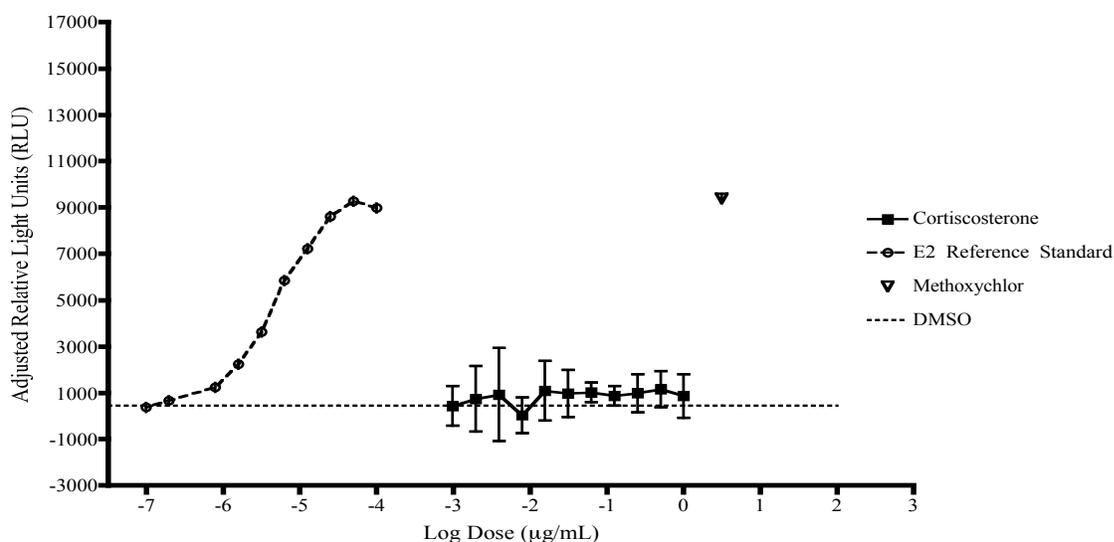
1570

1571 Results of averaged agonist experiments for corticosterone are shown in **Figure 10-25**.

1572  
 1573

PREDECISIONAL MATERIAL: DO NOT CITE, QUOTE OR DISTRIBUTE

1573 **Figure 10-25 Agonist Comprehensive Testing for N0004 – Corticosterone:**  
 1574 **Averaged Experiments<sup>1,2,3</sup>**



1575  
 1576

Abbreviations: E2 = 17 $\beta$ -estradiol; Methoxychlor = 3.13  $\mu$ g/mL methoxychlor control; DMSO = dimethyl sulfoxide.

1577 <sup>1</sup>Historical mean and standard deviation of the E2 reference standard.

1578 <sup>2</sup>Historical mean and standard deviation of the methoxychlor control.

1579 <sup>3</sup>Horizontal line represents the historical mean of DMSO vehicle control plus three times the standard

1580 deviation of the DMSO control mean. Values must be above this line in order to be considered positive for agonism.

1581

1582 Corticosterone was negative for agonism at all concentrations tested.

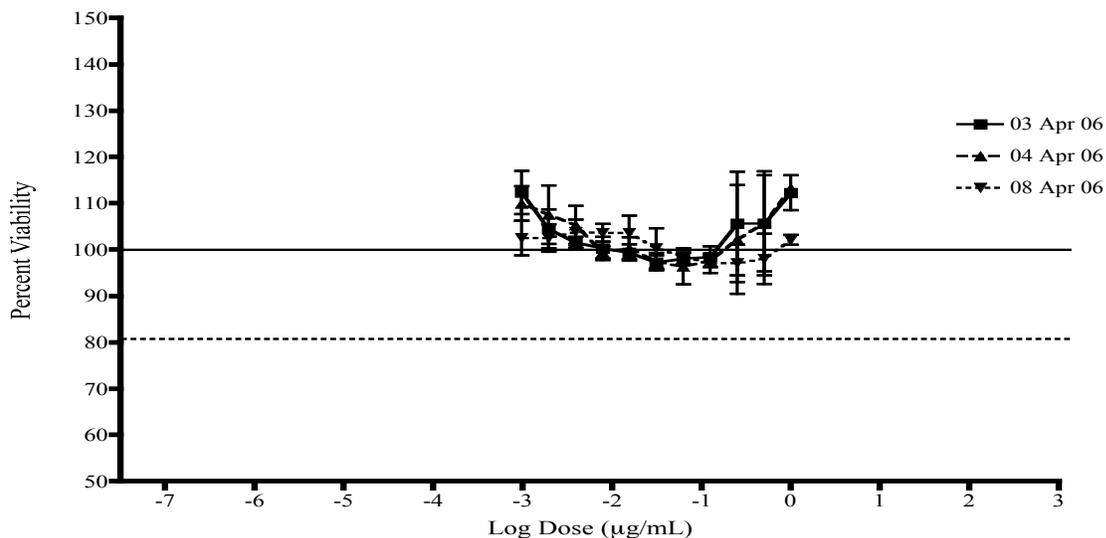
1583 Corticosterone was cytotoxic at the highest concentration tested (100  $\mu$ g/mL) in the range finder, but did  
 1584 not decrease cell viability at any concentration tested in comprehensive testing (**Figures 10-26, 10-27,**  
 1585 **and 10-28**).

1586

1587

PREDECISIONAL MATERIAL: DO NOT CITE, QUOTE OR DISTRIBUTE

1587 **Figure 10-26 CellTiter-Glo® Viability Assessment for N0004 – Corticosterone<sup>1,2</sup>**

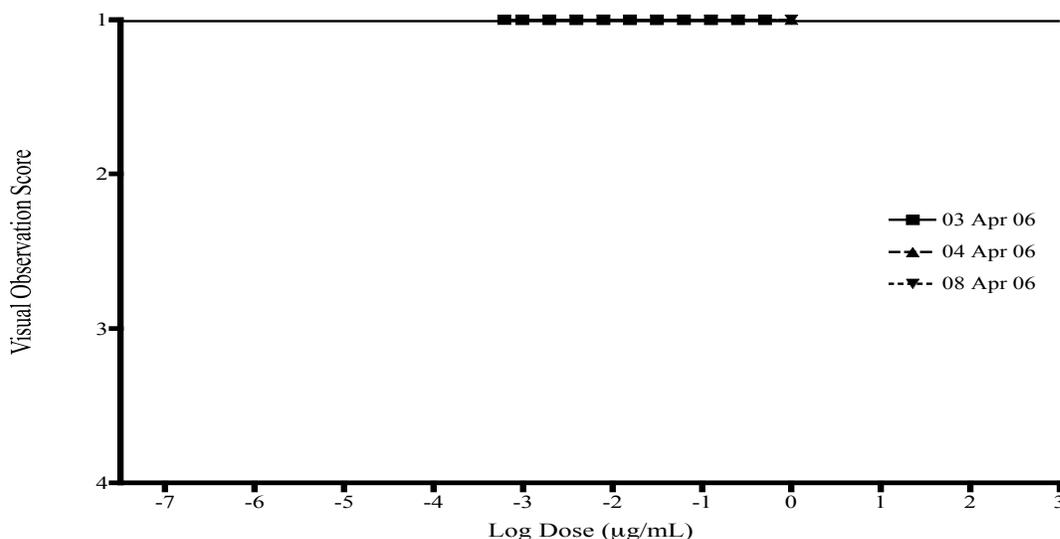


1588  
1589 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

1590  
1591 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability below this line are considered cytotoxic and are not included in the evaluation of agonist activity.

1592

1593 **Figure 10-27 Visual Observation Viability Assessment for N0004 – Corticosterone<sup>1</sup>**

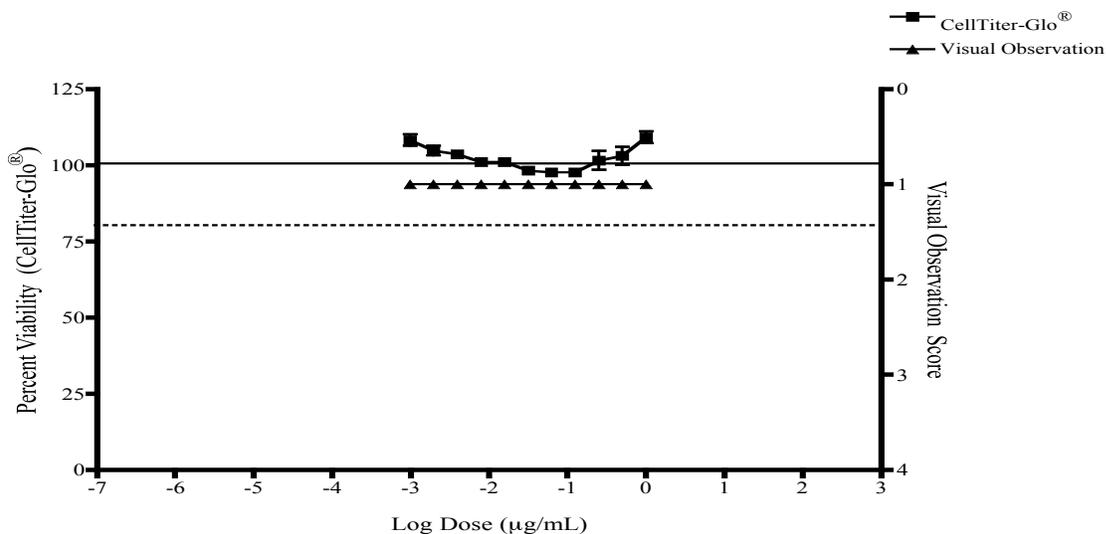


1594  
1595  
1596  
1597 <sup>1</sup>Wells containing cells that exhibit normal morphology and density are given a visual observation score of 1. Wells that are exposed to concentrations resulting in visual observation scores  $\geq 2$  are considered cytotoxic and are not included in the evaluation of antagonist activity.

1598

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1598 **Figure 10-28 Combined Qualitative and Quantitative**  
 1599 **Viability Assessment for N0004 – Corticosterone<sup>1,2</sup>**



1600

1601 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in DMSO control.

1602 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability  
 1603 below this line are considered cytotoxic and are not included in the evaluation of agonist activity.

1604

### 1605 **10.2.5N0005 – *o,p'*-DDT**

1606 *o,p'*-DDT was selected for agonist testing because it was listed as weakly positive for ER agonist activity  
 1607 in the ICCVAM Guidelines (ICCVAM 2003, 2006). It was also indicated as potentially cytotoxic  
 1608 (Freyberger and Schmuck 2004). The highest concentration of *o,p'*-DDT used in comprehensive testing  
 1609 was 10 µg/mL. This concentration was selected as the starting point for a double serial dilution because it  
 1610 was within a log dilution of the concentration giving the highest adjusted RLU value during range finder  
 1611 testing, and was not cytotoxic as was the high concentration (100 µg/mL). The concentrations of *o,p'*-  
 1612 DDT tested are listed in **Table 10-12**.

1613

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1613 **Table 10-12 Concentrations of *o,p'*-DDT used in**  
 1614 **Comprehensive Testing**

N0005 – <i>o,p'</i> -DDT (µg/mL)		
10	0.63	$3.91 \times 10^{-2}$
5	0.31	$1.95 \times 10^{-2}$
2.5	0.16	$9.77 \times 10^{-3}$
1.25	$7.81 \times 10^{-2}$	

1615 Abbreviations: *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-  
 1616 (*p*-chlorophenyl)ethane

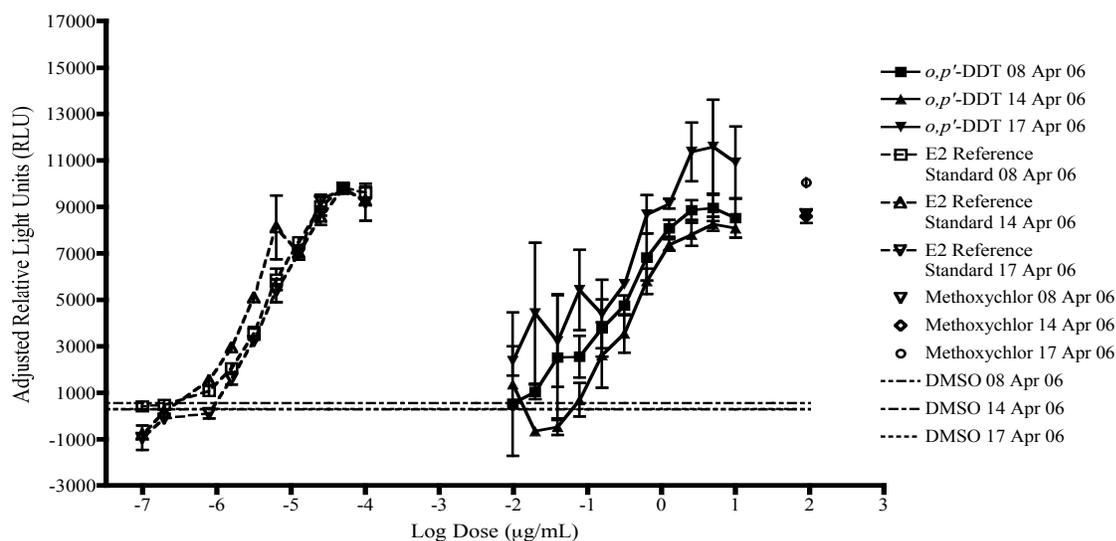
1617

1618 Results of individual agonist experiments for *o,p'*-DDT are shown in **Figure 10-29**.

1619

1620 **Figure 10-29 Agonist Comprehensive Testing for N0005 – *o,p'*-DDT:**

1621 **Individual Experiments<sup>1,2</sup>**



1622

1623

1624

Abbreviations: *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane; E2 = 17 $\beta$ -estradiol;  
 Methoxychlor = 3.13 µg/mL methoxychlor control; DMSO = dimethyl sulfoxide.

1625

1626

<sup>1</sup>Horizontal lines represent the mean of four DMSO control replicates plus three times the standard deviation of the DMSO control mean. Values must be above this line in order to be considered positive for agonism.

1627

1628

<sup>5</sup>The 3.13 µg/mL methoxychlor controls are not shown at the concentration at which they were tested. They have been placed on the graph in such a way as to maximize visibility of these controls.

1629

1630 *o,p'*-DDT showed agonist activity in all experiments conducted. EC<sub>50</sub> values for individual experiments

1631 are shown in **Table 10-13**.

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1632 **Table 10-13 Individual EC<sub>50</sub> Values for N0005 – *o,p'*-DDT**

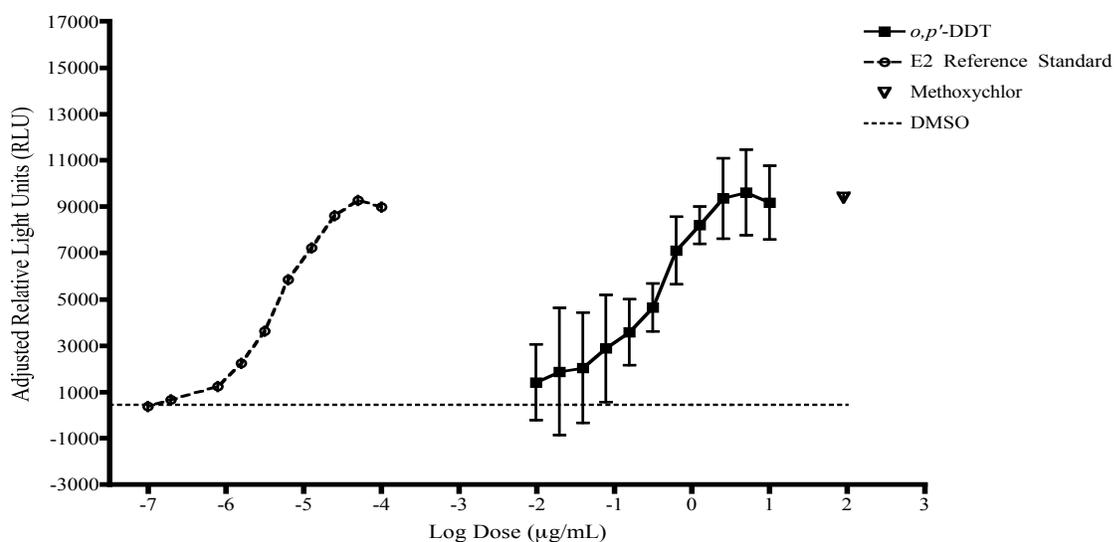
Experiment Date	EC <sub>50</sub> (µg/mL)
8 April 06	0.28
14 April 06	0.34
17 April 06	0.53

1633 Abbreviations: EC<sub>50</sub> = half-maximal effect concentration; *o,p'*-DDT =  
 1634 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane

1635

1636 Results of averaged agonist experiments for *o,p'*-DDT are shown in **Figure 10-30**.

1637

1638 **Figure 10-30 Agonist Comprehensive Testing for N0005 – *o,p'*-DDT:**1639 **Averaged Experiments<sup>1,2,3,4</sup>**

1640

1641 Abbreviations: *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane; E2 = 17β-estradiol;  
 1642 Methoxychlor = 3.13 µg/mL methoxychlor control; DMSO = Dimethyl Sulfoxide.

1643 <sup>1</sup>Historical mean and standard deviation of the E2 reference standard.1644 <sup>2</sup>Historical mean and standard deviation of the methoxychlor control).1645 <sup>3</sup>Horizontal line represents the historical mean of DMSO vehicle control plus three times the standard

1646 deviation of the DMSO control mean. Values must be above this line in order to be considered positive for agonism.

1647 <sup>4</sup>The 3.13 µg/mL methoxychlor controls are not shown at the concentration at which they were tested. They have been placed  
 1648 on the graph in such a way as to maximize visibility.

1649

1650 *o,p'*-DDT was positive for agonism at the majority of concentrations tested. The averaged EC<sub>50</sub> (**Table**1651 **10-14**) value was calculated as the mean of three experiments.

1652

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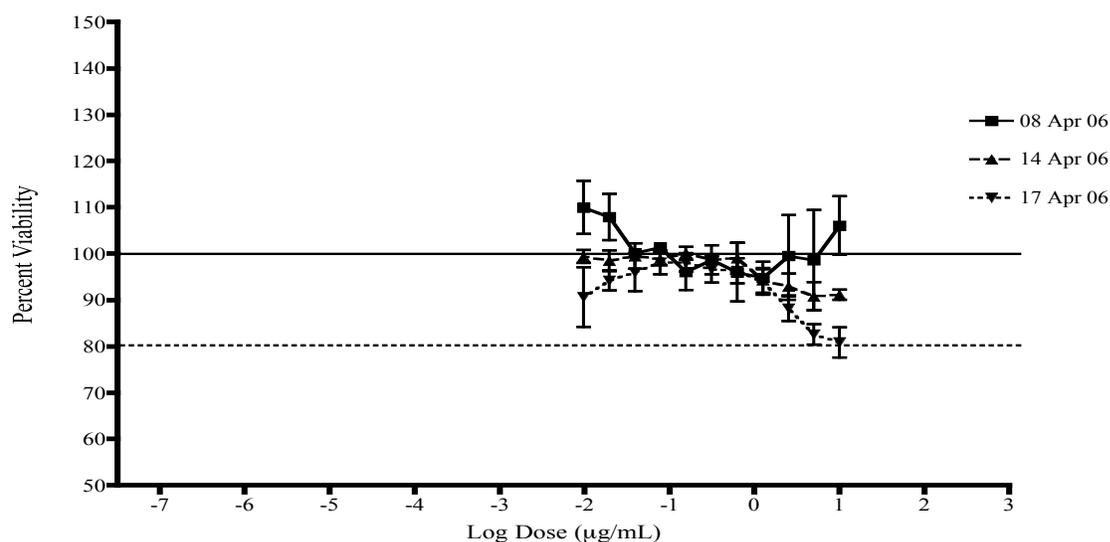
1653 **Table 10-14 Averaged EC<sub>50</sub> Value for N0005 – *o,p'*-DDT**

EC <sub>50</sub> (µg/mL)	STD DEV	CV
0.38	0.13	34%

1654 Abbreviations: EC<sub>50</sub> = half-maximal effect concentration; *o,p'*-DDT =  
 1655 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane;  
 1656 STD DEV = Standard Deviation; CV = Coefficient of Variation

1657  
 1658 *o,p'*-DDT was cytotoxic at the highest concentration tested (100 µg/mL) in the range finder, but did not  
 1659 decrease cell viability below 80% at any concentration tested in comprehensive testing (**Figures 10-31,**  
 1660 **10-32, and 10-33**).

1661

1662 **Figure 10-31 CellTiter-Glo® Viability Assessment for N0005 – *o,p'*-DDT<sup>1,2</sup>**

1663  
 1664 Abbreviations: *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane.

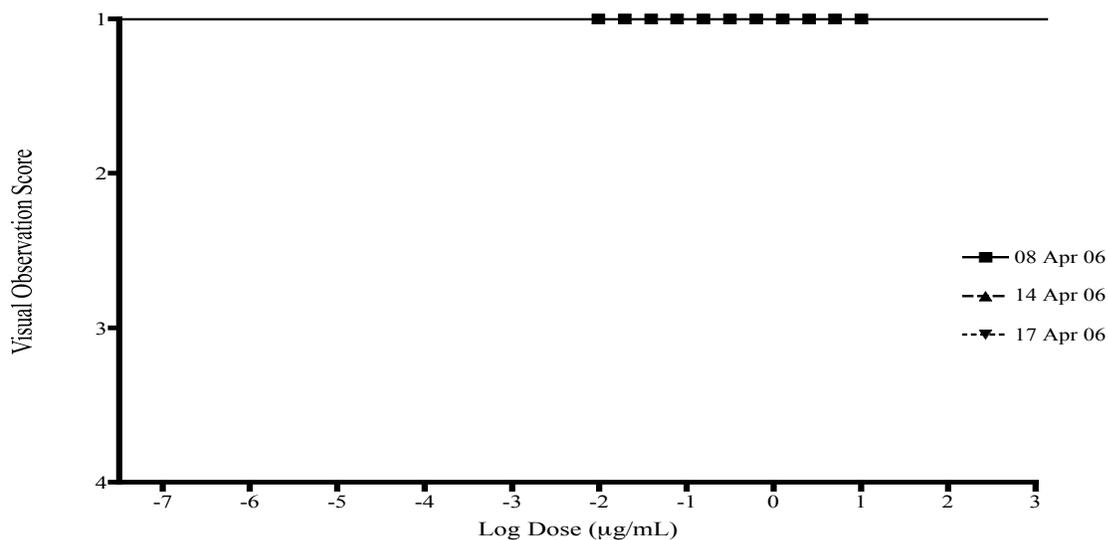
1665 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

1666 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability  
 1667 below this line are considered cytotoxic and are not included in the evaluation of agonist activity.

1668

PREDECISIONAL MATERIAL: DO NOT CITE, QUOTE OR DISTRIBUTE

1669 **Figure 10-32 Visual Observation Viability Assessment for N0005 – *o,p'*-DDT<sup>1</sup>**



1670

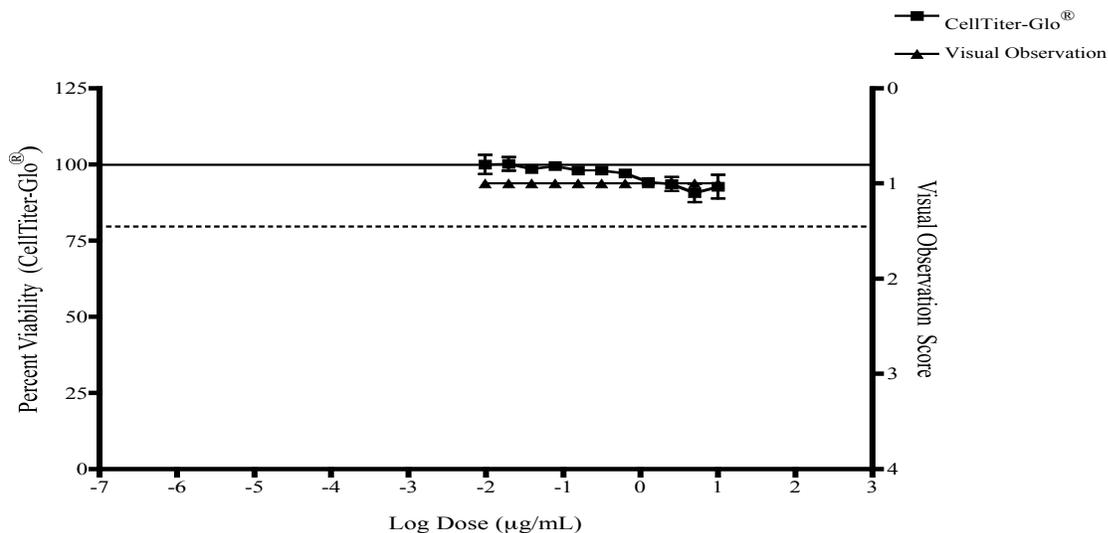
1671 Abbreviations: *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane.

1672 <sup>1</sup>Wells containing cells that exhibit normal morphology and density are given a visual observation score of 1. Wells  
 1673 that are exposed to concentrations resulting in visual observation scores ≥ 2 are considered cytotoxic and are not  
 1674 included in the evaluation of antagonist activity.

1675

1676 **Figure 10-33 Combined Qualitative and Quantitative Viability**

1677 **Assessment for N0005 – *o,p'*-DDT<sup>1,2</sup>**



1678

1679 Abbreviations: *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane.

1680 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

1681 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability  
 1682 below this line are considered cytotoxic and are not included in the evaluation of agonist activity.

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1683

1684 **10.2.6N0006 – Diethylstilbestrol**

1685 Diethylstilbestrol was selected for agonist testing because it was listed as strongly positive for ER agonist  
1686 activity in the ICCVAM Guidelines (ICCVAM 2003, 2006). The highest concentration of  
1687 diethylstilbestrol used in comprehensive testing was  $1.00 \times 10^{-4}$   $\mu\text{g/mL}$ . This concentration was selected  
1688 as the starting point for a double serial dilution because it was within a log dilution of the concentration  
1689 giving the highest adjusted RLU value during range finder testing and to ensure resolution of the top of  
1690 the concentration curve. The concentrations of diethylstilbestrol tested are listed in **Table 10-15**.

1691

1692 **Table 10-15 Concentrations of N0006 – Diethylstilbestrol**  
1693 **Used in Comprehensive Testing**

<b>N0006 – Diethylstilbestrol (<math>\mu\text{g/mL}</math>)</b>		
$1.00 \times 10^{-4}$	$6.25 \times 10^{-6}$	$3.91 \times 10^{-7}$
$5.00 \times 10^{-5}$	$3.13 \times 10^{-6}$	$1.95 \times 10^{-7}$
$2.50 \times 10^{-5}$	$1.56 \times 10^{-6}$	$9.77 \times 10^{-8}$
$1.25 \times 10^{-5}$	$7.81 \times 10^{-7}$	

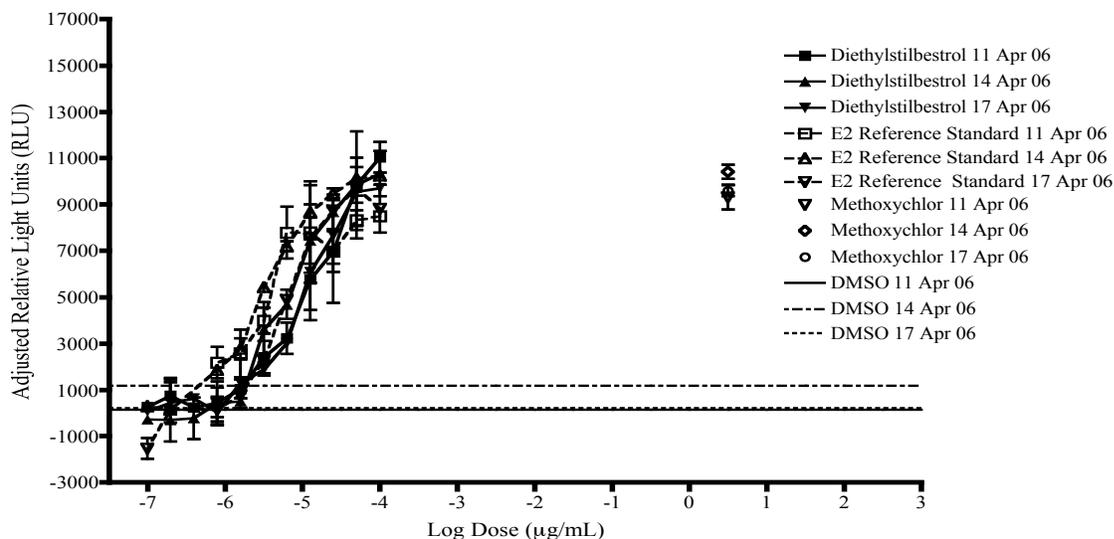
1694

1695 Results of individual agonist experiments for diethylstilbestrol are shown in **Figure 10-34**.

1696

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1696 **Figure 10-34 Agonist Comprehensive Testing for N0006 –**  
 1697 **Diethylstilbestrol: Individual Experiments<sup>1</sup>**



1698  
 1699 Abbreviations: E2 = 17β-estradiol; Methoxychlor = 3.13 µg/mL methoxychlor control; DMSO = dimethyl sulfoxide.

1700 <sup>1</sup>Horizontal lines represent the mean of four DMSO control replicates plus three times the standard deviation of the DMSO  
 1701 control mean. Values must be above this line in order to be considered positive for agonism.

1702  
 1703 Diethylstilbestrol showed agonist activity in all experiments conducted. EC<sub>50</sub> values for individual  
 1704 experiments are shown in **Table 10-16**.

1705  
 1706 **Table 10-16 Individual EC<sub>50</sub> Values for N0006 – Diethylstilbestrol**

Experiment Date	EC <sub>50</sub> (µg/mL)
8 April 06	2.02 x 10 <sup>-5</sup>
14 April 06	6.60 x 10 <sup>-6</sup>
17 April 06	1.09 x 10 <sup>-5</sup>

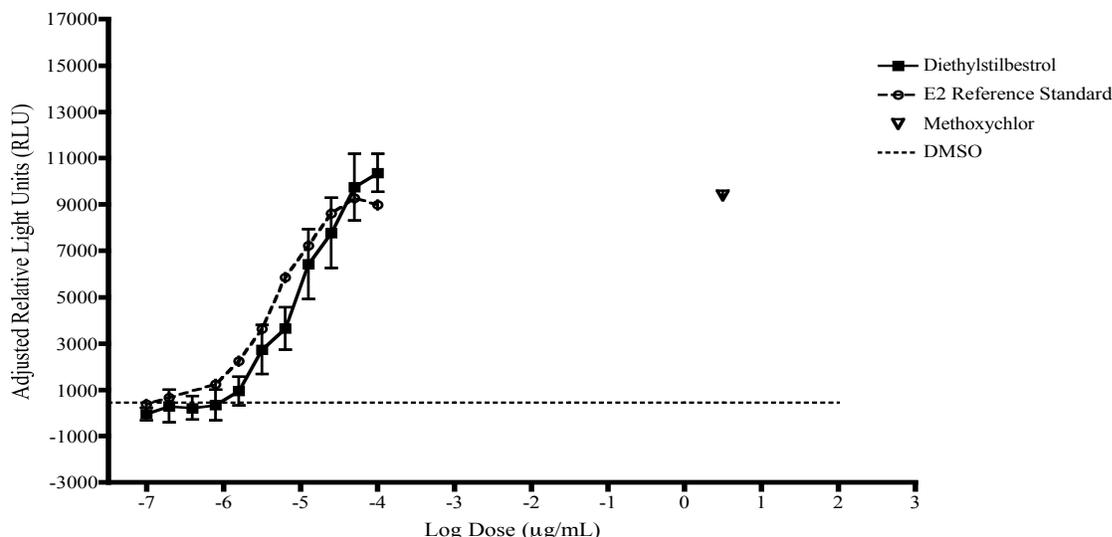
1707 Abbreviations: EC<sub>50</sub> = half-maximal effect concentration

1708  
 1709 Results of averaged agonist experiments for diethylstilbestrol are shown in **Figure 10-35**.

1710  
 1711

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1711 **Figure 10-35 Agonist Comprehensive Testing for N0006 –**  
 1712 **Diethylstilbestrol: Averaged Experiments<sup>1,2,3</sup>**



1713 Abbreviations: E2 = 17β-estradiol; Methoxychlor = 3.13 µg/mL methoxychlor control; DMSO = dimethyl sulfoxide.

1715 <sup>1</sup>Historical mean and standard deviation of the E2 reference standard.

1716 <sup>2</sup>Historical mean and standard deviation of the methoxychlor control.

1717 <sup>3</sup>Horizontal line represents the historical mean of DMSO vehicle control plus three times the standard  
 1718 deviation of the DMSO control mean. Values must be above this line in order to be considered  
 1719 positive for agonism.

1720  
 1721 Diethylstilbestrol was positive for agonism at the majority of concentrations tested. The averaged EC<sub>50</sub>  
 1722 (Table 10-17) value was calculated as the mean of three experiments.

1724 **Table 10-17 Averaged EC<sub>50</sub> Value for N0006 – Diethylstilbestrol**

EC <sub>50</sub> (µg/mL)	STD DEV	CV
1.26 x 10 <sup>-5</sup>	7.00 x 10 <sup>-6</sup>	55%

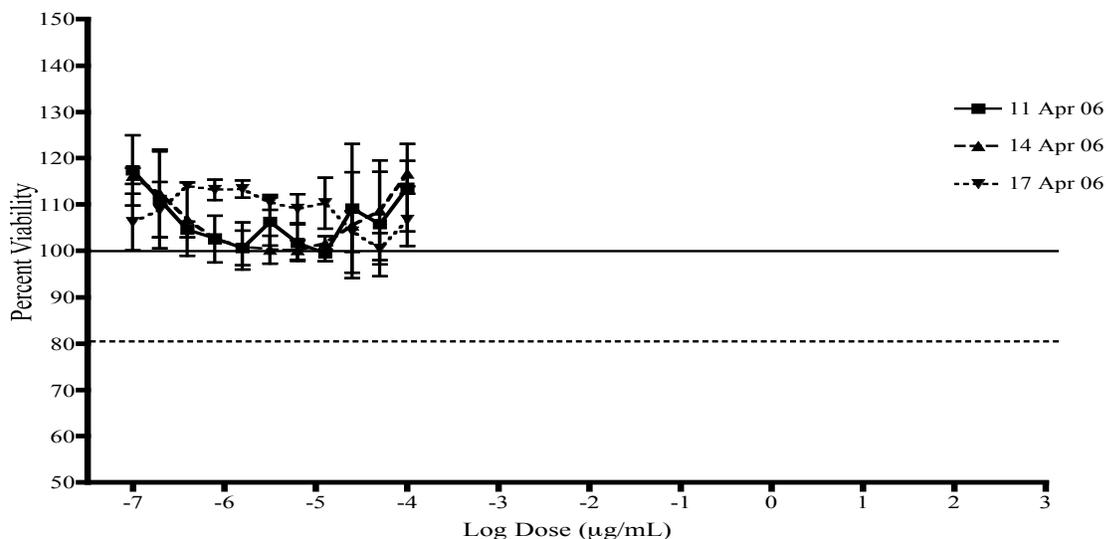
1725 Abbreviations: EC<sub>50</sub> = half-maximal effect concentration; STD DEV = Standard  
 1726 Deviation of the Mean; CV = Coefficient of Variation

1727  
 1728 Diethylstilbestrol was cytotoxic at the highest concentration tested (100 µg/mL) in the range finder, did  
 1729 not cause a decrease in cell viability at any concentration tested in comprehensive testing (Figures 10-36,  
 1730 10-37 and 10-38).

1731  
 1732

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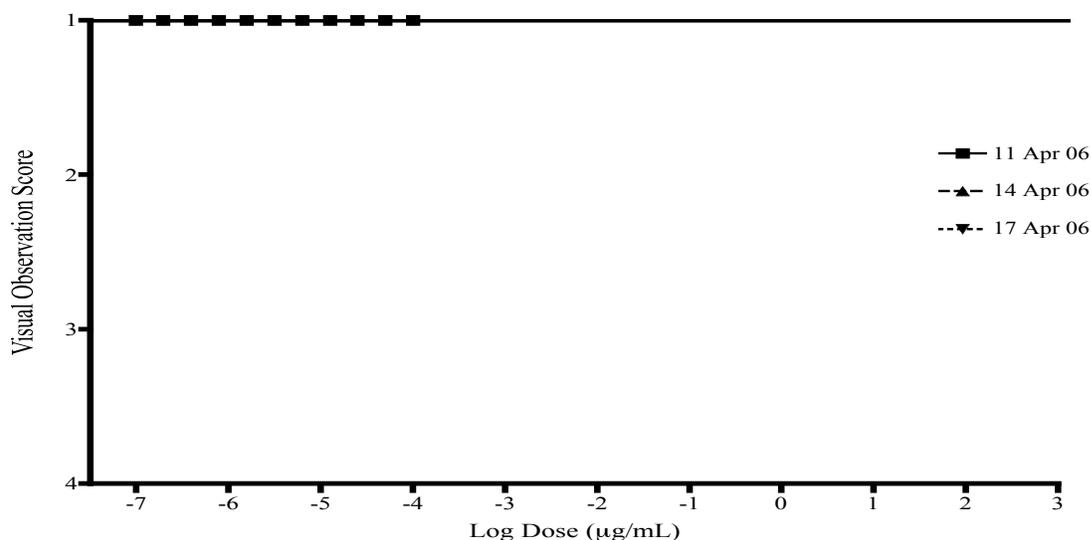
1732 **Figure 10-36 CellTiter-Glo® Viability Assessment for N0006 –**  
 1733 **Diethylstilbestrol<sup>1,2</sup>**



1734  
 1735 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in DMSO control.

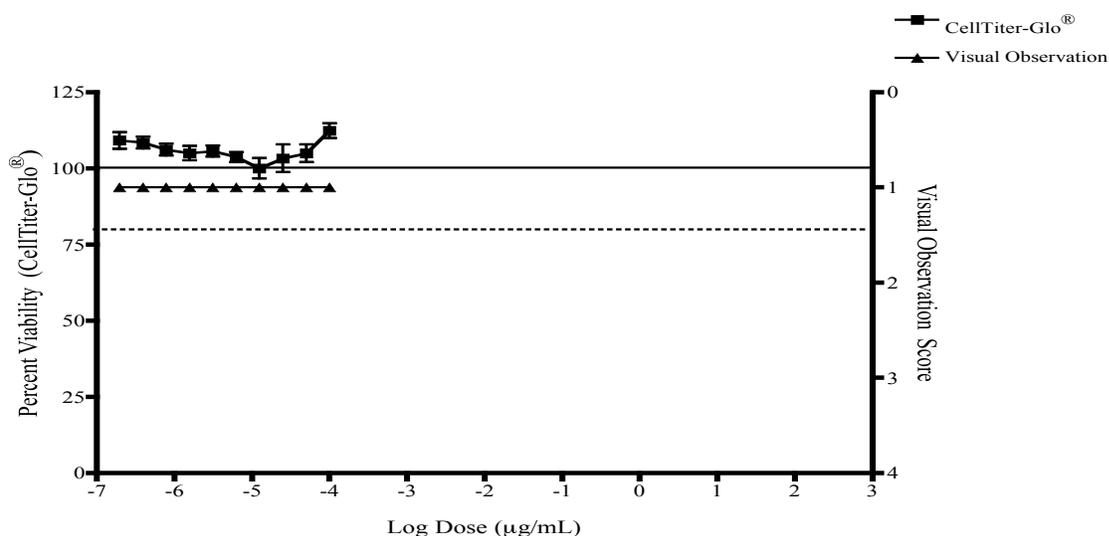
1736  
 1737 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability below this line are considered cytotoxic and are not included in the evaluation of agonist activity.

1738  
 1739 **Figure 10-37 Visual Observation Viability Assessment for N0006 –**  
 1740 **Diethylstilbestrol<sup>1</sup>**



1741  
 1742 <sup>1</sup>Wells containing cells that exhibit normal morphology and density are given a visual observation score of 1. Wells  
 1743 that are exposed to concentrations resulting in visual observation scores  $\geq 2$  are considered cytotoxic and are not  
 1744 included in the evaluation of antagonist activity.

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1745 **Figure 10-38 Combined Qualitative and Quantitative Viability**1746 **Assessment for N0006 – Diethylstilbestrol<sup>1,2</sup>**

1747

1748 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.1749 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability  
1750 below this line are considered cytotoxic and are not included in the evaluation of agonist activity.

1751

1752 **10.2.7N0007 – EE**

1753 EE was selected for agonist testing because it was listed as strongly positive for ER agonist activity in the  
1754 ICCVAM Guidelines (ICCVAM 2003, 2006). The highest concentration of EE used in comprehensive  
1755 testing was  $1.00 \times 10^{-4}$  µg/mL. This concentration was selected as the starting point for a double serial  
1756 dilution because it was within a log dilution of the concentration giving the highest adjusted RLU value  
1757 during range finder testing and to ensure resolution of the top of the concentration curve. The  
1758 concentrations of EE tested are listed in **Table 10-18**.

1759

PREDECISIONAL MATERIAL: DO NOT CITE, QUOTE OR DISTRIBUTE

1759 **Table 10-18 Concentrations of N0007 - EE Used**  
 1760 **in Comprehensive Testing**

N0007 – EE (µg/mL)		
$1.00 \times 10^{-4}$	$6.25 \times 10^{-6}$	$3.91 \times 10^{-7}$
$5.00 \times 10^{-5}$	$3.13 \times 10^{-6}$	$1.95 \times 10^{-7}$
$2.50 \times 10^{-5}$	$1.56 \times 10^{-6}$	$9.77 \times 10^{-8}$
$1.25 \times 10^{-5}$	$7.81 \times 10^{-7}$	

1761 Abbreviations: EE = 17 $\alpha$ -ethinyl estradiol

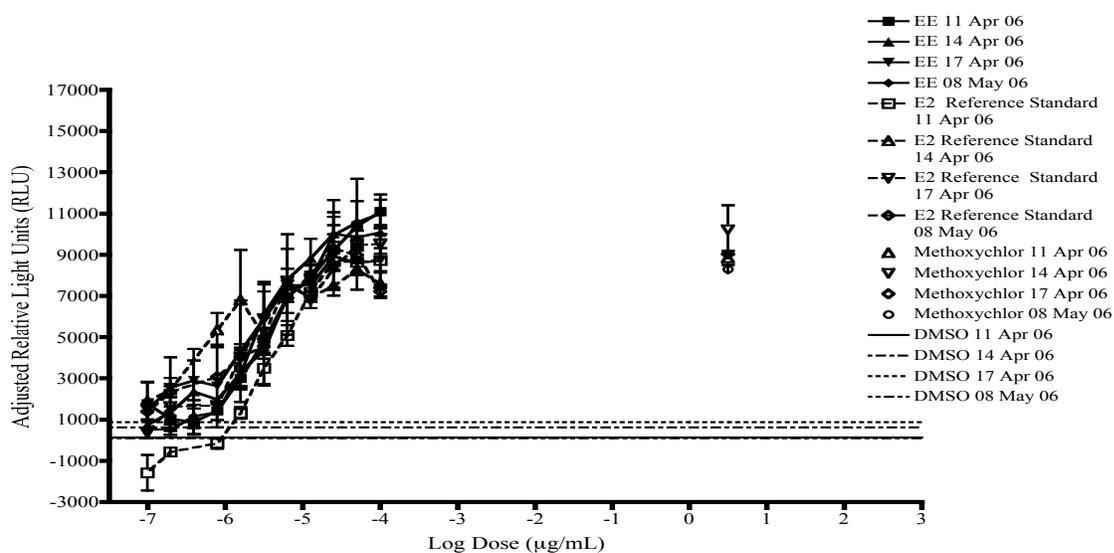
1762

1763 Results of individual agonist experiments for EE are shown in **Figure 10-39**.

1764

1765 **Figure 10-39 Agonist Comprehensive Testing for N0007 – EE:**

1766 **Individual Experiments<sup>1</sup>**



1767

1768 Abbreviations: EE = 17 $\alpha$ -ethinyl estradiol; E2 = 17 $\beta$ -estradiol; Methoxychlor = 3.13 µg/mL methoxychlor control;  
 1769 DMSO = dimethyl sulfoxide.

1770 <sup>1</sup>Horizontal lines represent the mean of four DMSO control replicates plus three times the standard deviation of the DMSO  
 1771 control mean. Values must be above this line in order to be considered positive for agonism.

1772

1773 Four separate comprehensive tests were conducted for EE. The experiment conducted on 4 April 06 had  
 1774 an entire serial dilution omitted due to experimenter error (**Section 13.1.4**) and was not used to calculate  
 1775 an EC<sub>50</sub> value. Therefore, an additional EE comprehensive test was conducted. EE showed agonist  
 1776 activity in all of the experiments that were conducted. EC<sub>50</sub> values are shown in **Table 10-19**.

1777

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1777 **Table 10-19 Individual EC<sub>50</sub> Values for N0007 – EE**

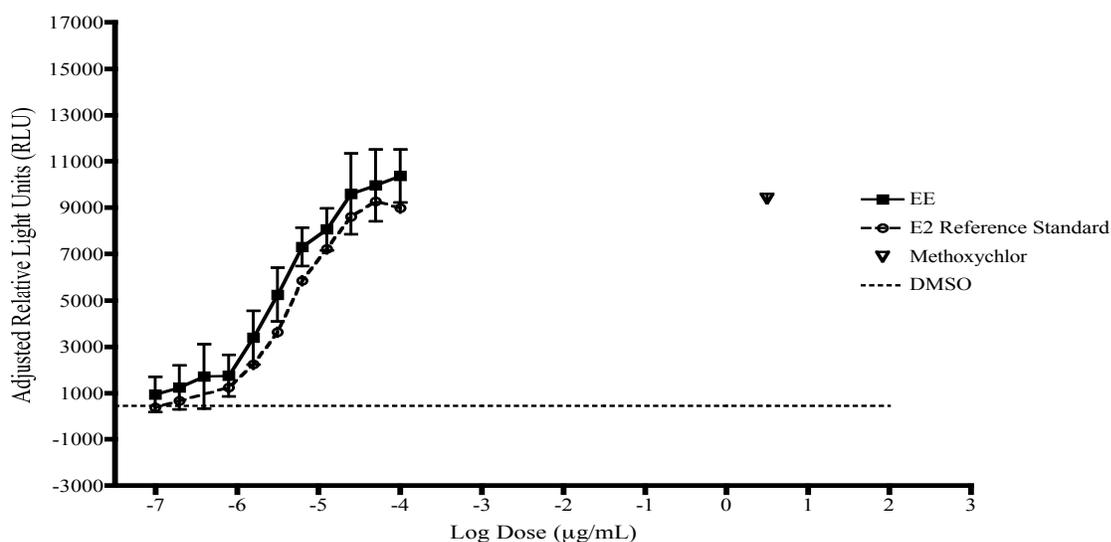
Experiment Date	EC <sub>50</sub> (µg/mL)
11 April 06	5.07 x 10 <sup>-6</sup>
14 April 06	3.00 x 10 <sup>-6</sup>
17 April 06	Not Calculated
8 May 06	4.90 x 10 <sup>-6</sup>

1778 Abbreviations: EC<sub>50</sub> = half-maximal effect concentration;  
 1779 EE = 17α-ethinyl estradiol

1780

1781 Results of averaged agonist experiments for EE are shown in **Figure 10-40**.

1782

1783 **Figure 10-40 Agonist Comprehensive Testing for N0007 – EE:**1784 **Averaged Experiments<sup>1,2,3</sup>**

1785 Abbreviations: EE = 17α-ethinyl estradiol; E2 = 17β-estradiol; Methoxychlor = 3.13 µg/mL methoxychlor control;  
 1786 DMSO = dimethyl sulfoxide.  
 1787

1788 <sup>1</sup>Historical mean and standard deviation of the E2 reference standard.1789 <sup>2</sup>Historical mean and standard deviation of the methoxychlor control.1790 <sup>3</sup>Horizontal line represents the historical mean of DMSO vehicle control plus three times the standard

1791 deviation of the DMSO control mean. Values must be above this line in order to be considered positive for agonism.

1792

1793

1794 EE was positive for agonism at the majority of concentrations tested. The averaged EC<sub>50</sub> (**Table 10-20**)

1795 value was calculated as the mean of three experiments.

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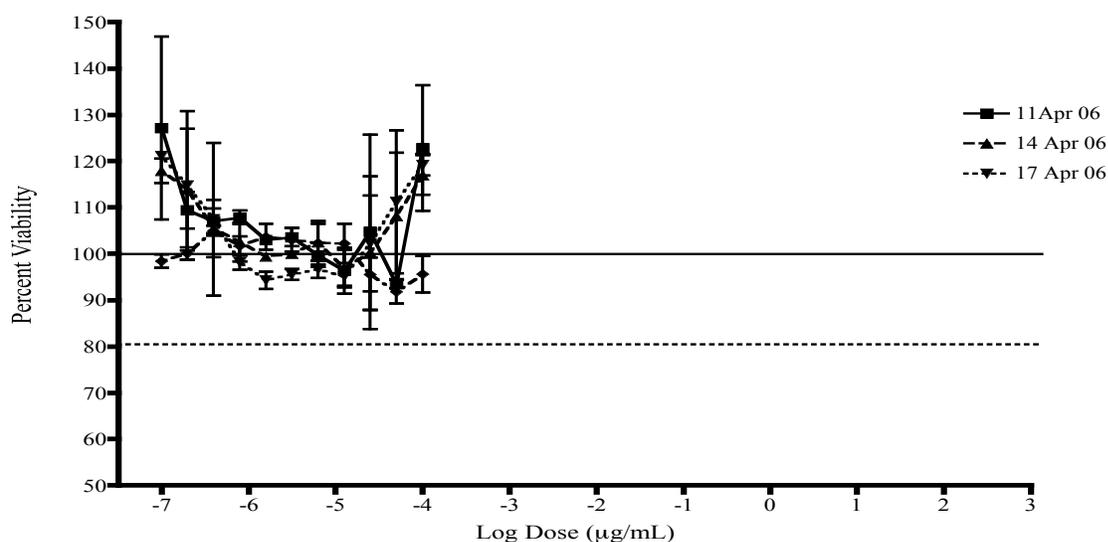
1796 **Table 10-20 Averaged EC<sub>50</sub> Value for N0007 – EE**

EC <sub>50</sub> (µg/mL)	STD DEV	CV
$3.87 \times 10^{-6}$	$1.31 \times 10^{-6}$	34%

1797 Abbreviations: CV = Coefficient of Variation ;EC<sub>50</sub> = half-maximal effect concentration; EE =  
 1798 17α-ethinyl estradiol; STD DEV = Standard Deviation of the Mean

1799  
 1800 EE was cytotoxic at the highest concentration tested (100 µg/mL) in the range finder, but did not cause a  
 1801 decrease in cell viability at any concentration tested in comprehensive testing (**Figures 10-41, 10-42 and**  
 1802 **10-43**).

1803

1804 **Figure 10-41 CellTiter-Glo® Viability Assessment for N0007 – EE<sup>1,2</sup>**

1805  
 1806 Abbreviations: EE = 17α-ethinyl estradiol

1807 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

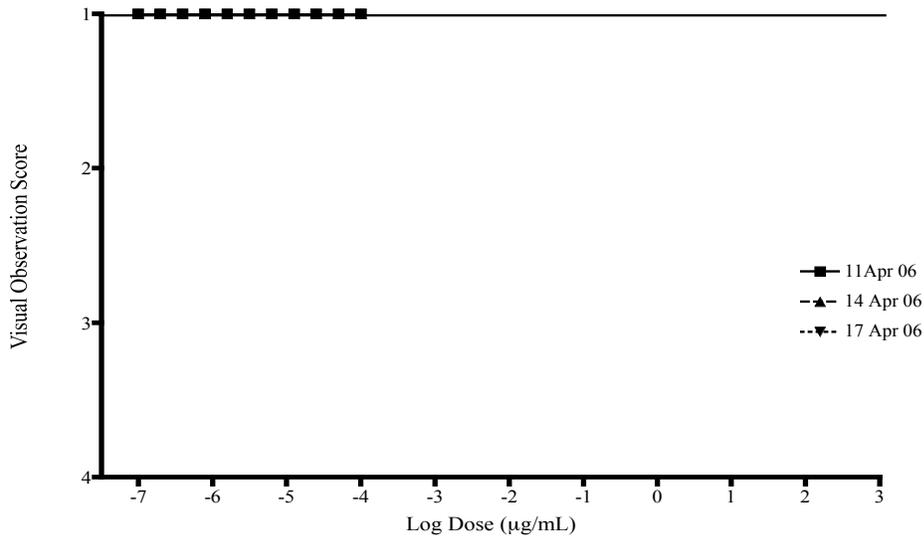
1808 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability  
 1809 below this line are considered cytotoxic and are not included in the evaluation of agonist activity.

1810

1811

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1811 **Figure 10-42 Visual Observation Viability Assessment for N0007 – EE<sup>1</sup>**



1812

1813 Abbreviations: EE = 17 $\alpha$ -ethinyl estradiol

1814 <sup>1</sup>Wells containing cells that exhibit normal morphology and density are given a visual observation score of 1. Wells  
1815 that are exposed to concentrations resulting in visual observation scores  $\geq 2$  are considered cytotoxic and are not  
1816 included in the evaluation of antagonist activity.

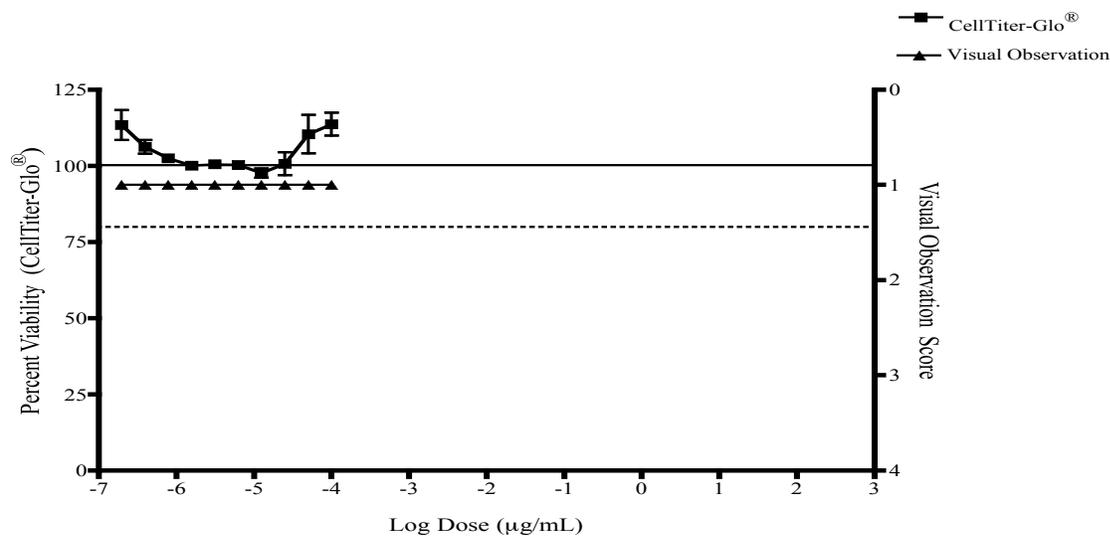
1817

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1817

1818 **Figure 10-43 Combined Qualitative and Quantitative Viability**

1819

**Assessments for N0007 – EE<sup>1,2</sup>**

1820

1821 EE = 17 $\alpha$ -ethinyl estradiol1822 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.1823 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability  
1824 below this line are considered cytotoxic and are not included in the evaluation of agonist activity.

1825

1826 **10.2.8N0008 – Flavone**

1827 Flavone was selected for agonist testing because it was listed as weakly positive for ER agonist activity in  
1828 the ICCVAM Guidelines (ICCVAM 2003, 2006). The initial highest concentration of flavone used in  
1829 comprehensive testing was 25 µg/mL. This concentration was selected as the starting point for a double  
1830 serial dilution because it was within a log dilution of the concentration giving the highest adjusted RLU  
1831 value during range finder testing. However, initial comprehensive testing indicated that 25 µg/mL would  
1832 not induce a maximum estrogenic response in the assay. Therefore, the highest concentration of flavone  
1833 used for comprehensive testing was increased to 50 µg/mL. The concentrations of flavone tested are listed  
1834 in **Table 10-21**.

1835

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1835 **Table 10-21 Concentrations of N0008 – Flavone Used in Comprehensive Testing**

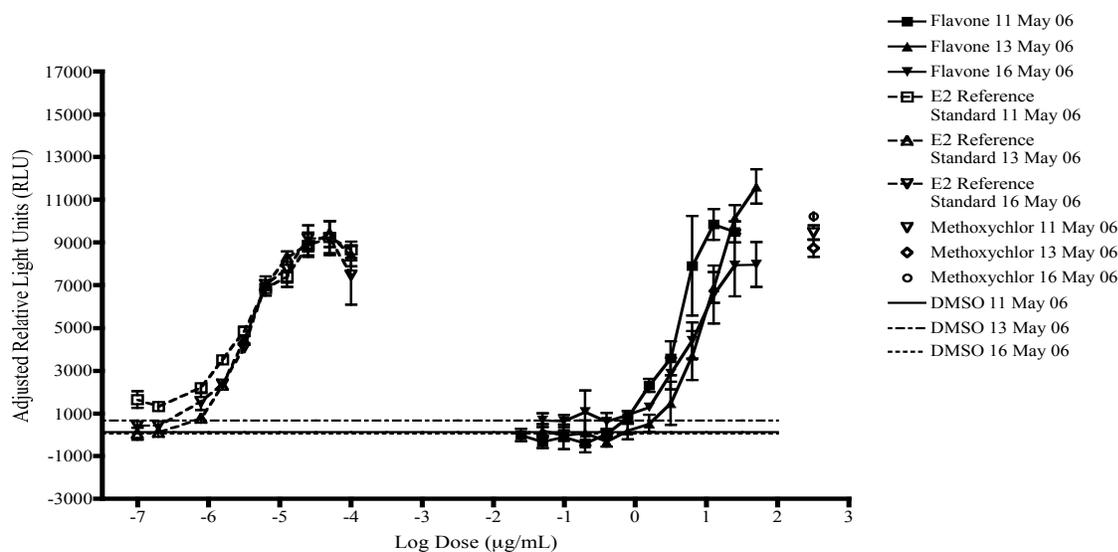
N0008 – Flavone (µg/mL)		
50 <sup>#</sup>	3.13	0.2
25 <sup>*</sup>	1.56	9.77 x 10 <sup>-2</sup>
12.5	0.78	4.88 x 10 <sup>-2</sup>
6.25	0.39	2.44 x 10 <sup>-2</sup>

1836 <sup>#</sup>Final starting concentration for flavone testing.1837 <sup>\*</sup>Initial starting concentration for flavone testing.

1838

1839 Results of individual agonist experiments for flavone are shown in **Figure 10-44**.

1840

1841 **Figure 10-44 Agonist Comprehensive Testing for N0008 – Flavone: Individual Experiments<sup>1,2</sup>**

1842

1843 Abbreviations: E2 = 17β-estradiol; Methoxychlor = 3.13 µg/mL methoxychlor control; DMSO = dimethyl sulfoxide.

1844 <sup>1</sup>Horizontal lines represent the mean of four DMSO control replicates plus three times the standard deviation of the DMSO  
1845 control mean. Values must be above this line in order to be considered positive for agonism.1846 <sup>2</sup>The 3.13 µg/mL methoxychlor controls are not shown at the concentration at which they were tested. They have been  
1847 placed on the graph in such a way as to maximize visibility.

1848

1849 Flavone showed agonist activity at the majority of concentrations tested. EC<sub>50</sub> values for individual  
1850 experiments are shown in **Table 10-22**.

1851

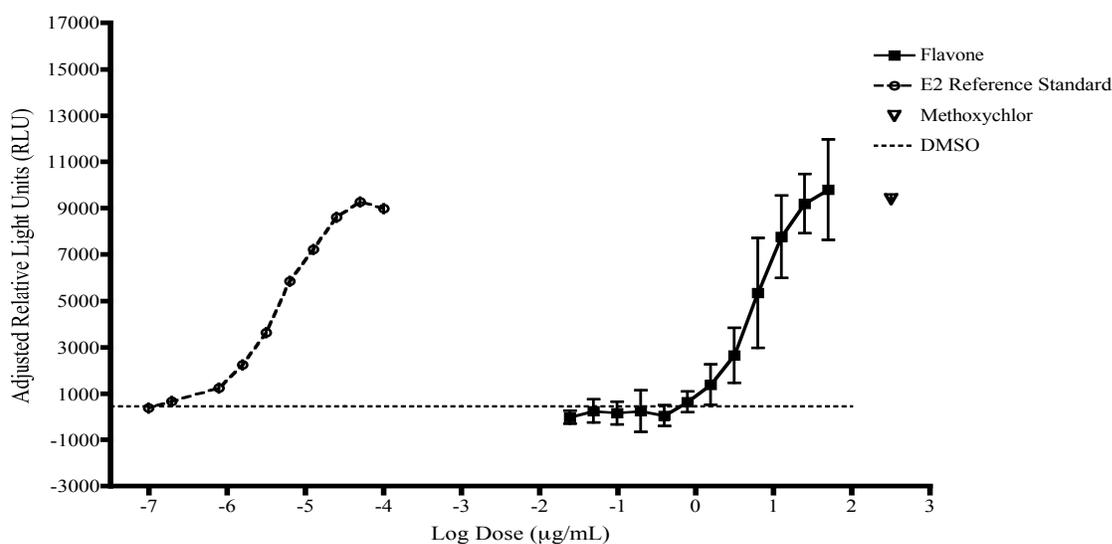
PREDECISIONAL MATERIAL: DO NOT CITE, QUOTE OR DISTRIBUTE

1852 **Table 10-22 Individual EC<sub>50</sub> Values for N0008 – Flavone**

Experiment Date	EC <sub>50</sub> (µg/mL)
11 May 06	3.64
13 May 06	11
16 May 06	6.13

1853 Abbreviations: EC<sub>50</sub> = half-maximal effect concentration;

1854

1855 Results of averaged agonist experiments for flavone are shown in **Figure 10-45**.1856 **Figure 10-45 Agonist Comprehensive Testing for N0008 – Flavone:**1857 **Averaged Experiments<sup>1,2,3,4</sup>**

1858

1859 Abbreviations: E2 = 17β-estradiol; Methoxychlor = 3.13 µg/mL methoxychlor control; DMSO = dimethyl sulfoxide.

1860 <sup>1</sup>Historical mean and standard deviation of the E2 reference standard.1861 <sup>2</sup>Historical mean and standard deviation of the methoxychlor control.1862 <sup>3</sup>Horizontal line represents the historical mean of DMSO vehicle control plus three times the standard

1863 deviation of the DMSO control mean. Values must be above this line in order to be considered positive for agonism.

1864 <sup>4</sup>The 3.13 µg/mL methoxychlor controls are not shown at the concentration at which they were tested. They have been placed on the graph in  
1865 such a way as to maximize visibility.

1866

1867 Flavone was positive for agonism at the majority of concentrations tested. The averaged EC<sub>50</sub> (**Table 10-**  
1868 **23**) value was calculated as the mean of three experiments.

1869

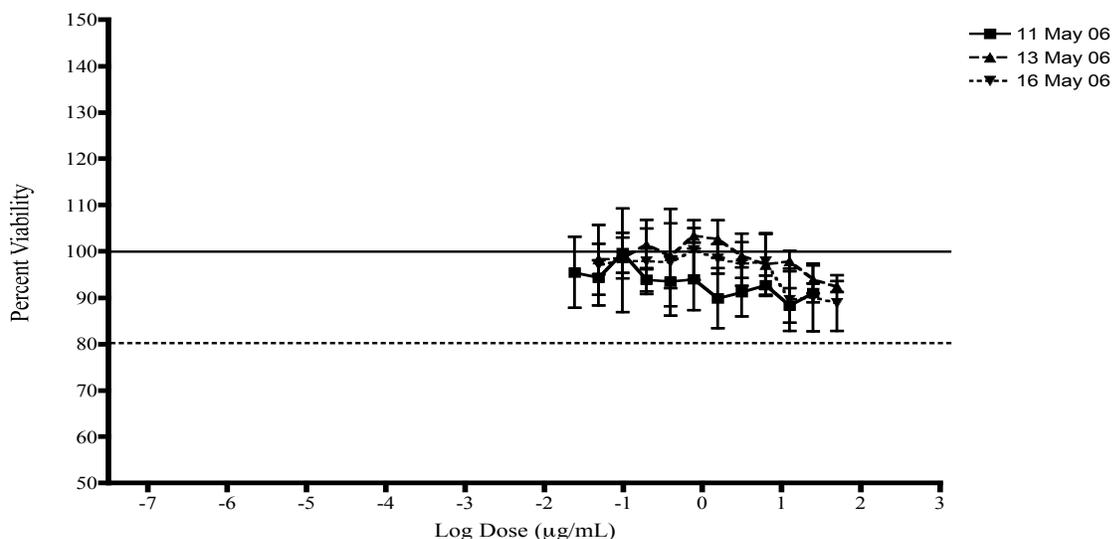
PREDECISIONAL MATERIAL: DO NOT CITE, QUOTE OR DISTRIBUTE

1869 **Table 10-23 Averaged EC<sub>50</sub> Value for N0008 – Flavone**

EC <sub>50</sub> (µg/mL)	STD DEV	CV
6.88	3.67	53%

1870 Abbreviations: EC<sub>50</sub> = half-maximal effect concentration; STD DEV =  
 1871 Standard Deviation of the Mean; CV = Coefficient of Variation

1872  
 1873 Flavone was cytotoxic at the highest concentration tested (100 µg/mL) in the range finder, but did not  
 1874 decrease cell viability at any concentration tested in comprehensive testing (Figures 10-46, 10-47 and 10-  
 1875 48).

1876 **Figure 10-46 CellTiter-Glo® Viability Assessment for N0008 – Flavone<sup>1,2</sup>**

1877  
 1878 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

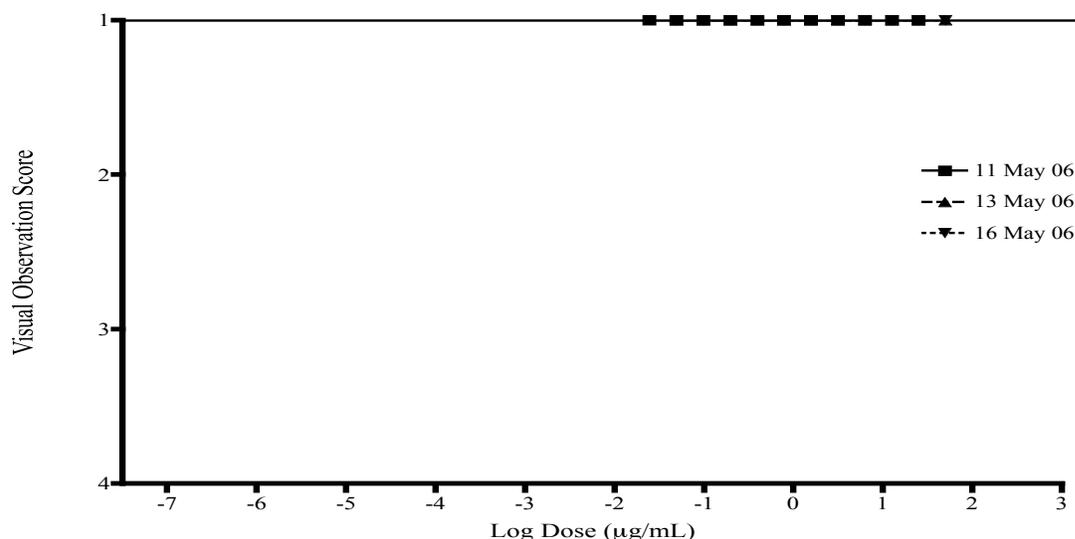
1879  
 1880 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability  
 below this line are considered cytotoxic and are not included in the evaluation of agonist activity.

1881

1882

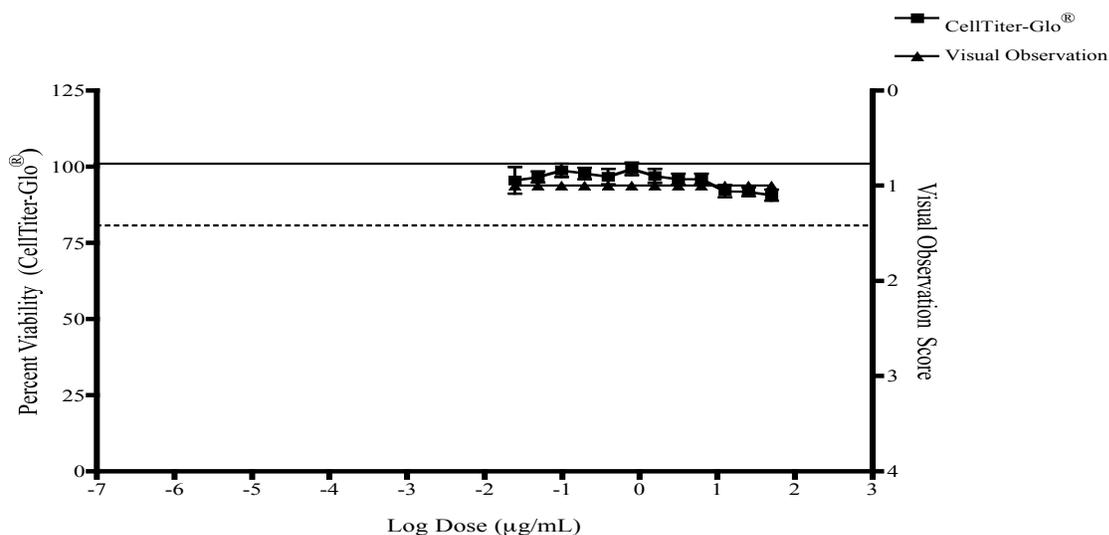
PREDECISIONAL MATERIAL: DO NOT CITE, QUOTE OR DISTRIBUTE

1883 **Figure 10-47 Visual Observation Viability Assessment for**  
 1884 **N0008 – Flavone<sup>1</sup>**



1885  
 1886 <sup>1</sup>Wells containing cells that exhibit normal morphology and density are given a visual observation score of 1. Wells  
 1887 that are exposed to concentrations resulting in visual observation scores  $\geq 2$  are considered cytotoxic and are not  
 1888 included in the evaluation of antagonist activity.

1889 **Figure 10-48 Combined Qualitative and Quantitative Viability**  
 1890 **Assessment for N0008 – Flavone<sup>1,2</sup>**



1891  
 1892 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

1893 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability  
 1894 below this line are considered cytotoxic and are not included in the evaluation of agonist activity.

1895

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## 1896 **11.0 General Procedure for Antagonist Testing**

1897 Antagonist range finder experiments were conducted with substances tested in log concentrations. Results  
 1898 from range finder testing were then used to select starting concentrations for comprehensive testing of  
 1899 coded substances. Antagonist range finder and comprehensive testing were conducted on 96-well plates.  
 1900 The reference standard (i.e., raloxifene) was tested in duplicate at nine concentrations in combination with  
 1901 E2 at  $2.5 \times 10^{-5}$   $\mu\text{g/mL}$  (**Table 11-1**). Three replicate wells for the DMSO control and weak positive  
 1902 control (i.e., flavone) were included on each plate. In order to avoid edging effects<sup>4</sup>, wells on the  
 1903 perimeter of the plate were not used for experiments. These wells did not contain cells but did contain cell  
 1904 culture media to prevent drying out of experimental wells.

1905 **Table 11-1 Concentrations of Raloxifene in Ral/E2 Reference Standard Used**  
 1906 **in Range Finder and Comprehensive Testing**

Raloxifene Concentrations ( $\mu\text{g/mL}$ )		
$1.25 \times 10^{-2}$	$1.56 \times 10^{-3}$	$1.95 \times 10^{-4}$
$6.25 \times 10^{-3}$	$7.81 \times 10^{-4}$	$9.77 \times 10^{-5}$
$3.13 \times 10^{-3}$	$3.91 \times 10^{-4}$	$4.88 \times 10^{-5}$

1907 Abbreviations: Ral/E2 = concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$   $\mu\text{g/mL}$   $17\beta$ -estradiol

1908  
 1909 Luminescence of treated, reference standard, and control wells was corrected by subtracting the averaged  
 1910 luminescence of the solvent controls from the RLU measured in each well. Data was transferred into  
 1911 PRISM<sup>®</sup> statistical software, graphed, and evaluated for positive or negative response. For substances that  
 1912 inhibited estrogenic activity, the concentration of test substance that caused a half-maximal inhibition of  
 1913 estrogenic response ( $\text{IC}_{50}$ ) was calculated using a Hill function analysis. The Hill function is a four-  
 1914 parameter logistic mathematical model relating the substance concentration to the relative light units in a  
 1915 sigmoidal shape:

$$1916 \quad Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log \text{IC}_{50} - X) \text{HillSlope}}}$$

1917 where Y= response (i.e., relative light units), X is the logarithm of concentration, Bottom is the minimum  
 1918 response, Top is the maximum response,  $\log \text{IC}_{50}$  is the logarithm of X as the response midway between

<sup>4</sup>Edging effects are variations in response seen in the outermost wells in a tissue culture plate. These variations are believed to be due to variations in temperature, evaporation, etc., that may occur in these wells that would ultimately affect cellular growth and health.

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1919 Top and Bottom, and HillSlope describes the steepness of the curve. The model calculates the best fit for  
1920 the Top, Bottom, HillSlope, and IC<sub>50</sub> parameters.

1921

1922 Acceptance or rejection of a test was based on evaluation of reference standard and control results from  
1923 each experiment. Results were compared to quality controls for these parameters derived from the  
1924 historical database established during development and standardization of the BG1LUC ER TA  
1925 antagonist protocol. The quality control parameters are as follows:

- 1926 • Reduction – Plate reduction (i.e., the highest Ral/E2 reference standard RLU value divided  
1927 by the lowest Ral/E2 reference standard RLU value) must be greater than three-fold.
- 1928 • Reference standard results – Calculated Ral/E2 reference standard IC<sub>50</sub> values must be within  
1929 2.5 times the standard deviation of the historical database IC<sub>50</sub> mean values.
- 1930 • DMSO control results - DMSO control RLU values must be within 2.5 times the standard  
1931 deviation of the historical database DMSO control mean RLU values.
- 1932 • Flavone and E2 control results – Flavone and E2 control RLU values must be within 2.5  
1933 times the standard deviation of the historical database flavone and E2 control mean RLU  
1934 values.

1935

## 1936 **12.0 Antagonist testing**

1937 The substances selected for antagonist testing were butylbenzyl phthalate (BBP), DBA, flavone,  
1938 genistein, *p*,*n*-nonylphenol (nonylphenol), progesterone, *o*,*p*'-DDT, and tamoxifen (**Table 12-1**). These  
1939 substances were selected from the subset of minimum substances recommended for validation of *in vitro*  
1940 ER assays in the ICCVAM Guidelines (ICCVAM 2003, 2006). They were selected to represent a range of  
1941 ER antagonist activity classification (including those that are negative for antagonism) and to evaluate  
1942 substances with properties that may be problematic (e.g., limited solubility, cytotoxicity).

1943

1944 Because they were insoluble in cell culture media containing 1% DMSO, none of the selected substances  
1945 could be tested at the recommended limit concentration (1 mg/mL). Therefore, the limit concentration for  
1946 protocol standardization was set at 100 µg/mL, one log concentration lower than the intended limit  
1947 concentration. However, an error in the process of making serial dilutions resulted in use of an actual  
1948 limit concentration of 50 µg/mL for range finder testing.

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1949 **Table 12-1 Test Substances for Antagonist Testing**

Code	Substance Name	CASRN	ER TA Antagonist Activity <sup>1,2,3</sup>	Additional Basis for Selection <sup>4</sup>
N0009	Butylbenzyl phthalate	85-68-7	-	
N0010	Dibenzo [a,h] anthracene	53-70-3	##	
N0011	Genistein	446-72-0	#	Insoluble
N0012	Flavone	525-82-6	###	
N0013	<i>p</i> -n-nonylphenol	104-40-5	#	
N0014	Progesterone	57-83-0	-	
N0015	<i>o,p'</i> -DDT	789-02-6	#	Cytotoxic
N0016	Tamoxifen	10540-29-1	###	Cytotoxic

1950 Abbreviations: CASRN = Chemical Abstracts Service Registry Number; *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-  
1951 chlorophenyl)ethane; ER = estrogen receptor; TA = transcriptional activation

1952 <sup>1</sup>Data on antagonist activities were derived from ICCVAM (2006)

1953 <sup>2</sup>### Indicates that the substance was uniformly positive in multiple assays; ## indicates that the substance  
1954 was positive in the majority of assays in which it was tested; # indicates that the substance was positive in the  
1955 single assay in which it was tested; #- indicates the substance was positive in one assay but was also negative in  
1956 one or more assays; - indicates that the substance was uniformly negative in multiple assays

1957 <sup>3</sup>Antag = Antagonist

1958 <sup>4</sup>Information on solubility and cytotoxicity were derived from the scientific literature.

1959

1960 **12.1 Antagonist Range Finding**

1961 Antagonist range finding for coded substances consisted of eight-point, logarithmic serial dilutions, with  
1962 each concentration tested in conjunction with a fixed concentration of E2 ( $2.50 \times 10^{-3}$   $\mu\text{g/mL}$ ) in a single  
1963 well of the 96-well plate. Each range finder experiment was conducted once. All antagonist range finder  
1964 experiments used the same concentrations of test substance (**Table 12-2**).

1965

1966 **Table 12-2 Antagonist Range Finder Concentrations**  
1967 **for Coded Substances<sup>1</sup>**

Range Finder Concentrations ( $\mu\text{g/mL}$ )		
50	$5.00 \times 10^{-2}$	$5.00 \times 10^{-5}$
5	$5.00 \times 10^{-3}$	$5.00 \times 10^{-6}$
0.5	$5.00 \times 10^{-4}$	

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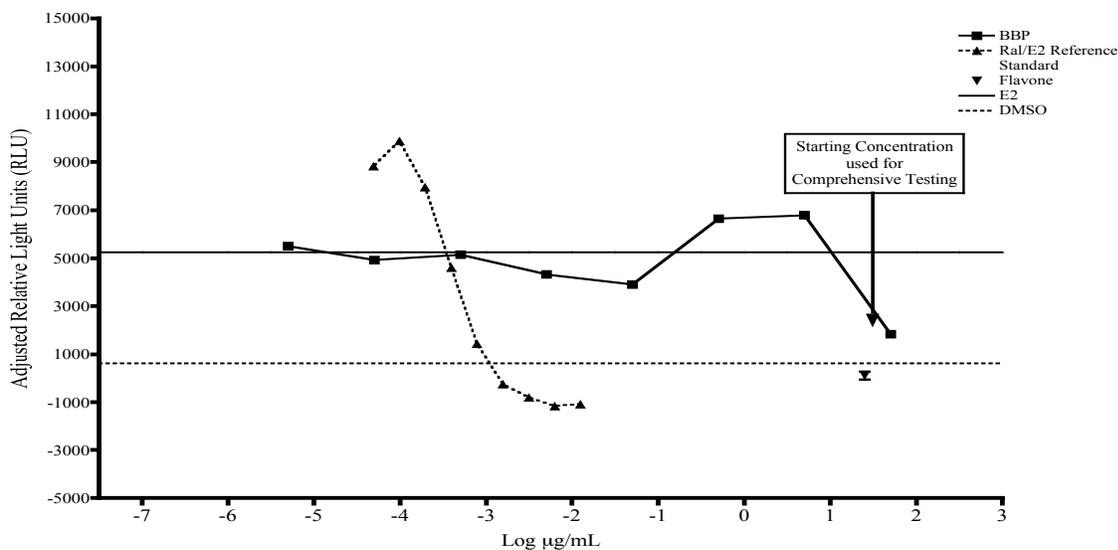
1968  
1969 <sup>1</sup>All concentrations of test substance were run in conjunction with  
2.50 x 10<sup>-3</sup> µg/mL E2.

1970

1971 Results for antagonist range finder experiments are presented in **Figures 12-1** through **12-8**.

1972

1973 **Figure 12-1 Antagonist Range Finder for N0009 – BBP<sup>1,2</sup>**



1974

1975 Abbreviations: BBP = Butylbenzyl phthalate; Ral/E2 Reference Standard = varying concentrations of raloxifene with a fixed  
1976 concentration of 2.5 x 10<sup>-5</sup> µg/mL 17β-estradiol; Flavone = 25 µg/mL Flavone + 2.5 x 10<sup>-5</sup> µM 17β-estradiol; E2 = 2.5 x 10<sup>-5</sup> µg/mL  
1977 17β-estradiol; DMSO = dimethyl sulfoxide.

1978 <sup>1</sup>Solid horizontal line represents the mean of three E2 control replicates plus three times the standard deviation  
1979 of the E2 control mean. Values must be below this line without any significant decreases in cell viability in order  
1980 to be considered positive for antagonism.

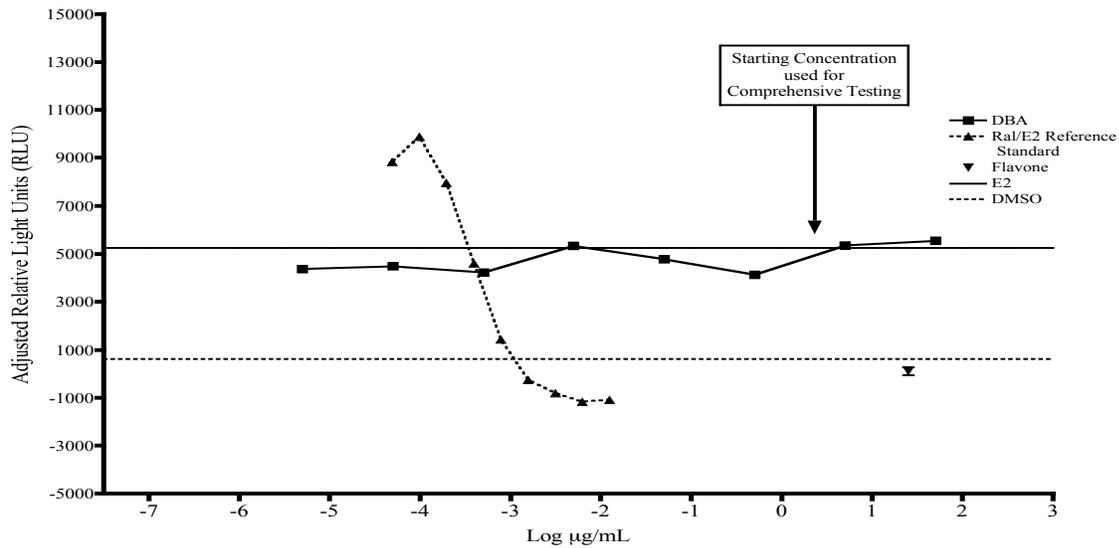
1981 <sup>2</sup>Dashed horizontal line represents the mean of three DMSO control replicates plus three times the standard  
1982 deviation of the DMSO control mean.

1983

1984

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1984 **Figure 12-2 Antagonist Range Finder for N0010 – DBA<sup>1,2</sup>**



1985

1986

1987

1988

Abbreviations: DBA = Dibenzo [a,h] anthracene; Ral/E2 Reference Standard = varying concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$  µg/mL 17β-estradiol; Flavone = 25 µg/mL Flavone +  $2.5 \times 10^{-5}$  µM 17β-estradiol; E2 =  $2.5 \times 10^{-5}$  µg/mL 17β-estradiol; DMSO = dimethyl sulfoxide.

1989

1990

1991

<sup>1</sup>Solid horizontal line represents the mean of three E2 control replicates plus three times the standard deviation of the E2 control mean. Values must be below this line without any significant decreases in cell viability in order to be considered positive for antagonism.

1992

1993

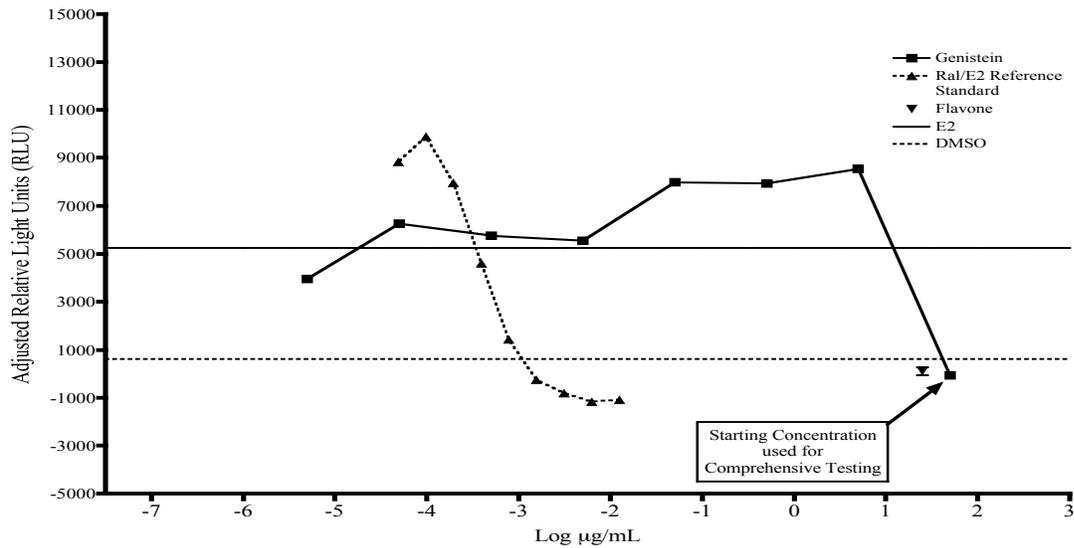
<sup>2</sup>Dashed horizontal line represents the mean of three DMSO control replicates plus three times the standard deviation of the DMSO control mean.

1994

1995

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1995 **Figure 12-3 Antagonist Range Finder for N0011 – Genistein<sup>1,2</sup>**



1996

1997  
1998  
1999

Abbreviations: Ral/E2 Reference Standard = varying concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$  µg/mL 17β-estradiol; Flavone = 25 µg/mL Flavone +  $2.5 \times 10^{-5}$  µM 17β-estradiol; E2 =  $2.5 \times 10^{-5}$  µg/mL 17β-estradiol; DMSO = dimethyl sulfoxide.

2000  
2001  
2002

<sup>1</sup>Solid horizontal line represents the mean of three E2 control replicates plus three times the standard deviation of the E2 control mean. Values must be below this line without any significant decreases in cell viability in order to be considered positive for antagonism.

2003  
2004

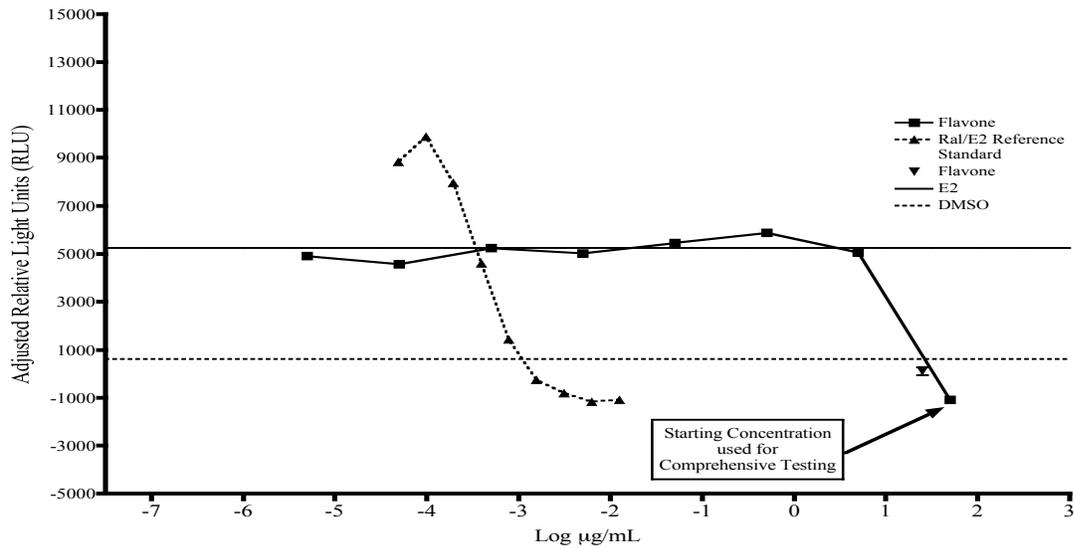
<sup>2</sup>Dashed horizontal line represents the mean of three DMSO control replicates plus three times the standard deviation of the DMSO control mean.

2005

2006

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2006 **Figure 12-4 Antagonist Range Finder for N0012 – Flavone<sup>1,2</sup>**



2007

2008 Abbreviations: Ral/E2 Reference Standard = varying concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$  µg/mL  
 2009  $17\beta$ -estradiol; Flavone = 25 µg/mL Flavone +  $2.5 \times 10^{-5}$  µM  $17\beta$ -estradiol; E2 =  $2.5 \times 10^{-5}$  µg/mL  $17\beta$ -estradiol; DMSO =  
 2010 dimethyl sulfoxide.

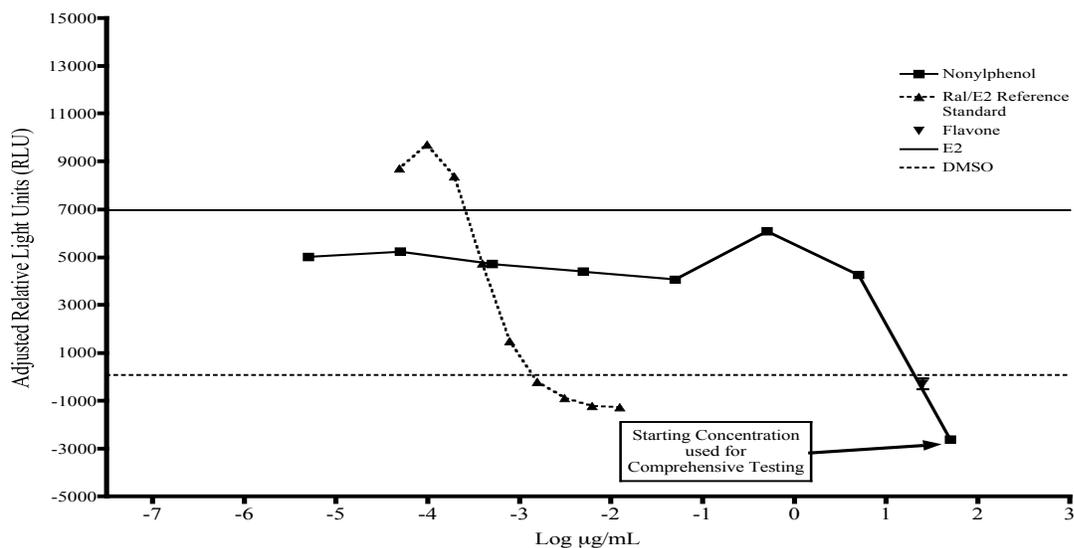
2011 <sup>1</sup>Solid horizontal line represents the mean of three E2 control replicates plus three times the standard  
 2012 deviation of the E2 control mean. Values must be below this line without any significant decreases in cell  
 2013 viability in order to be considered positive for antagonism.

2014 <sup>2</sup>Dashed horizontal line represents the mean of three DMSO control replicates plus three times the standard  
 2015 deviation of the DMSO control mean.

2016  
 2017

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2017 **Figure 12-5 Antagonist Range Finder for N0013 – Nonylphenol<sup>1,2</sup>**



2018

2019 Abbreviations: Ral/E2 Reference Standard = varying concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$  µg/mL  
 2020  $17\beta$ -estradiol; Flavone = 25 µg/mL Flavone +  $2.5 \times 10^{-5}$  µM  $17\beta$ -estradiol; E2 =  $2.5 \times 10^{-5}$  µg/mL  $17\beta$ -estradiol; DMSO =  
 2021 dimethyl sulfoxide.

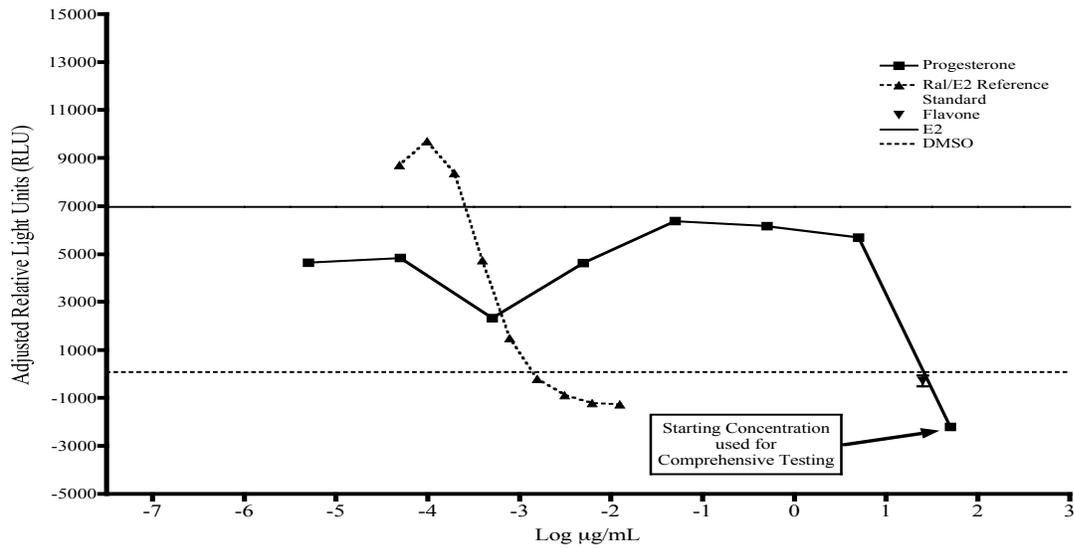
2022 <sup>1</sup>Solid horizontal line represents the mean of three E2 control replicates plus three times the standard  
 2023 deviation of the E2 control mean. Values must be below this line without any significant decreases in cell  
 2024 viability in order to be considered positive for antagonism.

2025 <sup>2</sup>Dashed horizontal line represents the mean of three DMSO control replicates plus three times the standard  
 2026 deviation of the DMSO control mean.

2027  
 2028

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2028 **Figure 12-6 Antagonist Range Finder for N0014 – Progesterone<sup>1,2</sup>**



2029

2030 Abbreviations: Ral/E2 Reference Standard = varying concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$  µg/mL  
 2031  $17\beta$ -estradiol; Flavone = 25 µg/mL Flavone +  $2.5 \times 10^{-5}$  µM  $17\beta$ -estradiol; E2 =  $2.5 \times 10^{-5}$  µg/mL  $17\beta$ -estradiol; DMSO =  
 2032 dimethyl sulfoxide.

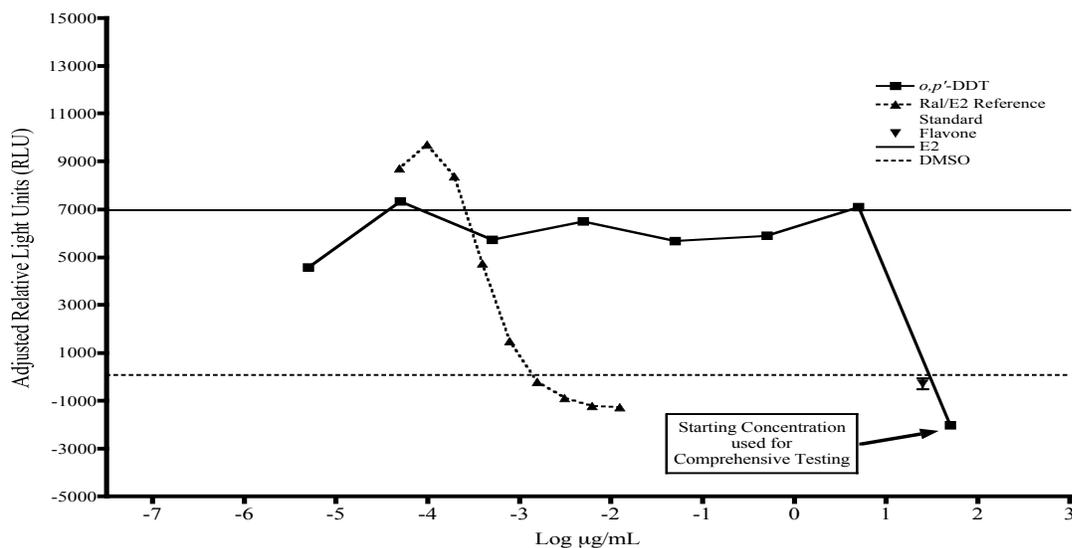
2033 <sup>1</sup>Solid horizontal line represents the mean of three E2 control replicates plus three times the standard  
 2034 deviation of the E2 control mean. Values must be below this line without any significant decreases in cell  
 2035 viability in order to be considered positive for antagonism.

2036 <sup>2</sup>Dashed horizontal line represents the mean of three DMSO control replicates plus three times the standard  
 2037 deviation of the DMSO control mean.

2038

2039

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2039 **Figure 12-7 Antagonist Range Finder for N0015 – *o,p'*-DDT<sup>1,2</sup>**

2040

2041 Abbreviations: *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane; Ral/E2 Reference Standard = varying  
 2042 concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$  µg/mL 17β-estradiol; Flavone = 25 µg/mL Flavone +  $2.5 \times 10^{-5}$   
 2043 µM 17β-estradiol; E2 =  $2.5 \times 10^{-5}$  µg/mL 17β-estradiol; DMSO = dimethyl sulfoxide.

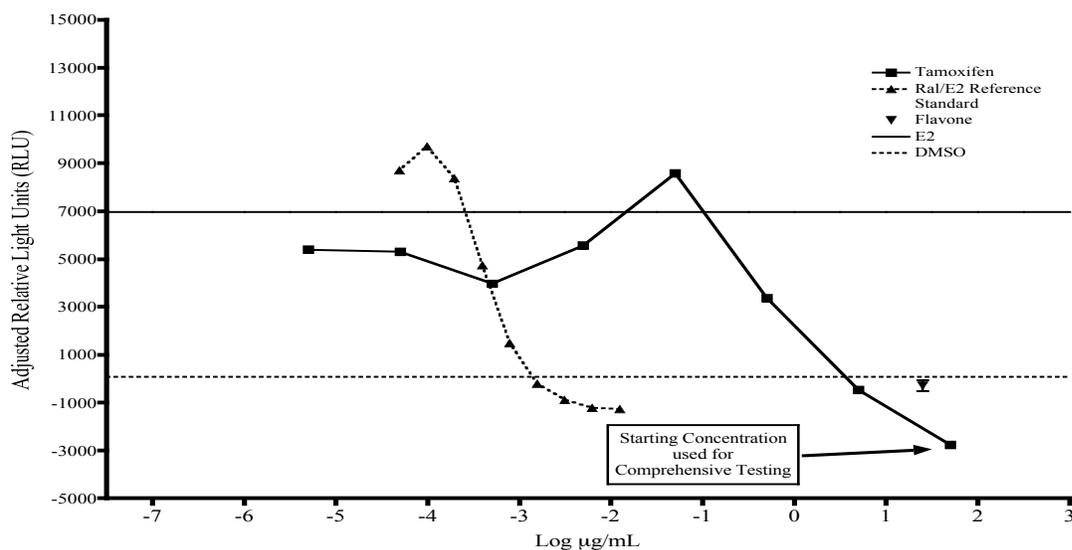
2044 <sup>1</sup>Solid horizontal line represents the mean of three E2 control replicates plus three times the standard  
 2045 deviation of the E2 control mean. Values must be below this line without any significant decreases in cell  
 2046 viability in order to be considered positive for antagonism.

2047 <sup>2</sup>Dashed horizontal line represents the mean of three DMSO control replicates plus three times the standard  
 2048 deviation of the DMSO control mean.

2049

2050

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2050 **Figure 12-8 Antagonist Range Finder for N0016 – Tamoxifen<sup>1,2</sup>**

2051

2052 Abbreviations: Ral/E2 Reference Standard = varying concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$  µg/mL  
 2053  $17\beta$ -estradiol; Flavone = 25 µg/mL Flavone +  $2.5 \times 10^{-5}$  µM  $17\beta$ -estradiol; E2 =  $2.5 \times 10^{-5}$  µg/mL  $17\beta$ -estradiol; DMSO =  
 2054 dimethyl sulfoxide.

2055 <sup>1</sup>Solid horizontal line represents the mean of three E2 control replicates plus three times the standard  
 2056 deviation of the E2 control mean. Values must be below this line without any significant decreases in cell  
 2057 viability in order to be considered positive for antagonism.

2058 <sup>2</sup>Dashed horizontal line represents the mean of three DMSO control replicates plus three times the standard  
 2059 deviation of the DMSO control mean.

2060

2061 Visual observations for cell viability were conducted for all experimental plates just prior to BG1LUC ER  
 2062 TA evaluation. Cell viability testing (i.e., CellTiter-Glo<sup>®</sup>) was conducted in parallel plates on the same  
 2063 day. Comparisons of cell viability data from CellTiter-Glo<sup>®</sup> assays and visual observations are shown in  
 2064 **Table 12-3.**

2065

2066

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2066 **Table 12-3 CellTiter-Glo® and Visual Observation Data for Antagonist Range Finder**  
 2067 **Experiments**

Substance	Concentration (µg/mL)	CellTiter-Glo®	Visual Observation Score <sup>1</sup>
N0009 - BBP	50	111%	1
	5	102%	1
	0.5	116%	1
	$5.00 \times 10^{-2}$	83%	1
	$5.00 \times 10^{-3}$	96%	1
	$5.00 \times 10^{-4}$	99%	1
	$5.00 \times 10^{-5}$	98%	1
	$5.00 \times 10^{-6}$	113%	1
N0010 - DBA	50	103%	1
	5	104%	1
	0.5	105%	1
	$5.00 \times 10^{-2}$	120%	1
	$5.00 \times 10^{-3}$	104%	1
	$5.00 \times 10^{-4}$	85%	1
	$5.00 \times 10^{-5}$	88%	1
	$5.00 \times 10^{-6}$	92%	1
N0011 - Genistein	50	85%	1
	5	104%	1
	0.5	103%	1
	$5.00 \times 10^{-2}$	112%	1
	$5.00 \times 10^{-3}$	109%	1
	$5.00 \times 10^{-4}$	123%	1
	$5.00 \times 10^{-5}$	117%	1
	$5.00 \times 10^{-6}$	81%	1
N0012 – Flavone	<b>50<sup>2</sup></b>	<b>65%</b>	<b>2</b>
	5	89%	1
	0.5	93%	1
	$5.00 \times 10^{-2}$	96%	1
	$5.00 \times 10^{-3}$	93%	1
	$5.00 \times 10^{-4}$	95%	1
	$5.00 \times 10^{-5}$	100%	1
	$5.00 \times 10^{-6}$	103%	1

2068 Abbreviations: BBP = Butylbenzyl phthalate; DBA = Dibenzo[*a,h*]anthracene; *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-  
 2069 chlorophenyl)ethane;

2070 <sup>1</sup>Visual observations are scored using the scale provide in **Table 7-1**

2071 <sup>2</sup>Bolded text indicates substances and concentrations that caused a decrease in cell viability below 80%

2072

2073

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2073 **Table 12-3 (Continued) CellTiter-Glo® and Visual Observation Data for Antagonist Range Finder**  
 2074 **Experiments**

Substance	Concentration (µg/mL)	CellTiter-Glo®	Visual Observation Score <sup>1</sup>
N0013 -Nonylphenol	<b>50</b>	<b>8%</b>	<b>4</b>
	5	104%	1
	0.5	111%	1
	$5.00 \times 10^{-2}$	101%	1
	$5.00 \times 10^{-3}$	101%	1
	$5.00 \times 10^{-4}$	100%	1
	$5.00 \times 10^{-5}$	111%	1
	$5.00 \times 10^{-6}$	107%	1
N0014 - Progesterone	<b>50</b>	<b>45%</b>	<b>3</b>
	5	103%	1
	0.5	105%	1
	$5.00 \times 10^{-2}$	117%	1
	$5.00 \times 10^{-3}$	112%	1
	$5.00 \times 10^{-4}$	101%	1
	$5.00 \times 10^{-5}$	101%	1
	$5.00 \times 10^{-6}$	104%	1
N0015 - <i>o,p'</i> -DDT	<b>50</b>	<b>23%</b>	<b>3</b>
	5	99%	1
	0.5	107%	1
	$5.00 \times 10^{-2}$	108%	1
	$5.00 \times 10^{-3}$	111%	1
	$5.00 \times 10^{-4}$	107%	1
	$5.00 \times 10^{-5}$	115%	1
	$5.00 \times 10^{-6}$	93%	1
N0016 - Tamoxifen	<b>50</b>	<b>5%</b>	<b>4</b>
	5	90%	1
	0.5	99%	1
	$5.00 \times 10^{-2}$	108%	1
	$5.00 \times 10^{-3}$	103%	1
	$5.00 \times 10^{-4}$	106%	1
	$5.00 \times 10^{-5}$	109%	1
	$5.00 \times 10^{-6}$	105%	1

2075 Abbreviations: BBP = Butylbenzyl phthalate; DBA = Dibenzo[*a,h*]anthracene; *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-  
 2076 chlorophenyl)ethane;

2077 <sup>1</sup>Visual observations are scored using the scale provide in **Table 7-1**

2078 <sup>2</sup>Bolded text indicates substances and concentrations that caused a decrease in cell viability below 80%

2079

2080 Five of the eight substances caused a decrease in cell viability (observed with both visual observations  
 2081 and CellTiter-Glo®) at the highest concentration used for range finder testing.

2082

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2083 **12.2 Antagonist Comprehensive Testing**2084 **12.2.1 N0009 – BBP**

2085 BBP was selected for antagonist testing because it was listed as negative for ER antagonist activity in the  
 2086 ICCVAM Guidelines (ICCVAM 2003, 2006). 50 µg/mL was selected as the starting concentration for the  
 2087 double serial dilution used for comprehensive testing because it gave the lowest adjusted RLU value  
 2088 during range finder testing. The concentrations of BBP tested are listed in **Table 12-4**.

2089

2090 **Table 12-4 Concentrations of N0009 – BBP**  
 2091 **Used in Comprehensive Testing**

N0009 – BBP (µg/mL)		
50	3.13	0.2
25	1.56	9.77 x 10 <sup>-2</sup>
12.5	0.78	4.88 x 10 <sup>-2</sup>
6.25	0.39	

2092 Abbreviations: BBP = butylbenzyl phthalate

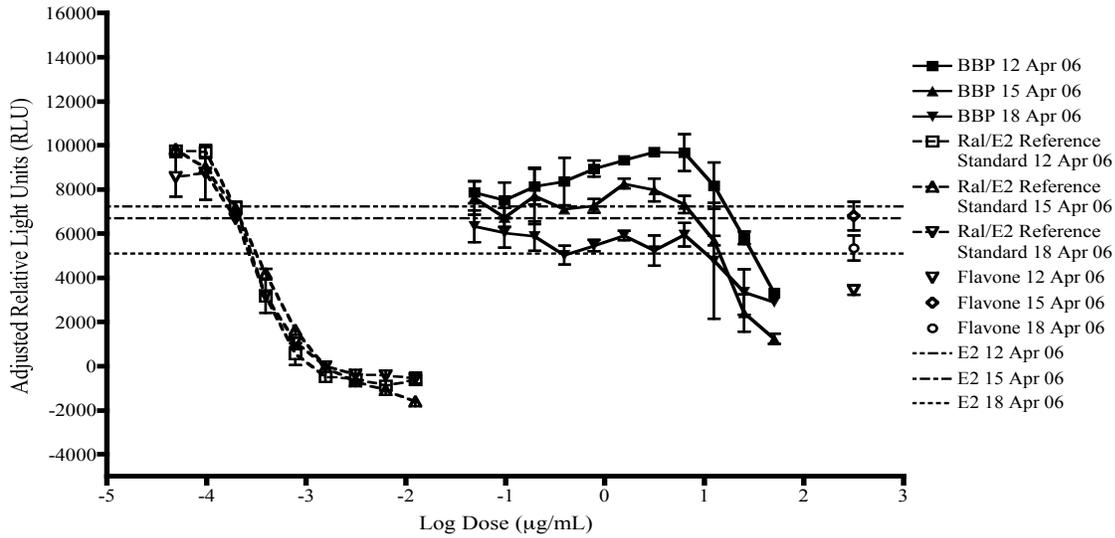
2093

2094 Results of individual antagonist experiments for BBP are shown in **Figure 12-9**. BBP showed potential  
 2095 antagonist activity at the two highest concentrations tested (25 and 50 µg/mL).

2096

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2096 **Figure 12-9 Antagonist Comprehensive Testing for N0009 – BBP:**  
 2097 **Individual Experiments<sup>1,2</sup>**



2098  
 2099 Abbreviations: BBP = butylbenzyl phthalate; Ral/E2 Reference Standard = concentrations of raloxifene with a fixed  
 2100 concentration of  $2.5 \times 10^{-5}$  µg/mL 17β-estradiol; Flavone = 25 µg/mL flavone control; E2 = 17β-estradiol.

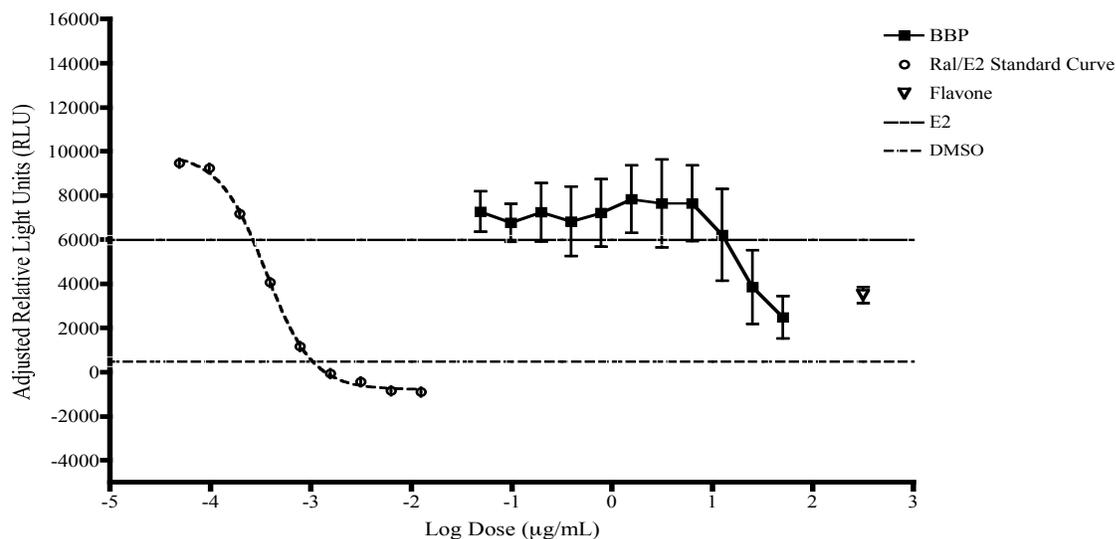
2101 <sup>1</sup>Horizontal lines represent the mean of three E2 control replicates minus three times the standard deviation  
 2102 of the E2 control mean. Values must be below the line without any significant decreases in cell viability in  
 2103 order to be considered positive for antagonism.

2104 <sup>2</sup>The 25 µg/mL flavone controls are not shown at the concentration at which they were tested. They  
 2105 have been placed on the graph in such a way as to maximize visibility.

2106  
 2107 Results of averaged antagonist experiments for BBP are shown in **Figure 12-10**.  
 2108  
 2109

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2109 **Figure 12-10 Antagonist Comprehensive Testing for N0009 –**  
 2110 **BBP: Averaged Experiments<sup>1,2,3,4,5,6</sup>**



2111

2112 Abbreviations: BBP = Butylbenzyl phthalate; Ral/E2 Reference Standard = concentrations of raloxifene with a fixed

2113 concentration of  $2.5 \times 10^{-5}$  µg/mL 17β-estradiol; Flavone = 25 µg/mL flavone control; E2 = 17β-estradiol;

2114 DMSO = dimethyl sulfoxide.

2115 <sup>1</sup>Historical mean and standard deviation of the Ral/E2 reference standard.2116 <sup>2</sup>Historical mean and standard deviation of the flavone control.2117 <sup>3</sup>Historical mean and standard deviation of the E2 control.2118 <sup>4</sup>Solid horizontal line represents the historical mean of the E2 control minus three times the  
 2119 standard deviation of the E2 control mean. Values must be below the line without any significant decreases in cell  
 2120 viability in order to be considered positive for antagonism.2121 <sup>5</sup>Dashed horizontal line represents the historical mean of the DMSO control minus three times the  
 2122 standard deviation of the DMSO control mean.2123 <sup>6</sup>The 25 µg/mL flavone controls are not shown at the concentration at which they were tested. They  
 2124 have been placed on the graph in such a way as to maximize visibility.

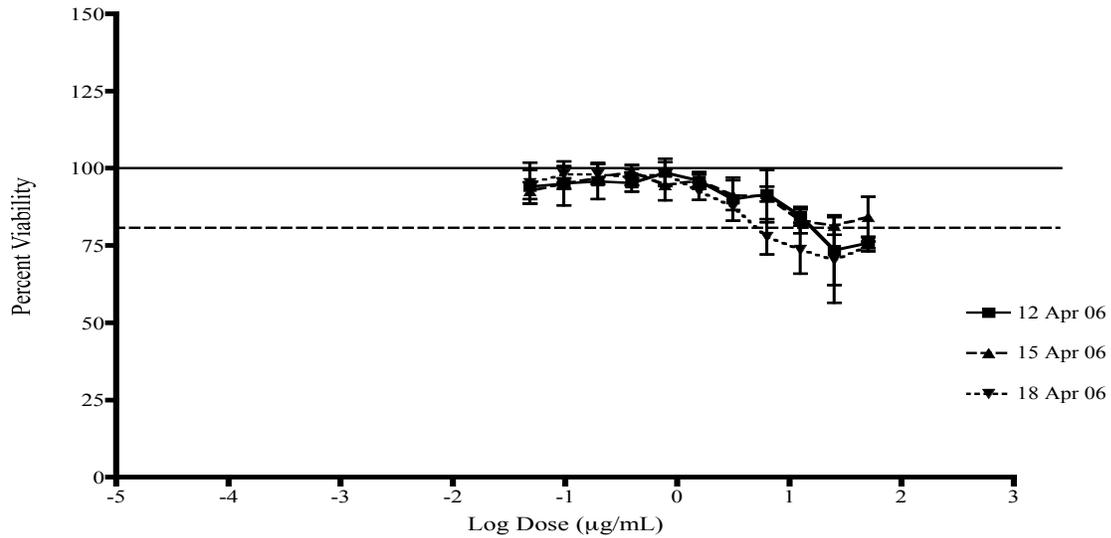
2125  
 2126 BBP showed potential antagonist activity at the two highest concentrations tested (25 and 50 µg/mL), but  
 2127 cell viability, as assessed by CellTiter-Glo® (**Figure 12-11**) was below the 80% limit (78 and 77 percent  
 2128 respectively), with visual observation scores of 2 (**Figure 12-12**). Therefore, the ER TA response may  
 2129 have been due to cytotoxicity rather than ER mediated antagonism. A comparison of CellTiter-Glo® data  
 2130 and visual observation scores are presented in **Figure 12-13**.

2131

2132

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2132 **Figure 12-11 CellTiter-Glo® Viability Assessment for N0009 – BBP<sup>1,2</sup>**



2133 Abbreviations: BBP = Butylbenzyl phthalate

2134 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

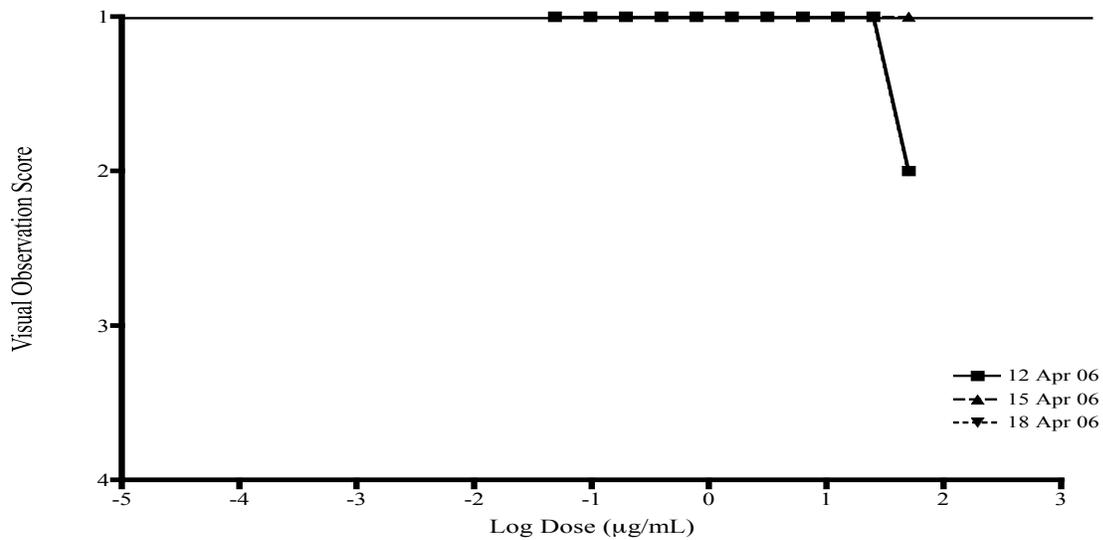
2135 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability

2136 below this line are considered cytotoxic and are not included in the evaluation of antagonist activity.

2137

2138

2139 **Figure 12-12 Visual Observation Viability Assessment for N0009 – BBP<sup>1</sup>**



2140

2141 Abbreviations: BBP = Butylbenzyl phthalate

2142 <sup>1</sup>Wells containing cells that exhibit normal morphology and density are given a visual observation score

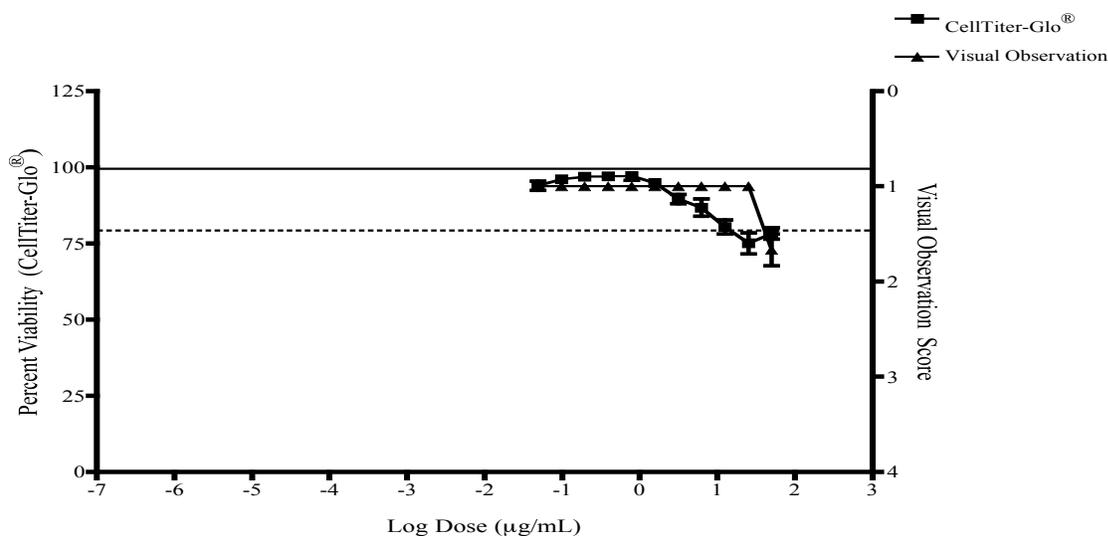
2143 of 1; wells containing cells that exhibit altered morphology and have small gaps between cells are given a

2144 visual observation score of 2. Wells that are exposed to concentrations resulting in visual observation

2145 scores  $\geq 2$  are considered cytotoxic and are not included in the evaluation of antagonist activity.

2146

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2146 **Figure 12-13 Combined Qualitative and Quantitative Viability**2147 **Assessments for N0009 – BBP<sup>1,2</sup>**

2148

2149 Abbreviations: BBP = Butylbenzyl phthalate

2150 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.2151 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability  
2152 below this line are considered cytotoxic and are not included in the evaluation of antagonist activity.

2153

2154 **12.2.2N0010 – DBA**

2155 DBA was selected for antagonist testing because it was listed in the ICCVAM Guidelines (ICCVAM  
2156 2003, 2006) as positive for ER antagonist activity in the majority of assays in which it was performed. 5  
2157 µg/mL was selected as the starting concentration for the double serial dilution used for comprehensive  
2158 testing because it was one log dilution higher than the concentration giving the peak adjusted RLU value  
2159 for the V shaped concentration curve found in range finder testing. The concentrations of DBA tested are  
2160 listed in **Table 12-5**.

2161

2162

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2162 **Table 12-5 Concentrations of N0010 – DBA**  
 2163 **used in Comprehensive Testing**

N0010 – DBA ( $\mu\text{g/mL}$ )		
5	0.31	$1.95 \times 10^{-2}$
2.5	0.16	$9.77 \times 10^{-3}$
1.25	$7.81 \times 10^{-2}$	$4.88 \times 10^{-3}$
0.63	$3.91 \times 10^{-2}$	

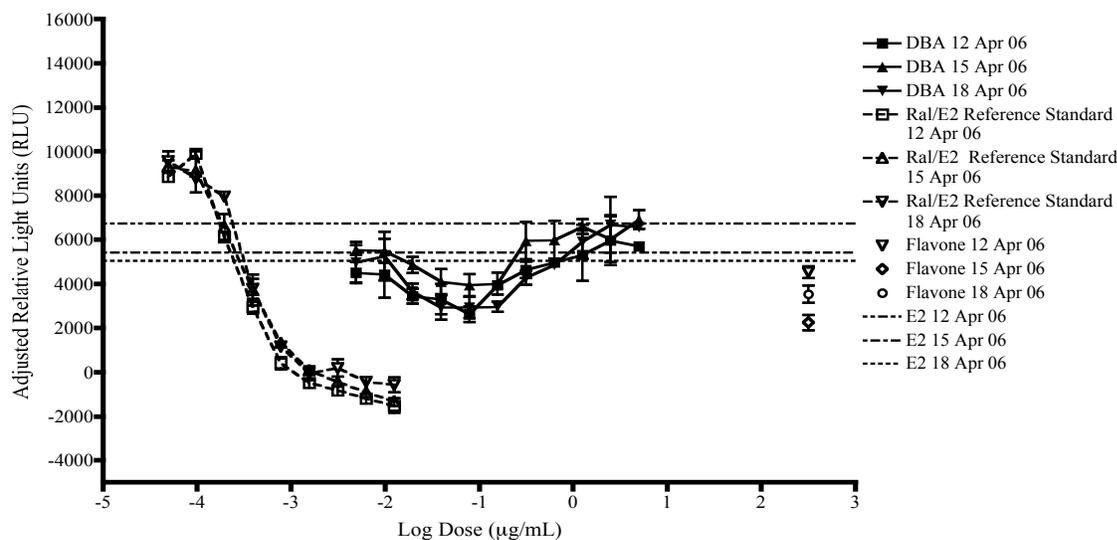
2164 Abbreviations: DBA = dibenzo[*a,h*]anthracene

2165

2166 Results of individual antagonist experiments for DBA are shown in **Figure 12-14**.

2167

2168 **Figure 12-14 Antagonist Comprehensive Testing for N0010 – DBA:**  
 2169 **Individual Experiments<sup>1,2</sup>**



2170

2171 Abbreviations: DBA = dibenzo[*a,h*]anthracene; Ral/E2 reference standard = concentrations of raloxifene with a fixed  
 2172 concentration of  $2.5 \times 10^{-5}$   $\mu\text{g/mL}$   $17\beta$ -estradiol; Flavone = 25  $\mu\text{g/mL}$  flavone control; E2 =  $17\beta$ -estradiol.

2173 <sup>1</sup>Horizontal lines represent the mean of three E2 control replicates minus three times the standard deviation  
 2174 of the E2 control mean. Values must be below the line without any significant decreases in cell viability in  
 2175 order to be considered positive for antagonism

2176 <sup>2</sup>The 25  $\mu\text{g/mL}$  flavone controls are not shown at the concentration at which they were tested. They  
 2177 have been placed on the graph in such a way as to maximize visibility.

2178

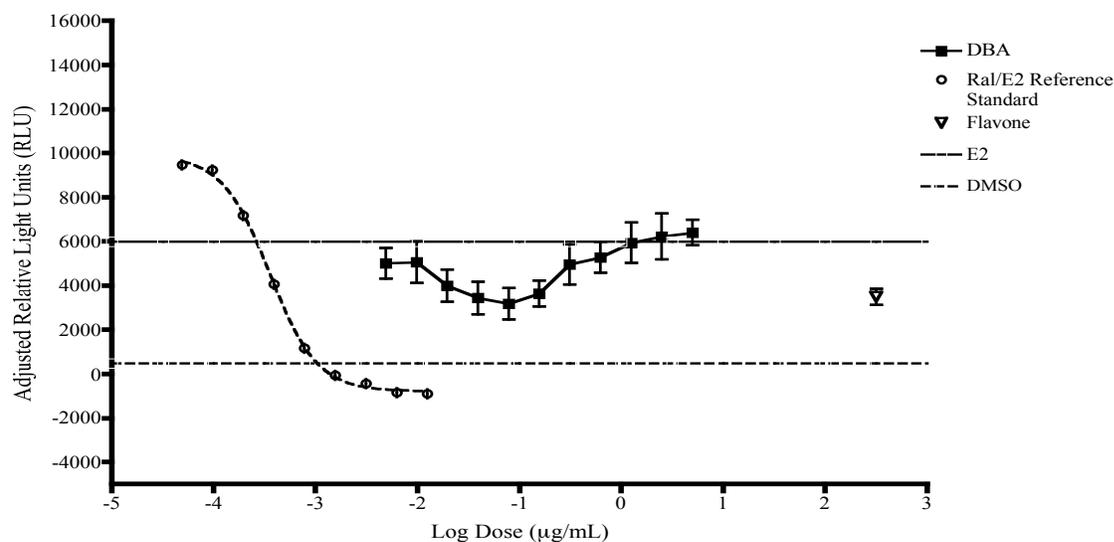
2179 DBA at concentrations between  $1.95 \times 10^{-2}$  and  $7.81 \times 10^{-2}$   $\mu\text{g/mL}$  showed a decrease below the E2 line.

2180 However, the concentration-response curve for DBA was biphasic and therefore an  $\text{IC}_{50}$  value could not

2181 be calculated. Results of averaged antagonist experiments for DBA are shown in **Figure 12-15**.

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2182

2183 **Figure 12-15 Antagonist Comprehensive Testing for N0010 – DBA:**2184 **Averaged Experiments<sup>1,2,3,4,5,6</sup>**

2185

2186

2187

2188

Abbreviations: DBA = Dibenzo[*a,h*]anthracene; Ral/E2 reference standard = concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$   $\mu\text{g/mL}$  17 $\beta$ -estradiol; Flavone = 25  $\mu\text{g/mL}$  flavone control; E2 = 17 $\beta$ -estradiol; DMSO = dimethyl sulfoxide.

2189 <sup>1</sup>Historical mean and standard deviation of the Ral/E2 reference standard.2190 <sup>2</sup>Historical mean and standard deviation of the flavone control2191 <sup>3</sup>Historical mean and standard deviation of the E2 control.

2192

2193

2194

<sup>4</sup>Solid horizontal line represents the historical mean of the E2 control minus three times the standard deviation of the E2 mean. Values must be below the line without any significant decreases in cell viability in order to be considered positive for antagonism.

2195

2196

<sup>5</sup>Dashed horizontal line represents the historical mean of the DMSO control minus three times the standard deviation of the DMSO control mean.

2197

2198

<sup>6</sup>The 25  $\mu\text{g/mL}$  flavone controls are not shown at the concentration at which they were tested. They have been placed on the graph in such a way as to maximize visibility.

2199

2200 DBA at concentrations between  $1.95 \times 10^{-2}$  and  $7.81 \times 10^{-2}$   $\mu\text{g/mL}$  showed a decrease below the E2 line.2201 However, the concentration-response curve for DBA was biphasic and an  $\text{IC}_{50}$  value could not be

2202 calculated.

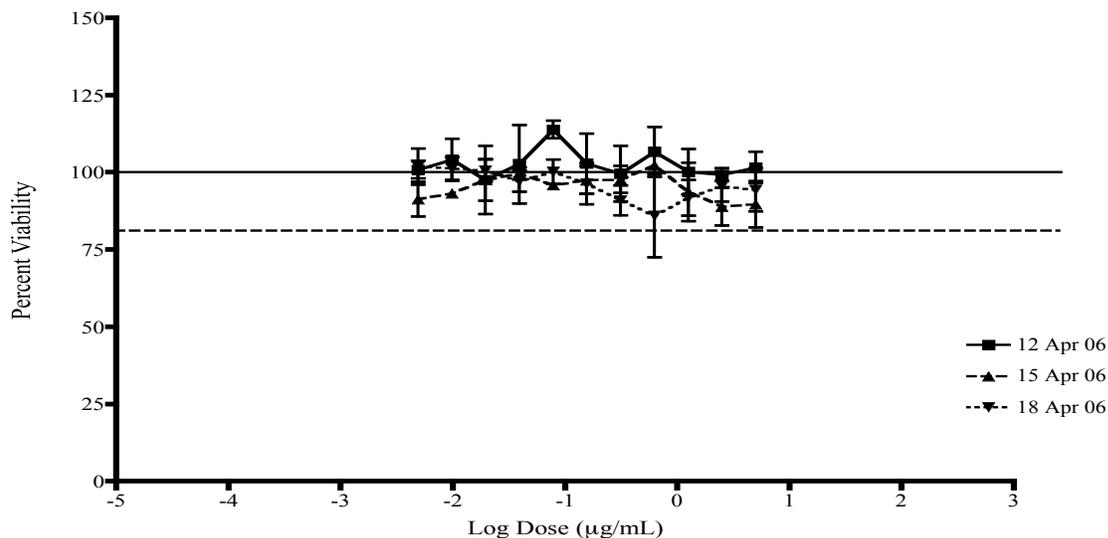
2203 DBA did not cause a decrease in the cell viability in range finder or comprehensive testing (**Figures 12-**2204 **16, 12-17, and 12-18**).

2205

2206

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2206 **Figure 12-16 CellTiter-Glo® Viability Assessment for N0010 – DBA<sup>1,2</sup>**



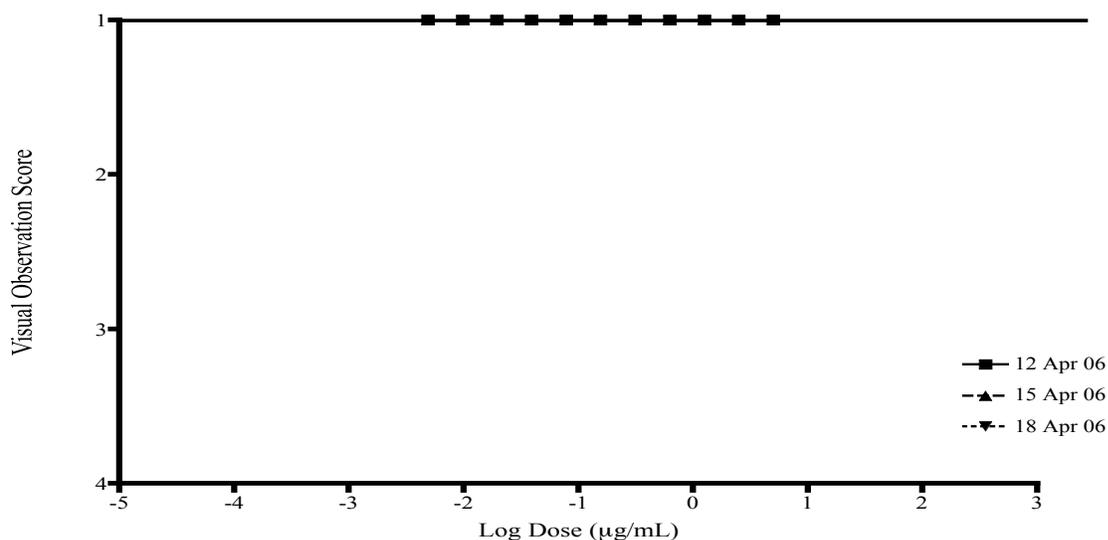
2207  
2208 Abbreviations: DBA = Dibenzo[*a,h*]anthracene

2209 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

2210 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability  
2211 below this line are considered cytotoxic and are not included in the evaluation of antagonist activity.

2212

2213 **Figure 12-17 Visual Observation Viability Assessment for N0010 – DBA<sup>1</sup>**

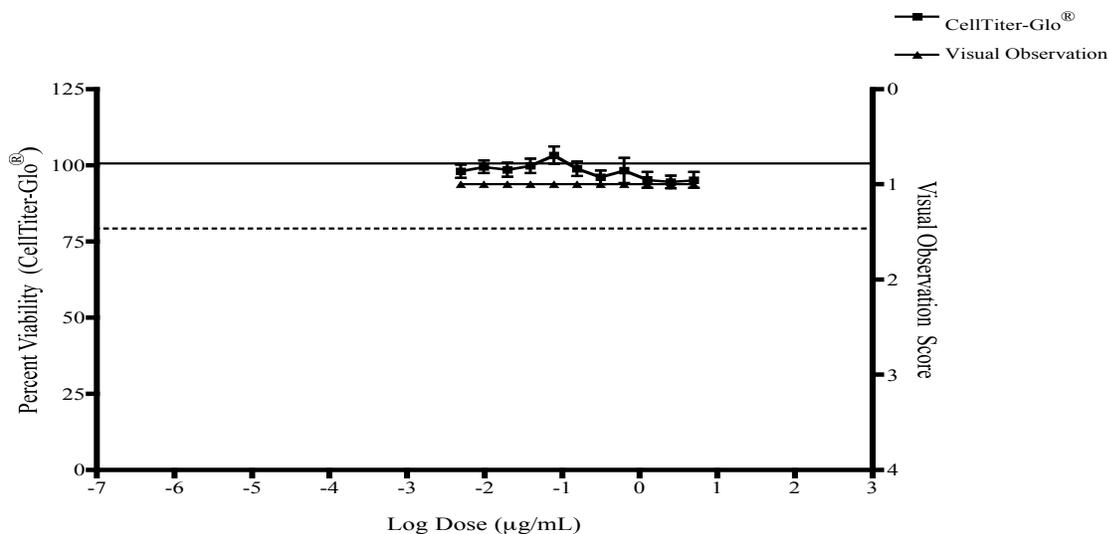


2214  
2215 Abbreviations: DBA = Dibenzo[*a,h*]anthracene

2216 <sup>1</sup>Wells containing cells that exhibit normal morphology and density are given a visual observation  
2217 score of 1; Wells that are exposed to concentrations resulting in visual observation scores ≥ 2 are  
2218 considered cytotoxic and are not included in the evaluation of antagonist activity.

2219  
2220

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2220 **Figure 12-18 Combined Qualitative and Quantitative Viability**2221 **Assessments for N0010 – DBA<sup>1,2</sup>**

2222

2223 Abbreviations: DBA = Dibenzo[*a,h*]anthracene2224 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.2225 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability  
2226 below this line are considered cytotoxic and are not included in the evaluation of antagonist activity.

2227

2228 **12.2.3N0011 – Genistein**

2229 Genistein was selected for antagonist testing because it was listed in the ICCVAM Guidelines (ICCVAM  
2230 2003, 2006) as positive for ER antagonist activity in the one assay in which it was tested, and because of  
2231 its potential problems with solubility in aqueous media. 50 µg/mL was selected as the starting  
2232 concentration for the double serial dilution used for comprehensive testing because it gave the lowest  
2233 adjusted RLU value during range finder testing. The concentrations of genistein tested are listed in **Table**  
2234 **12-6**.

2235

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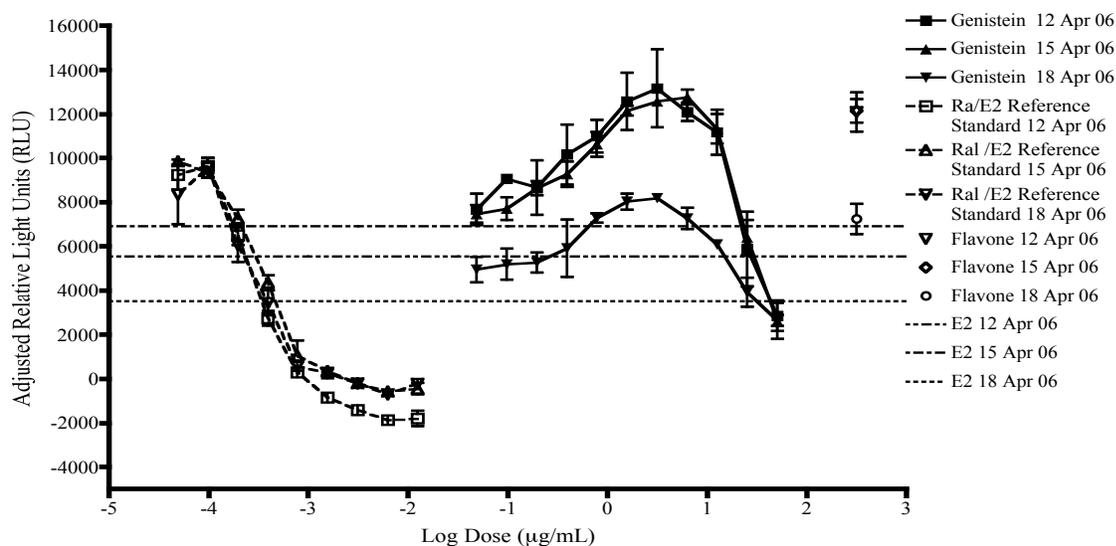
2235 **Table 12-6 Concentrations of N0011 – Genistein**  
 2236 **used in Comprehensive Testing**

N0011 – Genistein ( $\mu\text{g/mL}$ )		
50	3.13	0.2
25	1.56	$9.77 \times 10^{-2}$
12.5	0.78	$4.81 \times 10^{-2}$
6.25	0.39	

2237

2238 Results of individual antagonist experiments for genistein are shown in **Figure 12-19**.

2239

2240 **Figure 12-19 Antagonist Comprehensive Testing for N0011 –**2241 **Genistein: Individual Experiments<sup>1,2</sup>**

2242

2243

2244 Abbreviations: Ral/E2 Reference Standard = concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$   $\mu\text{g/mL}$ 17 $\beta$ -estradiol; Flavone = 25  $\mu\text{g/mL}$  flavone control; E2 = 17 $\beta$ -estradiol.

2245

2246

2247

<sup>1</sup>Horizontal lines represent the mean of three E2 control replicates minus three times the standard deviation of the E2 control mean. Values must be below the line without any significant decreases in cell viability in order to be considered positive for antagonism.

2248

2249

<sup>2</sup>The 25  $\mu\text{g/mL}$  flavone controls are not shown at the concentration at which they were tested. They have been placed on the graph in such a way as to maximize visibility.

2250

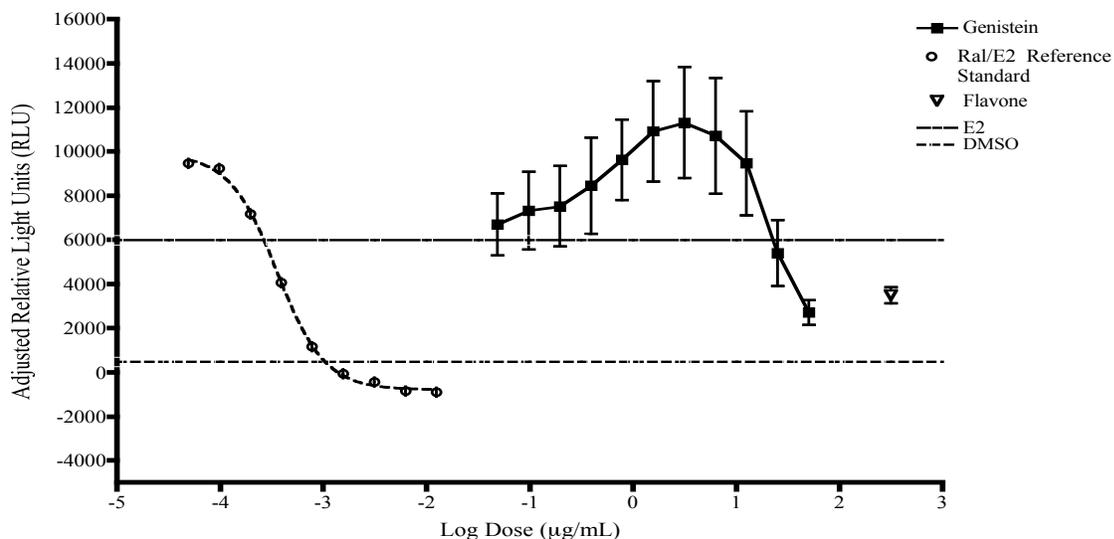
2251 Genistein showed potential antagonist activity at the highest concentration tested (50  $\mu\text{g/mL}$ ).2252 Results of averaged antagonist experiments for genistein are shown in **Figure 12-20**.

2253

2254

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2254 **Figure 12-20 Antagonist Comprehensive Testing for N0011 –**  
 2255 **Genistein: Averaged Experiments<sup>1,2,3,4,5,6</sup>**



2256

2257

2258

Abbreviations: Ral/E2 Reference Standard = concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$  µg/mL 17β-estradiol; Flavone = 25 µg/mL flavone control; E2 = 17β-estradiol; DMSO = dimethyl sulfoxide.

2259

<sup>1</sup>Historical mean and standard deviation of the Ral/E2 reference standard.

2260

<sup>2</sup>Historical mean and standard deviation of the flavone control

2261

<sup>3</sup>Historical mean and standard deviation of the E2 control.

2262

2263

2264

<sup>4</sup>Solid horizontal line represents the historical mean of the E2 control minus three times the standard deviation of the E2 control mean. Values must be below the line without any significant decreases in cell viability in order to be considered positive for antagonism.

2265

2266

<sup>5</sup>Dashed horizontal line represents the historical mean of the DMSO control minus three times the standard deviation of the DMSO control mean.

2267

2268

<sup>6</sup>The 25 µg/mL flavone controls are not shown at the concentration at which they were tested. They have been placed on the graph in such a way as to maximize visibility.

2269

2270 Genistein showed antagonist activity at the highest concentration tested (50 µg/mL). An IC<sub>50</sub> value could

2271 not be calculated because genistein did not reach saturation at the highest concentrations tested.

2272 Genistein did not cause a decrease in cell viability at any of the concentrations tested in either the range

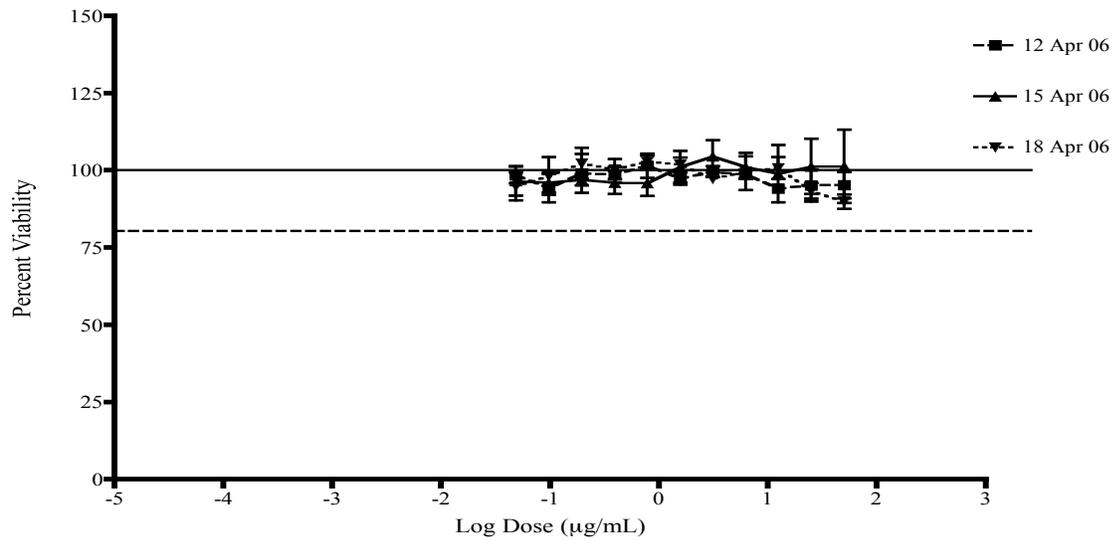
2273 finder or during comprehensive testing (**Figures 12-21, 12-22, and 12-23**).

2274

2275

PREDECISIONAL MATERIAL: DO NOT CITE, QUOTE OR DISTRIBUTE

2275 **Figure 12-21 CellTiter-Glo® Viability Assessment for N0011 – Genistein<sup>1,2</sup>**

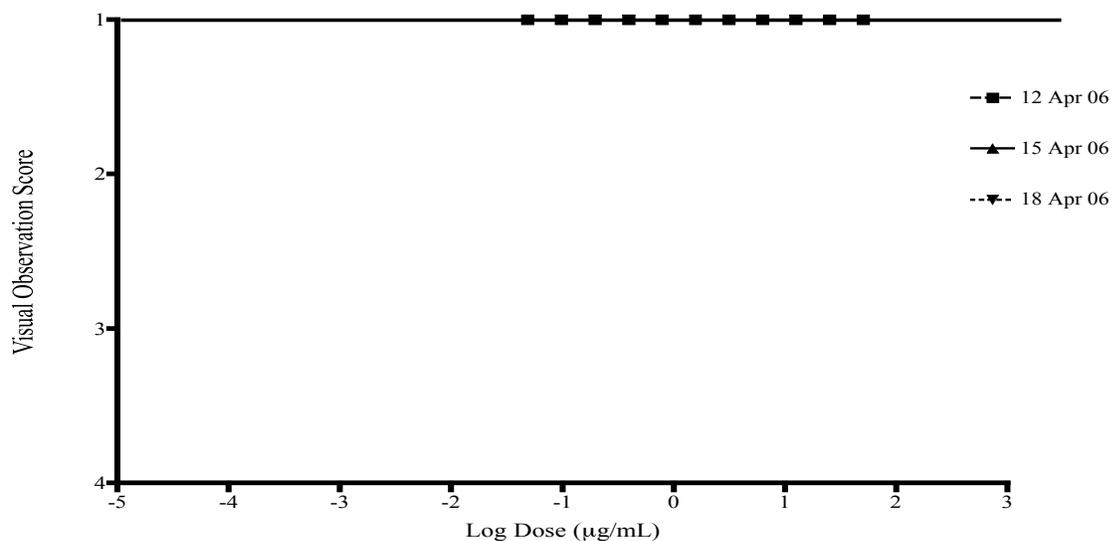


2276  
2277 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

2278  
2279 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability below this line are considered cytotoxic and are not included in the evaluation of antagonist activity.

2280

2281 **Figure 12-22 Visual Observation Viability Assessment for N0011 – Genistein<sup>1</sup>**

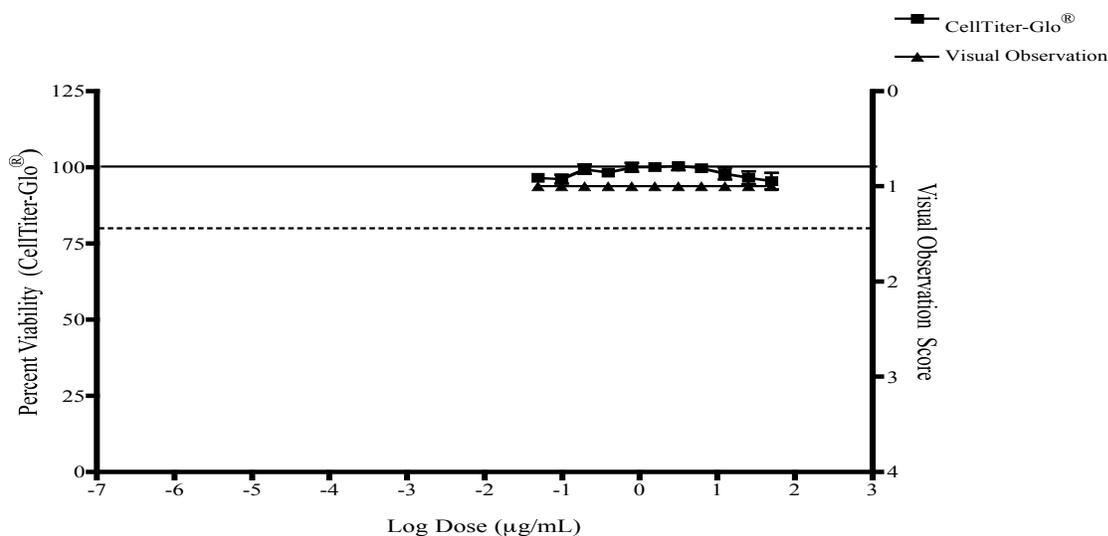


2282  
2283  
2284 <sup>1</sup>Wells containing cells that exhibit normal morphology and density are given a visual observation  
2285 score of 1. Wells that are exposed to concentrations resulting in visual observation scores  $\geq 2$  are  
considered cytotoxic and are not included in the evaluation of antagonist activity.

2286  
2287

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2287 **Figure 12-23 Combined Qualitative and Quantitative Viability**  
 2288 **Assessments for N0011 – Genistein<sup>1,2</sup>**



2289 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

2290 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability  
 2291 below this line are considered cytotoxic and are not included in the evaluation of antagonist activity.

2292

### 2293 12.2.4 N0012 – Flavone

2294 Flavone was selected for antagonist testing because it was listed in the ICCVAM Guidelines (ICCVAM  
 2295 2003, 2006) as uniformly positive for ER antagonist activity in multiple assays. 50 µg/mL was selected as  
 2296 the starting concentration for the double serial dilution used for comprehensive testing because it gave the  
 2297 lowest adjusted RLU value during range finder testing. The concentrations of flavone tested are listed in  
 2298 **Table 12-7**.

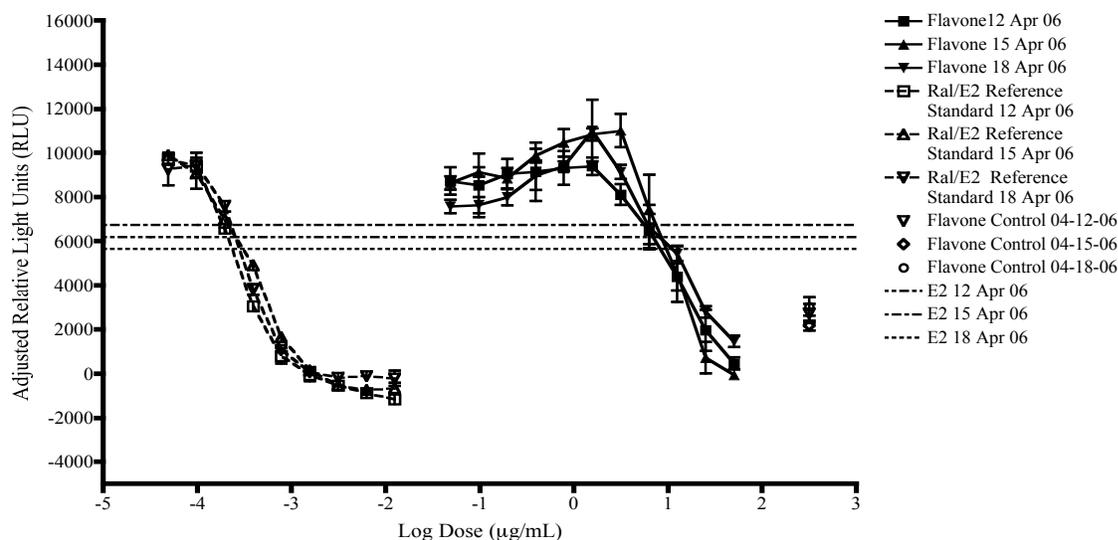
2300 **Table 12-7 Concentrations of N0012 – Flavone**  
 2301 **used in Comprehensive Testing**

N0012 – Flavone (µg/mL)		
50	3.13	0.2
25	1.56	$9.77 \times 10^{-2}$
12.5	0.78	$4.81 \times 10^{-2}$
6.25	0.39	

2302 Results of individual antagonist experiments for flavone are shown in **Figure 12-24**.  
 2303

PREDECISIONAL MATERIAL: DO NOT CITE, QUOTE OR DISTRIBUTE

2304

2305 **Figure 12-24 Antagonist Comprehensive Testing for N0012 – Flavone: Individual Experiments<sup>1,2</sup>**

2306

2307

2308

Abbreviations: Ral/E2 Reference Standard = concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$   $\mu\text{g/mL}$   $17\beta$ -estradiol; Flavone Control = 25  $\mu\text{g/mL}$  flavone control; E2 =  $17\beta$ -estradiol.

2309

2310

2311

2312

2313

<sup>1</sup>Horizontal lines represent the mean of three E2 control replicates minus three times the standard deviation of the E2 control mean. Values must be below the line without any significant decreases in cell viability in order to be considered positive for antagonism.

<sup>2</sup>The 25  $\mu\text{g/mL}$  flavone controls are not shown at the concentration at which they were tested. They have been placed on the graph in such a way as to maximize visibility.

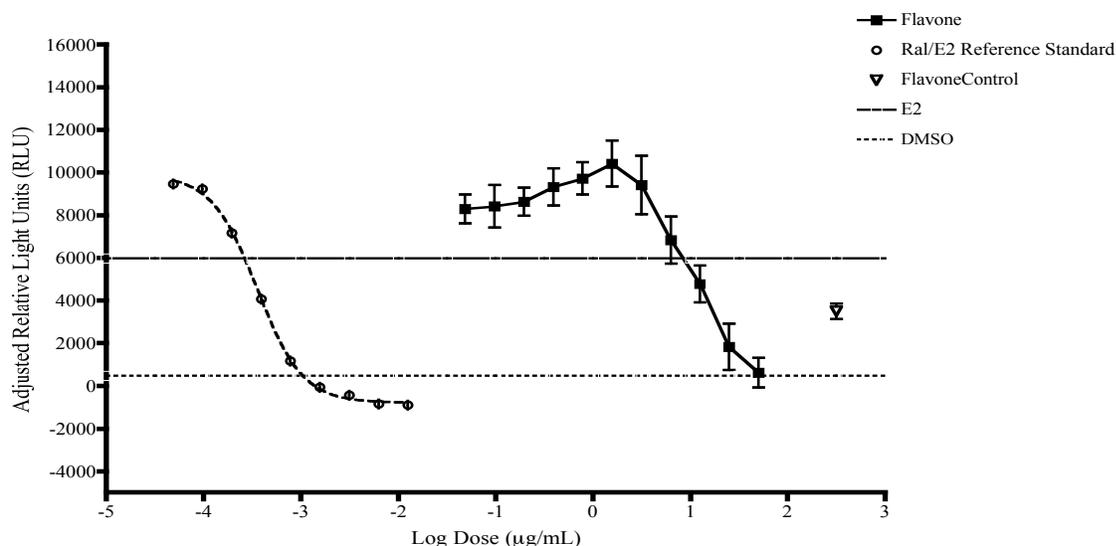
2314

2315 Flavone showed antagonist activity at the three highest concentrations tested (12.5, 25, and 50  $\mu\text{g/mL}$ ).2316 Results of averaged antagonist experiments for flavone are shown in **Figure 12-25**.

2317

PREDECISIONAL MATERIAL: DO NOT CITE, QUOTE OR DISTRIBUTE

2317 **Figure 12-25 Antagonist Comprehensive Testing for N0012 – Flavone: Averaged**  
 2318 **Experiments<sup>1,2,3,4,5,6</sup>**



2319  
 2320  
 2321

Abbreviations: Ral/E2 Reference Standard = concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$  µg/mL 17β-estradiol; Flavone Control = 25 µg/mL flavone control; E2 = 17β-estradiol; DMSO = dimethyl sulfoxide.

2322 <sup>1</sup>Historical mean and standard deviation of the Ral/E2 reference standard.

2323 <sup>2</sup>Historical mean and standard deviation of the flavone control

2324 <sup>3</sup>Historical mean and standard deviation of the E2 control.

2325 <sup>4</sup>Solid horizontal line represents the historical mean of the E2 control minus three times the standard  
 2326 deviation of the E2 control mean. Values must be below the line without any significant decreases in cell viability in  
 2327 order to be considered positive for antagonism.

2328 <sup>5</sup>Dashed horizontal line represents the historical mean of the DMSO control minus three times the  
 2329 standard deviation of the DMSO control mean.

2330 <sup>6</sup>The 25 µg/mL flavone controls are not shown at the concentration at which they were tested. They  
 2331 have been placed on the graph in such a way as to maximize visibility.

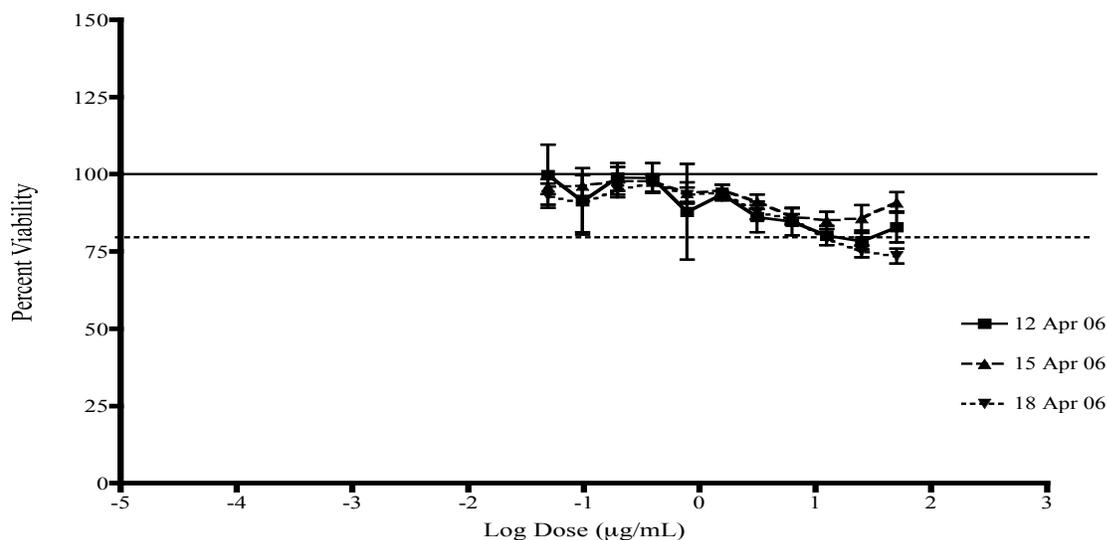
2332

2333 Flavone showed antagonist activity at 12.5, 25, and 50 µg/mL. An  $IC_{50}$  value for flavone could not be  
 2334 calculated because the flavone concentration-response curve did not reach saturation at the highest  
 2335 concentrations tested.

2336 Flavone did not reduce cell viability below 80% (**Figure 12-26**).

2337

PREDECISIONAL MATERIAL: DO NOT CITE, QUOTE OR DISTRIBUTE

2337 **Figure 12-26 CellTiter-Glo® Viability Assessment for N0012 – Flavone<sup>1,2</sup>**

2338

2339 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.2340 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability  
2341 below this line are considered cytotoxic and are not included in the evaluation of antagonist activity.

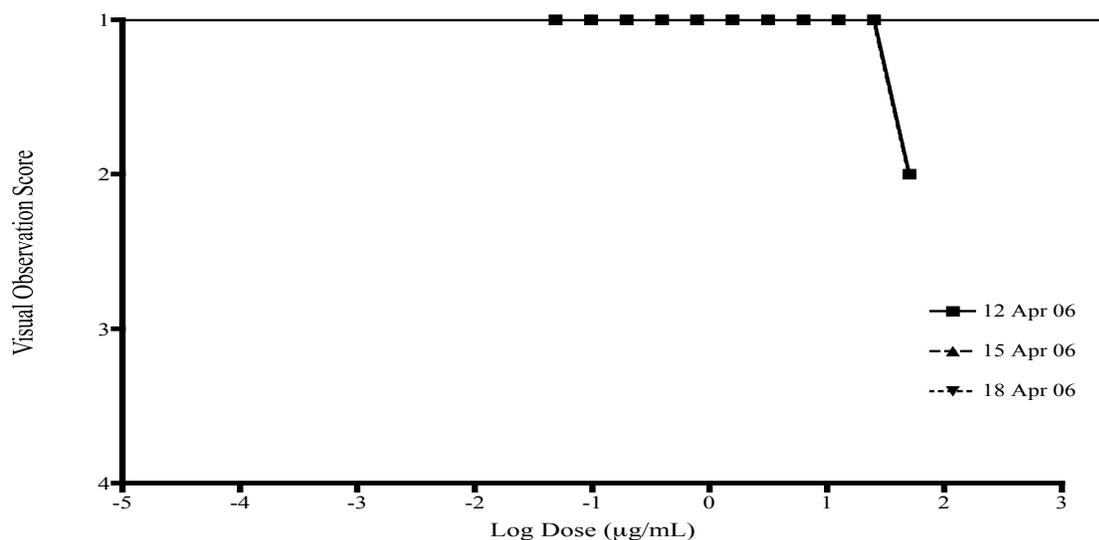
2342

2343 However, the results of the visual observation scoring (**Figure 12-27**) did not agree with those seen in  
 2344 CellTiter-Glo®. The visual observation scores indicated that flavone had a toxicity score of 2, indicating  
 2345 that cells were damaged. A comparison of CellTiter-Glo® data and visual observation scores is presented  
 2346 in **Figure 12-28**.

2347

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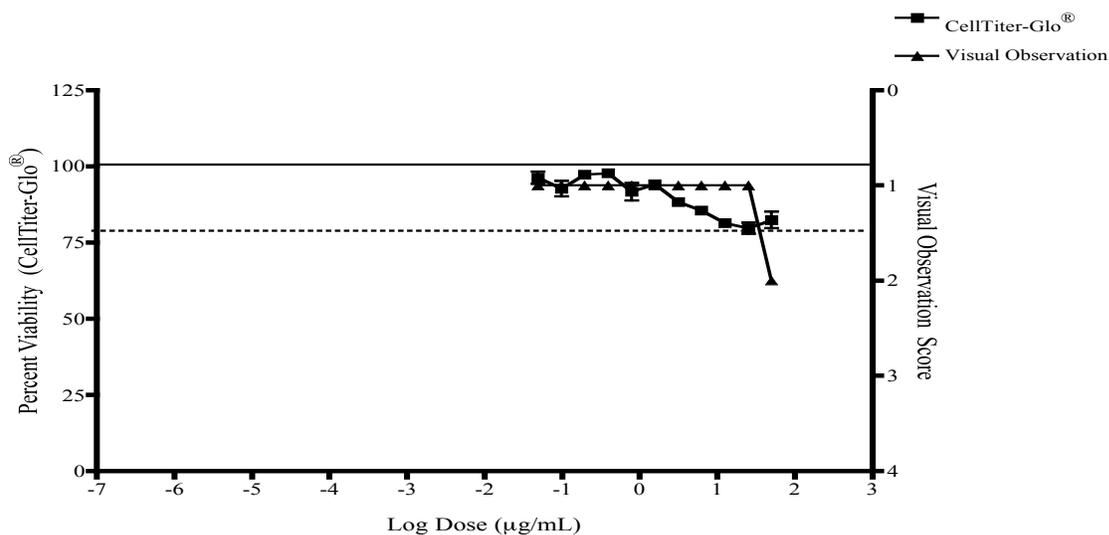
2347 **Figure 12-27 Visual Observation Viability Assessment for N0012 – Flavone<sup>1</sup>**



2348

2349 <sup>1</sup>Wells containing cells that exhibit normal morphology and density are given a visual observation  
 2350 score of 1; wells containing cells that exhibit altered morphology and have small gaps between  
 2351 cells are given a visual observation score of 2. Wells that are exposed to concentrations resulting in visual  
 2352 observation scores  $\geq 2$  are considered cytotoxic and are not included in the evaluation of antagonist activity.

2353 **Figure 12-28 Combined Qualitative and Quantitative Viability**  
 2354 **Assessments for N0012 – Flavone<sup>1,2</sup>**



2355

2356 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

2357 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability  
 2358 below this line are considered cytotoxic and are not included in the evaluation of antagonist activity.

2359

2360

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2360 **12.2.5N0013 – Nonylphenol**

2361 Nonylphenol was selected for antagonist testing because it was listed in the ICCVAM Guidelines  
 2362 (ICCVAM 2003, 2006) as positive for ER antagonist activity in the one assay in which it was tested. 50  
 2363 µg/mL was selected as the starting concentration for the double serial dilution used for comprehensive  
 2364 testing because it gave the lowest adjusted RLU value during range finder testing. The concentrations of  
 2365 nonylphenol tested are listed in **Table 12-8**.

2366

2367 **Table 12-8 Concentrations of N0013 – Nonylphenol**  
 2368 **Used in Comprehensive Testing**

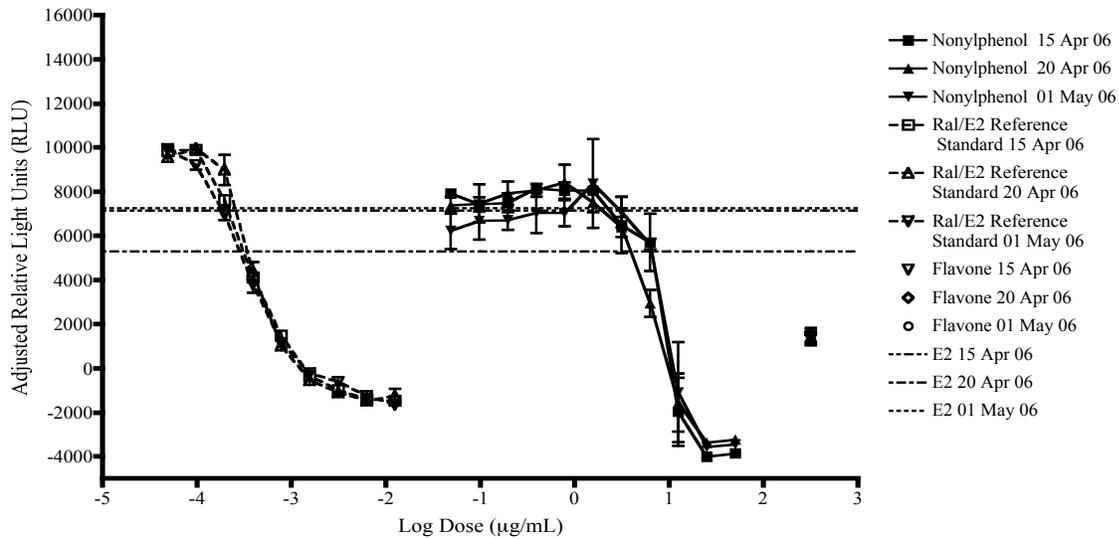
N0013 – Nonylphenol (µg/mL)		
50	3.13	0.2
25	1.56	9.77 x 10 <sup>-2</sup>
12.5	0.78	4.81 x 10 <sup>-2</sup>
6.25	0.39	

2369 Results of individual antagonist experiments for nonylphenol are shown in **Figure 12-29**. Nonylphenol  
 2370 showed potential antagonist activity at the three highest concentrations tested (12.5, 25, and 50 µg/mL).  
 2371

2372

PREDECISIONAL MATERIAL: DO NOT CITE, QUOTE OR DISTRIBUTE

2372 **Figure 12-29 Antagonist Comprehensive Testing for N0013 –**  
 2373 **Nonylphenol: Individual Experiments<sup>1,2</sup>**



2374  
 2375  
 2376

Abbreviations: Ral/E2 Reference Standard = concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$  µg/mL 17β-estradiol; Flavone = 25 µg/mL flavone control; E2 = 17β-estradiol; DMSO = dimethyl sulfoxide.

2377  
 2378  
 2379

<sup>1</sup>Horizontal lines represent the mean of three E2 control replicates minus three times the standard deviation of the E2 control mean. Values must be below the line without any significant decreases in cell viability in order to be considered positive for antagonism.

2380  
 2381

<sup>2</sup>The 25 µg/mL flavone controls are not shown at the concentration at which they were tested. They have been placed on the graph in such a way as to maximize visibility.

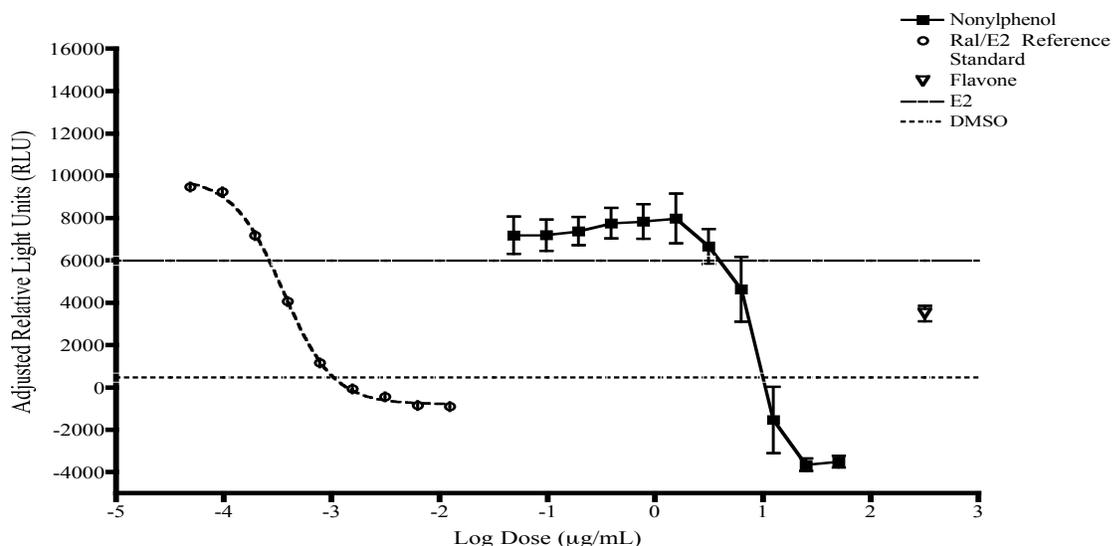
2382

2383 Results of averaged antagonist experiments for nonylphenol are shown in **Figure 12-30**.

2384

PREDECISIONAL MATERIAL: DO NOT CITE, QUOTE OR DISTRIBUTE

2384 **Figure 12-30 Antagonist Comprehensive Testing for N0013 –**  
 2385 **Nonylphenol: Averaged Experiments<sup>1,2,3,4,5,6</sup>**



2386  
 2387  
 2388

Abbreviations: Ral/E2 Reference Standard = concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$  µg/mL 17β-estradiol; Flavone = 25 µg/mL flavone control; E2 = 17β-estradiol; DMSO = dimethyl sulfoxide.

2389 <sup>1</sup>Historical mean and standard deviation of the Ral/E2 reference standard.

2390 <sup>2</sup>Historical mean and standard deviation of the flavone control

2391 <sup>3</sup>Historical mean and standard deviation of the E2 control.

2392 <sup>4</sup>Solid horizontal line represents the historical mean of the E2 control minus three times the standard  
 2393 deviation of the E2 control mean. Values must be below the line without any significant decreases in  
 2394 cell viability in order to be considered positive for antagonism.

2395 <sup>5</sup>Dashed horizontal line represents the historical mean of the DMSO control minus three times the  
 2396 standard deviation of the DMSO control mean.

2397 <sup>6</sup>The 25 µg/mL flavone controls are not shown at the concentration at which they were tested. They  
 2398 have been placed on the graph in such a way as to maximize visibility.

2399

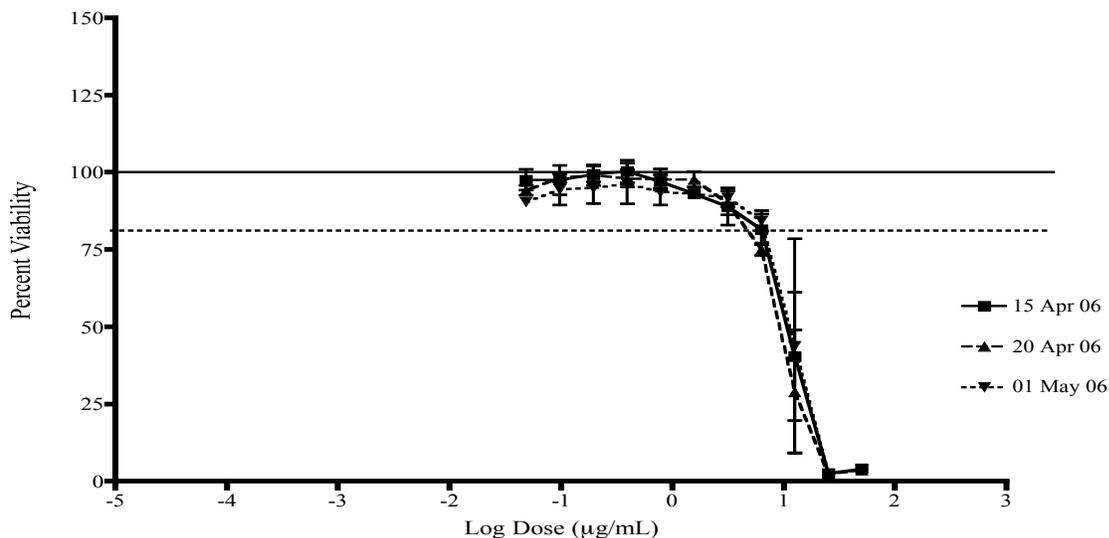
2400 On 20 April 06, one concentration of nonylphenol (6.25 µg/mL) induced a response that was less than the  
 2401 E2 response, without significant cytotoxicity. However, this response was only observed for a single  
 2402 concentration in a single experiment.

2403 Nonylphenol was cytotoxic at the three highest concentrations tested (Figures 12-31, 12-32, and 12-33),  
 2404 suggesting that the apparent antagonistic response may have been due to cytotoxicity rather than ER  
 2405 mediated antagonism.

2406

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2406 **Figure 12-31 CellTiter-Glo® Viability Assessment for N0013 – Nonylphenol<sup>1,2</sup>**

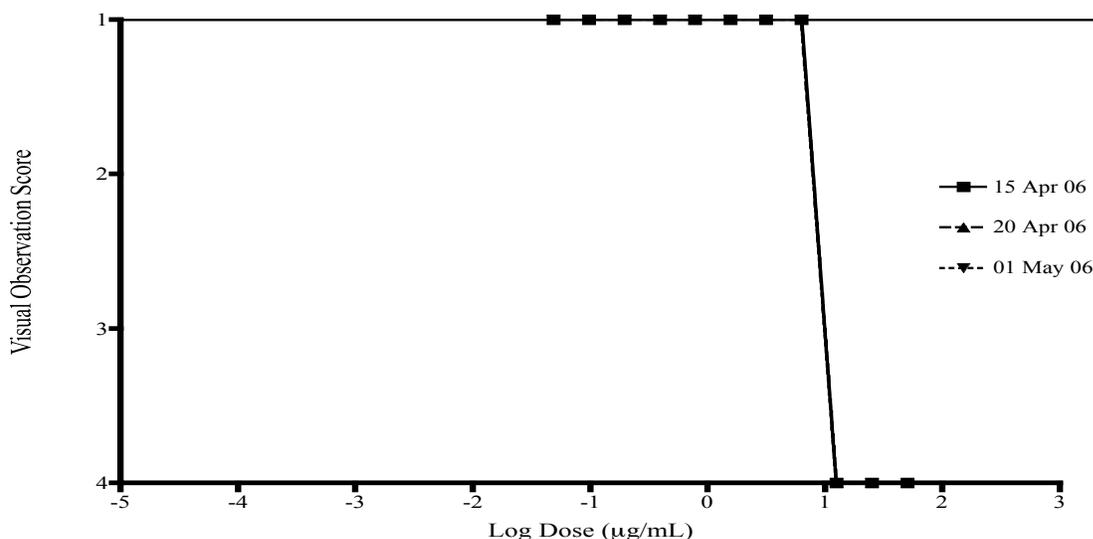


2407  
2408 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in DMSO control.

2409  
2410 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability below this line are considered cytotoxic and are not included in the evaluation of antagonist activity.

2411

2412 **Figure 12-32 Visual Observation Viability Assessment for N0013 – Nonylphenol<sup>1</sup>**



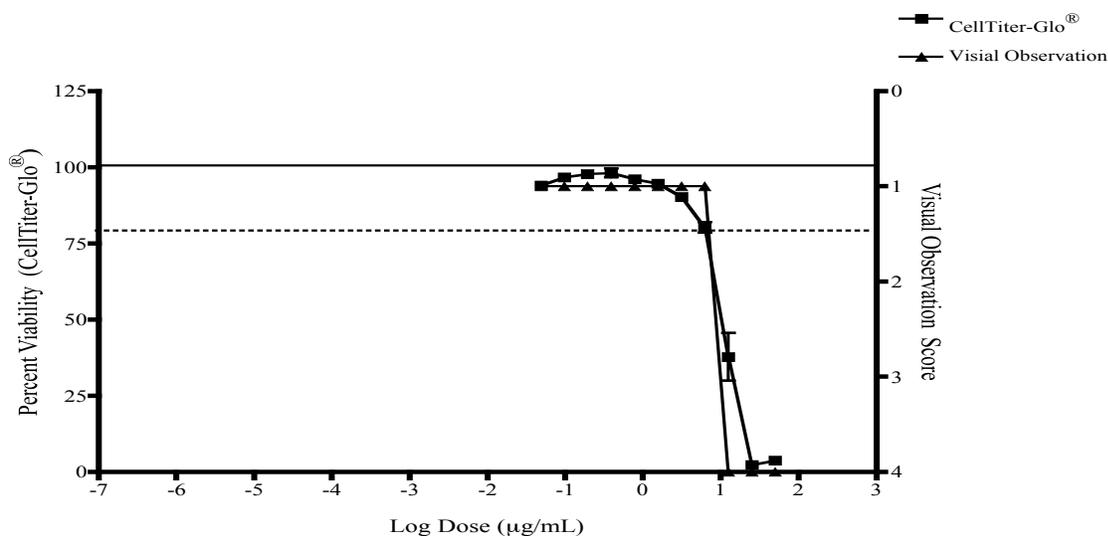
2413  
2414  
2415 <sup>1</sup>Wells containing cells that exhibit normal morphology and density are given a visual observation  
2416 score of 1; wells containing few or no visible cells are given a visual observation score of 2. Wells  
2417 that are exposed to concentrations resulting in visual observation scores ≥ 2 are considered cytotoxic  
and are not included in the evaluation of antagonist activity.

2418

2419

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2419 **Figure 12-33 Combined Qualitative and Quantitative Viability**  
 2420 **Assessments for N0013 – Nonylphenol<sup>1,2</sup>**



2421

2422 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in DMSO control.

2423 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability  
 2424 below this line are considered cytotoxic and are not included in the evaluation of antagonist activity.

2425

### 2426 12.2.6N0014 – Progesterone

2427 Progesterone was selected for antagonist testing because it was listed as negative for ER antagonist  
 2428 activity in the ICCVAM Guidelines (ICCVAM 2003, 2006). 50 µg/mL was selected as the starting  
 2429 concentration for the double serial dilution used for comprehensive testing because it gave the lowest  
 2430 adjusted RLU value during range finder testing. The concentrations of progesterone tested are listed in  
 2431 **Table 12-9.**

2432

2433

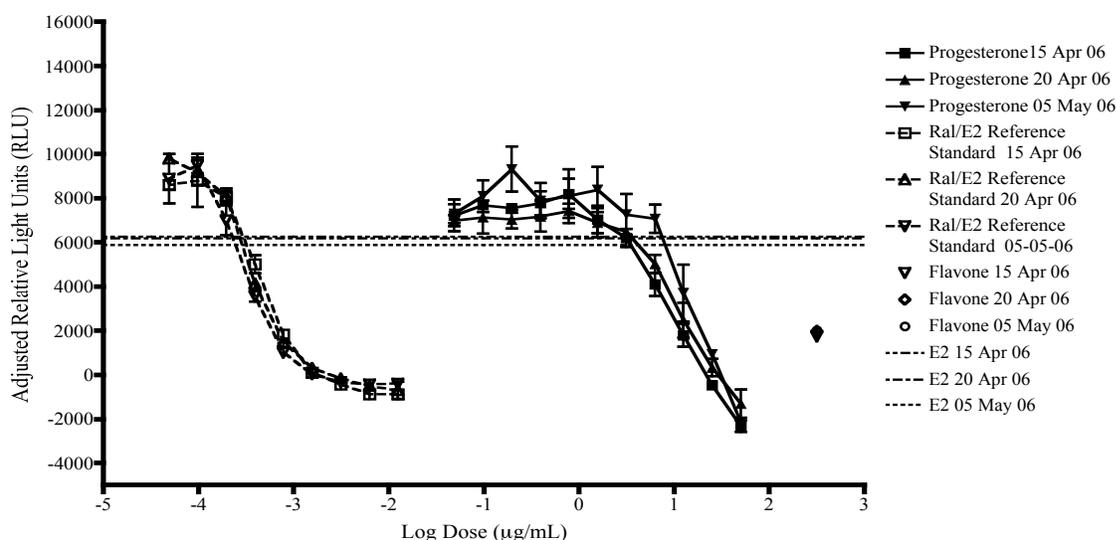
PREDECISIONAL MATERIAL: DO NOT CITE, QUOTE OR DISTRIBUTE

2433 **Table 12-9 Concentrations of N0014 – Progesterone**  
 2434 **Used in Comprehensive Testing**

N0014 – Progesterone (µg/mL)		
50	3.13	0.2
25	1.56	$9.77 \times 10^{-2}$
12.5	0.78	$4.81 \times 10^{-2}$
6.25	0.39	

2435  
 2436 Results of individual antagonist experiments for progesterone are shown in **Figure 12-34**.

2437  
 2438 **Figure 12-34 Antagonist Comprehensive Testing for N0014 –**  
 2439 **Progesterone: Individual Experiments<sup>1,2</sup>**



2440  
 2441 Abbreviations: Ral/E2 Reference Standard = concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$  µg/mL  
 2442 17β-estradiol; Flavone = 25 µg/mL flavone control; E2 = 17β-estradiol.

2443 <sup>1</sup>Horizontal lines represent the mean of three E2 control replicates minus three times the standard deviation  
 2444 of the E2 control mean. Values must be below the line without any significant decreases in cell viability in  
 2445 order to be considered positive for antagonism.

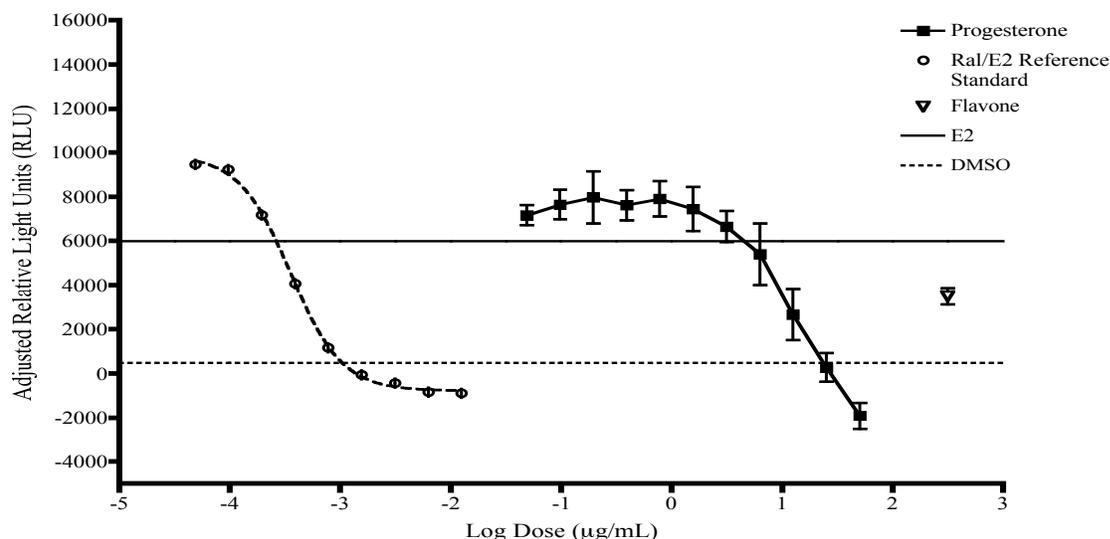
2446 <sup>2</sup>The 25 µg/mL flavone controls are not shown at the concentration at which they were tested. They  
 2447 have been placed on the graph in such a way as to maximize visibility.

2448  
 2449 Progesterone showed potential antagonist activity at the three highest concentrations tested (12.5, 25, and  
 2450 50 µg/mL).

2451 Results of averaged antagonist experiments for progesterone are shown in **Figure 12-35**.

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2452 **Figure 12-35 Antagonist Comprehensive Testing for N0014 –**  
 2453 **Progesterone: Averaged Experiments<sup>1,2,3,4,5,6</sup>**



2454

2455 Abbreviations: Ral/E2 Reference Standard = concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$  µg/mL  
 2456 17β-estradiol; Flavone = 25 µg/mL flavone control; E2 = 17β-estradiol; DMSO = dimethyl sulfoxide.

2457 <sup>1</sup>Historical mean and standard deviation of the Ral/E2 reference standard.

2458 <sup>2</sup>Historical mean and standard deviation of the flavone control

2459 <sup>3</sup>Historical mean and standard deviation of the E2 control.

2460 <sup>4</sup>Solid horizontal line represents the historical mean of the E2 control minus three times the standard  
 2461 deviation of the E2 control mean. Values must be below the line without any significant decreases in cell viability in  
 2462 order to be considered positive for antagonism.

2463 <sup>5</sup>Dashed horizontal line represents the historical mean of the DMSO control minus three times the  
 2464 standard deviation of the DMSO mean.

2465 <sup>6</sup>The 25 µg/mL flavone controls are not shown at the concentration at which they were tested. They  
 2466 have been placed on the graph in such a way as to maximize visibility.

2467

2468 Progesterone showed potential antagonist activity at 12.5, 25, and 50 µg/mL.

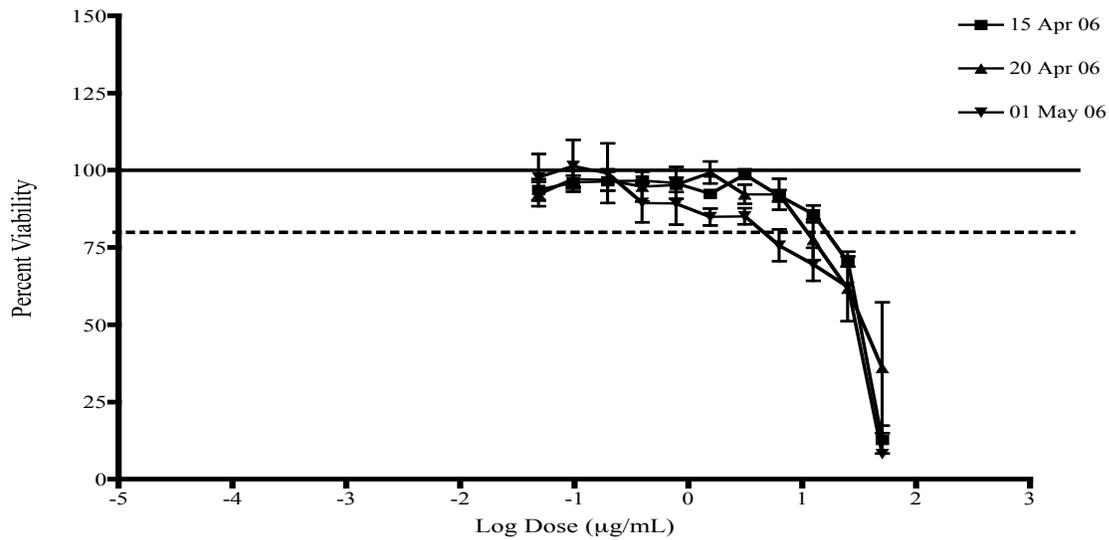
2469 Cell viability results for progesterone for CellTiter-Glo<sup>®</sup> and visual observations are shown in **Figures**  
 2470 **12-36, 12-37, and 12-38**. In the CellTiter-Glo<sup>®</sup> assay, progesterone caused reductions in cell viability at  
 2471 the three highest concentrations tested, suggesting that the apparent antagonistic response may have been  
 2472 due to cytotoxicity rather than ER mediated antagonism. These results are partially supported by the  
 2473 visual observation scoring, where cells exposed to 25 and 50 µg/mL progesterone showed moderate to  
 2474 severe cellular damage.

2475

2476

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2476 **Figure 12-36 CellTiter-Glo® Viability Assessment for N0014 – Progesterone<sup>1,2</sup>**

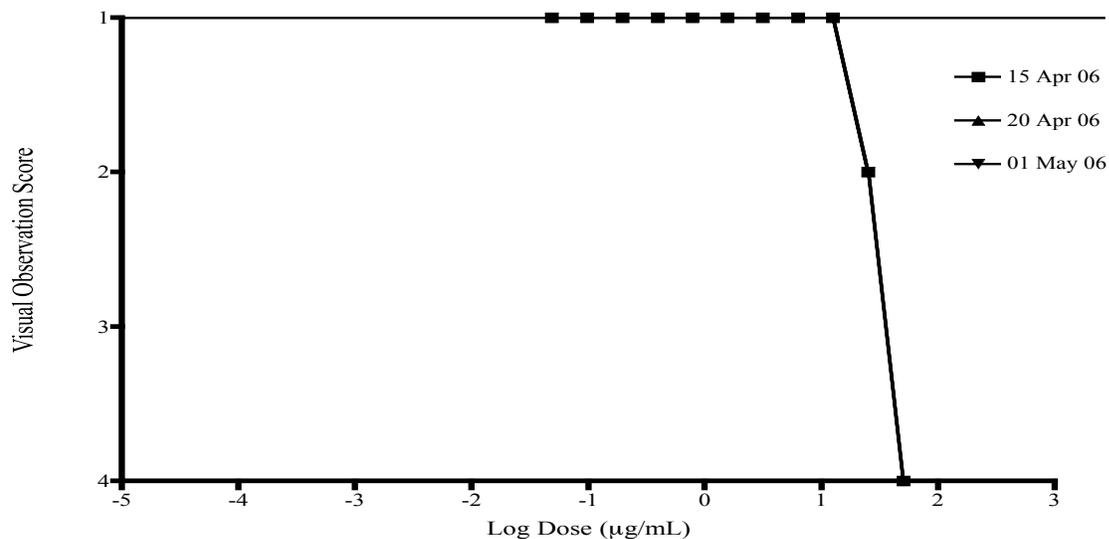


2477 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

2478 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability below this line are considered cytotoxic and are not included in the evaluation of antagonist activity.

2481

2482 **Figure 12-37 Visual Observation Viability Assessment for N0014 – Progesterone<sup>1</sup>**

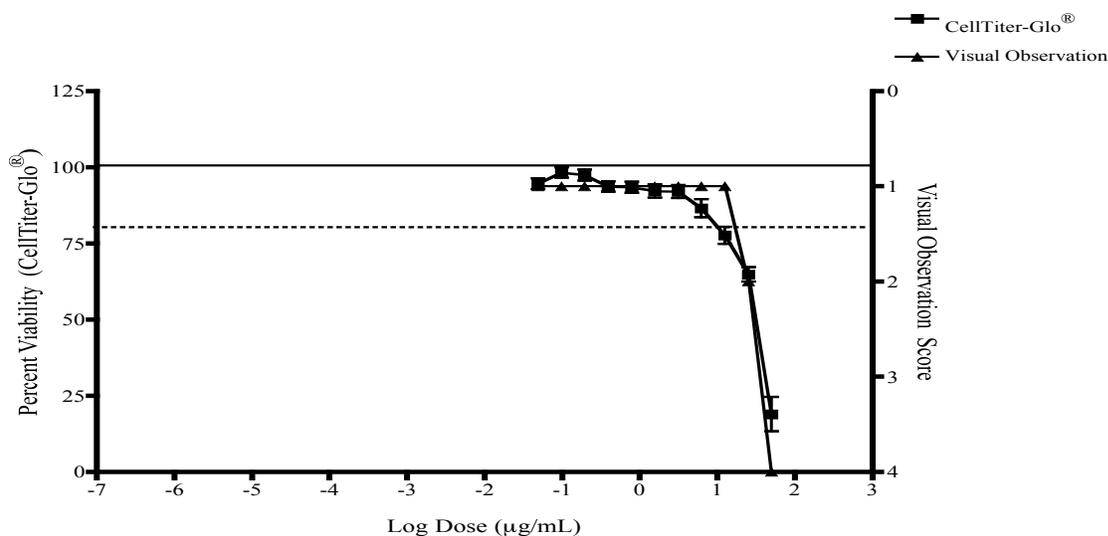


2483 <sup>1</sup>Wells containing cells that exhibit normal morphology and density are given a visual observation score of 1; wells containing cells that exhibit altered morphology and have small gaps between cells are given a visual observation score of 2; wells containing few or no visible cells are given a visual observation score of 4. Wells that are exposed to concentrations resulting in visual observation scores  $\geq 2$  are considered cytotoxic and are not included in the evaluation of antagonist activity.

2489

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2490 **Figure 12-38 Combined Qualitative and Quantitative Viability**2491 **Assessments for N0014 – Progesterone<sup>1,2</sup>**

2492

2493 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.2494 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability  
2495 below this line are considered cytotoxic and are not included in the evaluation of antagonist activity.

2496

2497 **12.2.7N0015 – *o,p'*-DDT**

2498 *o,p'*-DDT was selected for antagonist testing because it was listed in the ICCVAM Guidelines (ICCVAM  
2499 2003, 2006) as positive for ER antagonist activity in the one assay in which it was tested, and its potential  
2500 for cytotoxicity. 50 µg/mL was selected as the starting concentration for the double serial dilution used for  
2501 comprehensive testing because it gave the lowest adjusted RLU value during range finder testing. The  
2502 concentrations of *o,p'*-DDT tested are listed in **Table 12-10**.

2503

2504 **Table 12-10 Concentrations of N0015 - *o,p'*-DDT Used**  
2505 **in Comprehensive Testing**

N0015 – <i>o,p'</i> -DDT (µg/mL)		
50	3.13	0.2
25	1.56	9.77 x 10 <sup>-2</sup>
12.5	0.78	4.81 x 10 <sup>-2</sup>
6.25	0.39	

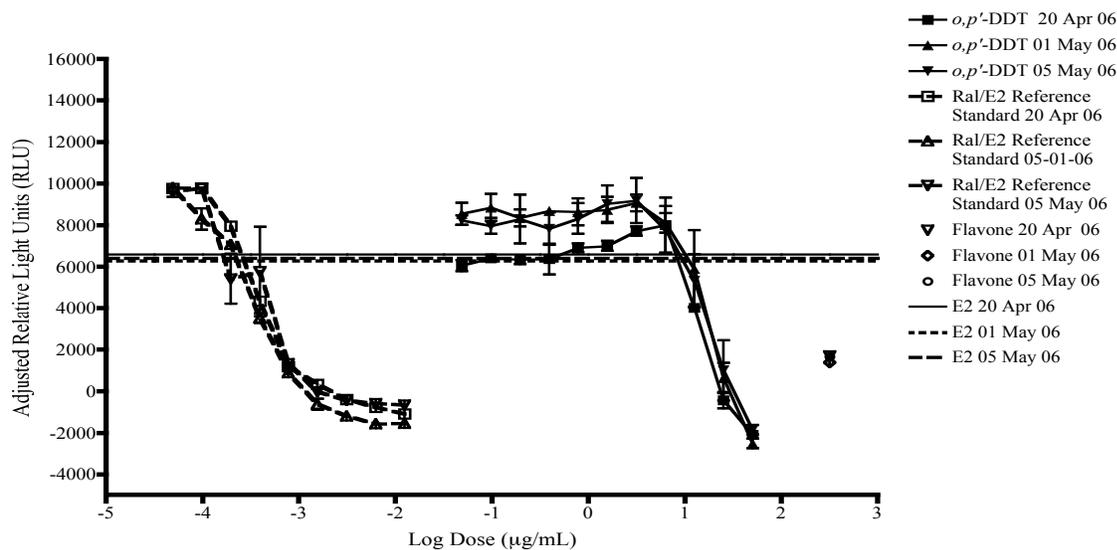
2506 Abbreviations: *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane

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2507

2508 Results of individual antagonist experiments for *o,p'*-DDT are shown in **Figure 12-39**.

2509

2510 **Figure 12-39 Antagonist Comprehensive Testing for N0015 –**2511 ***o,p'*-DDT: Individual Experiments<sup>1,2</sup>**

2512

2513

2514

2515

Abbreviations: *o,p'*-DDT and DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane; Ral/E2 Reference Standard = concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$  µg/mL 17β-estradiol; Flavone = 25 µg/mL flavone control; E2 = 17β-estradiol.

2516

2517

2518

<sup>1</sup>Horizontal lines represent the mean of three E2 control replicates minus three times the standard deviation of the E2 control mean. Values must be below the line without any significant decreases in cell viability in order to be considered positive for antagonism.

2519

2520

<sup>2</sup>The 25 µg/mL flavone controls are not shown at the concentration at which they were tested. They have been placed on the graph in such a way as to maximize visibility.

2521

2522 *o,p'*-DDT showed potential antagonist activity at the three highest concentrations tested (12.5, 25, and 50

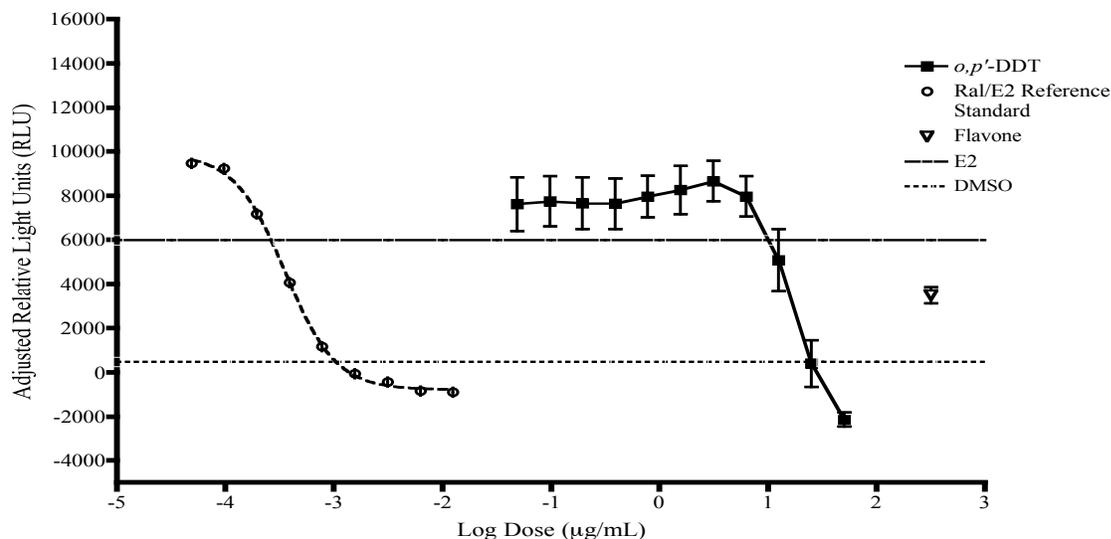
2523 µg/mL).

2524 Results of averaged antagonist experiments for *o,p'*-DDT are shown in **Figure 12-40**.

2525

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2525 **Figure 12-40 Antagonist Comprehensive Testing for N0015 –**  
 2526 ***o,p'*-DDT: Averaged Experiments<sup>1,2,3,4,5,6</sup>**



2527  
 2528  
 2529  
 2530

Abbreviations: *o,p'*-DDT and DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane; Ral/E2 Reference Standard = concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$  µg/mL 17β-estradiol; Flavone = 25 µg/mL flavone control; E2 = 17β-estradiol; DMSO = dimethyl sulfoxide.

2531 <sup>1</sup>Historical mean and standard deviation of the Ral/E2 reference standard.

2532 <sup>2</sup>Historical mean and standard deviation of the flavone control

2533 <sup>3</sup>Historical mean and standard deviation of the E2 control.

2534 <sup>4</sup>Solid horizontal line represents the historical mean of the E2 control minus three times the standard  
 2535 deviation of the E2 control mean. Values must be below the line without any significant decreases in cell viability in  
 2536 order to be considered positive for antagonism.

2537 <sup>5</sup>Dashed horizontal line represents the historical mean of the DMSO control minus three times the  
 2538 standard deviation of the DMSO mean.

2539 <sup>6</sup>The 25 µg/mL flavone controls are not shown at the concentration at which they were tested. They  
 2540 have been placed on the graph in such a way as to maximize visibility.

2541

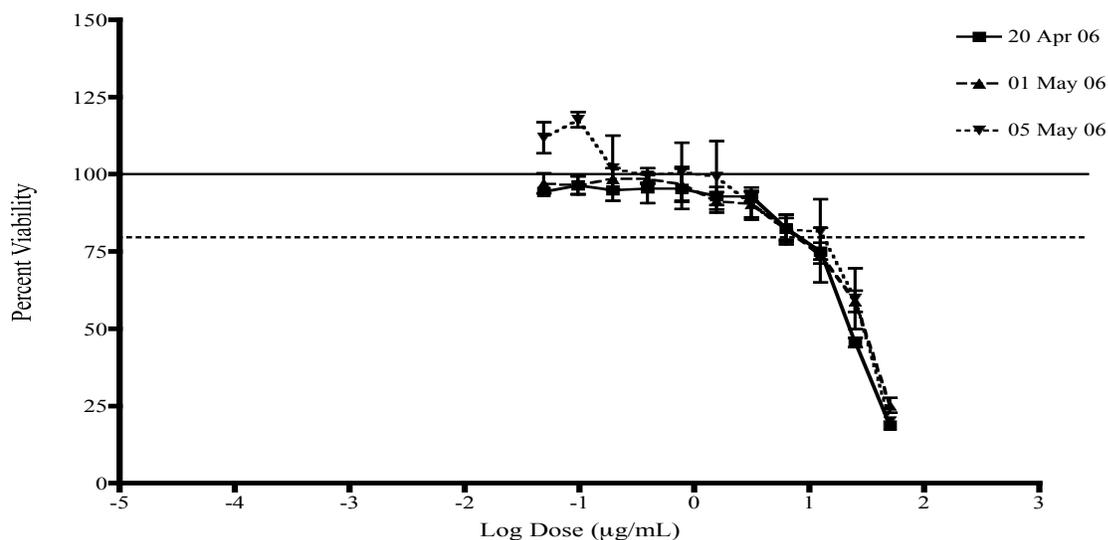
2542 *o,p'*-DDT showed potential antagonist activity at 12.5, 25, and 50 µg/mL.

2543 *o,p'*-DDT caused reductions in cell viability at the three highest concentrations tested, suggesting that the  
 2544 apparent antagonistic response may have been due to cytotoxicity rather than ER mediated antagonism  
 2545 (Figures 12-41, 12-42, and 12-43).

2546

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2546 **Figure 12-41 CellTiter-Glo® Viability Assessment for N0015 – *o,p'*-DDT<sup>1,2</sup>**



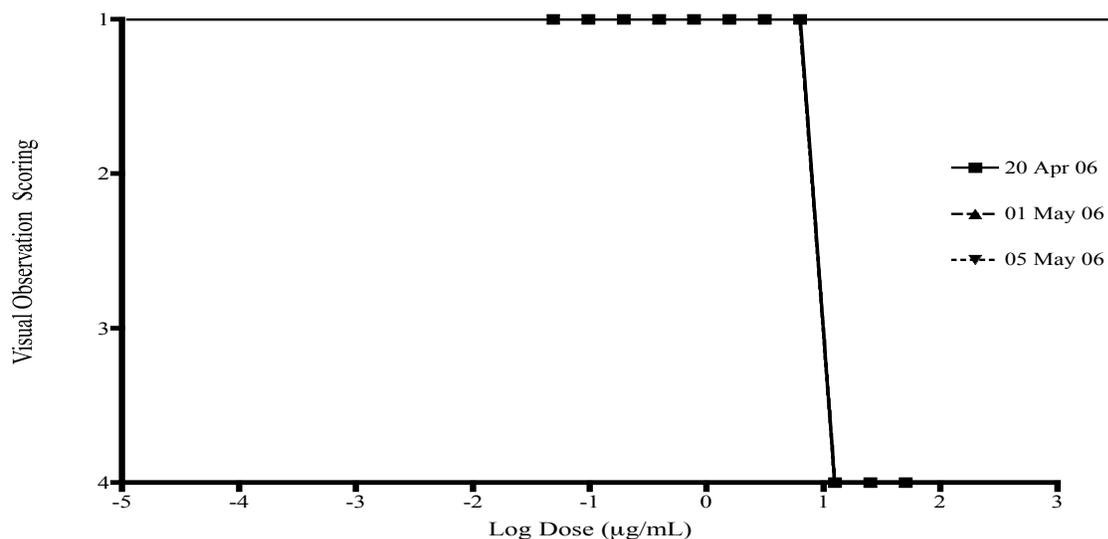
2547  
2548 *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane.

2549 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

2550 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability  
2551 below this line are considered cytotoxic and are not included in the evaluation of antagonist activity.

2552

2553 **Figure 12-42 Visual Observation Viability Assessment for N0015 – *o,p'*-DDT<sup>1</sup>**



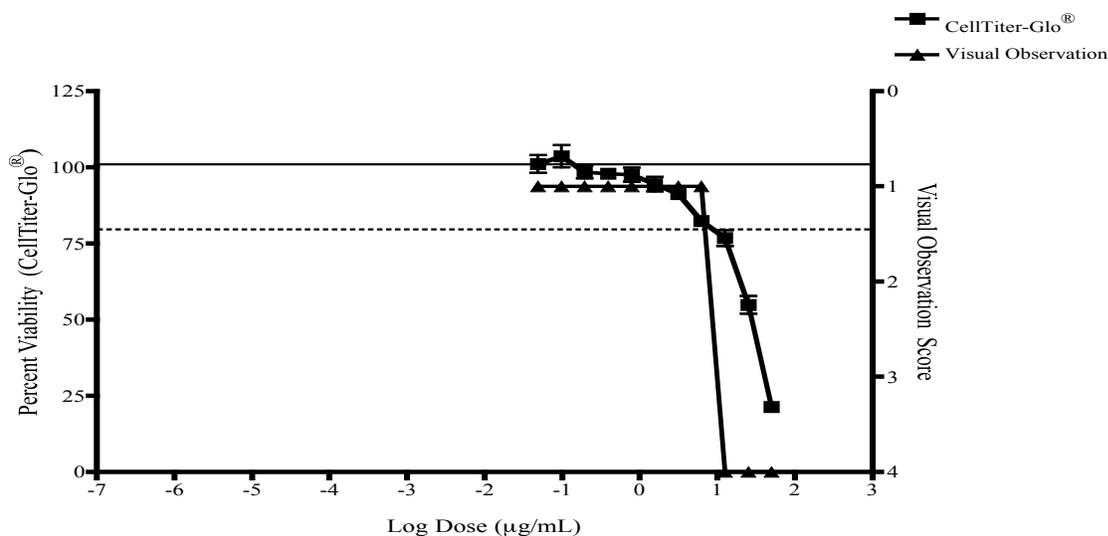
2554  
2555 *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane.

2556 <sup>1</sup>Wells containing cells that exhibit normal morphology and density are given a visual observation  
2557 score of 1; wells containing few or no visible cells are given a visual observation score of 4. Wells  
2558 that are exposed to concentrations resulting in visual observation scores ≥ 2 are considered  
2559 cytotoxic and are not included in the evaluation of antagonist activity.

2560

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2560 **Figure 12-43 Combined Qualitative and Quantitative**  
 2561 **Viability Assessments for N0015 – *o,p'*-DDT<sup>1,2</sup>**



2562 *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane.

2564 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

2565 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability  
 2566 below this line are considered cytotoxic and are not included in the evaluation of antagonist activity.

2567

### 2568 12.2.8N0016 – Tamoxifen

2569 Tamoxifen was selected for antagonist testing because it was listed in the ICCVAM Guidelines  
 2570 (ICCVAM 2003, 2006) as uniformly positive for ER antagonist activity in multiple assays. 5 µg/mL was  
 2571 selected as the starting concentration for the double serial dilution used for comprehensive testing because  
 2572 it gave the lowest adjusted RLU value during range finder testing. The experimenters changed the starting  
 2573 concentration to 50 µg/mL after conducting the first comprehensive experiment at 5 µg/mL in order to  
 2574 better define the top of the concentration-response curve. However, two independent experiments  
 2575 conducted 50 µg/mL resulted in excessive cytotoxicity. Therefore, the experimenters reverted back to 5  
 2576 µg/mL for the starting concentration and repeated the two experiments. The concentrations of tamoxifen  
 2577 tested are listed in **Table 12-11** (note: only results for experiments conducted using 5 µg/mL as the  
 2578 starting concentration for comprehensive testing are presented and discussed in this section of the report –  
 2579 see **Section 16.3** for results and discussion of the two experiments using 50 µg/mL as the starting  
 2580 concentration for comprehensive testing).

2581

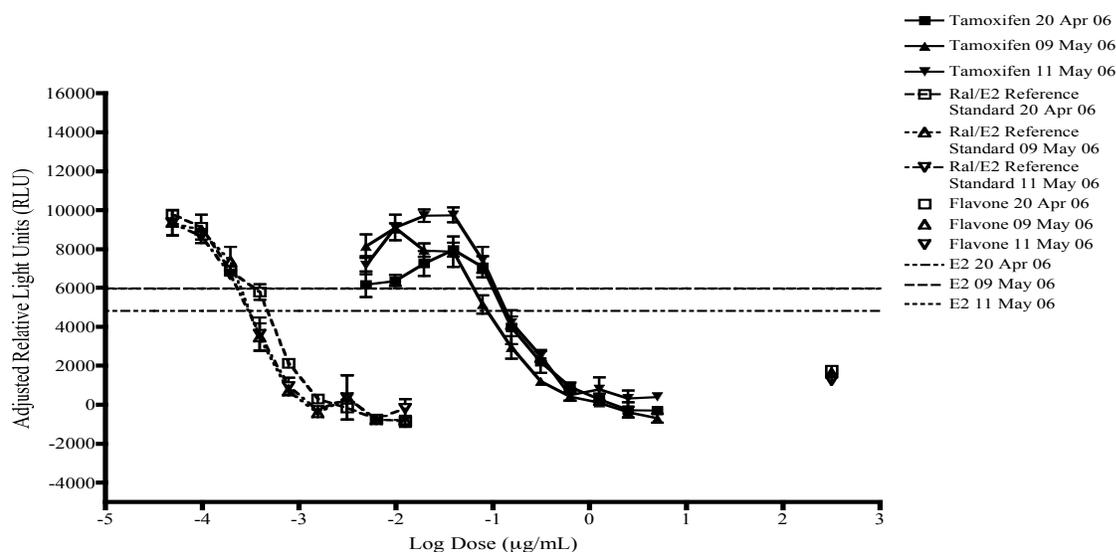
PREDECISIONAL MATERIAL: DO NOT CITE, QUOTE OR DISTRIBUTE

2581 **Table 12-11 Concentrations of N0016 – Tamoxifen**  
 2582 **used in Comprehensive Testing**

N0016 – Tamoxifen ( $\mu\text{g/mL}$ )		
5	0.31	$1.95 \times 10^{-2}$
2.5	0.16	$9.77 \times 10^{-3}$
1.25	$7.81 \times 10^{-2}$	$4.81 \times 10^{-3}$
0.63	$3.91 \times 10^{-2}$	

2583  
 2584 Results of individual antagonist experiments for tamoxifen are shown in **Figure 12-44**.

2585  
 2586 **Figure 12-44 Antagonist Comprehensive Testing for N0016 –**  
 2587 **Tamoxifen: Individual Experiments<sup>1,2</sup>**



2588  
 2589 Abbreviations: Ral/E2 Reference Standard = concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$   $\mu\text{g/mL}$   
 2590  $17\beta$ -estradiol; Flavone = 25  $\mu\text{g/mL}$  flavone control; E2 =  $17\beta$ -estradiol.

2591 <sup>1</sup>Horizontal lines represent the mean of three E2 control replicates minus three times the standard deviation  
 2592 of the E2 control mean. Values must be below the line without any significant decreases in cell viability in  
 2593 order to be considered positive for antagonism.

2594 <sup>2</sup>The 25  $\mu\text{g/mL}$  flavone controls are not shown at the concentration at which they were tested. They  
 2595 have been placed on the graph in such a way as to maximize visibility.

2596  
 2597 Tamoxifen showed antagonist activity at the majority of concentrations tested.  $\text{IC}_{50}$  values for tamoxifen  
 2598 experiments are reported in **Table 12-12**.

2599

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2599 **Table 12-12 Individual IC<sub>50</sub> Values for N0016 – Tamoxifen**

Experiment Date	IC <sub>50</sub> (µg/mL)
20 April 06	0.21
9 May 06	0.11
11 May 06	0.15

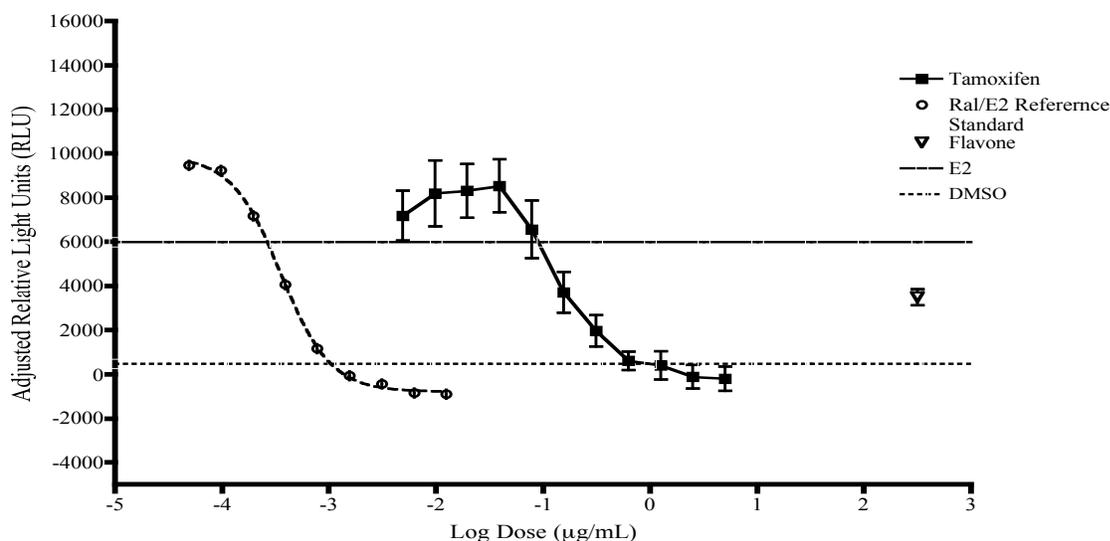
2600 Abbreviations: IC<sub>50</sub> = concentration of test substance that inhibits the reference estrogen  
 2601 response by 50%

2602

2603 Results of averaged antagonist experiments for tamoxifen are shown in **Figure 12-45**.

2604

2605 **Figure 12-45 Antagonist Comprehensive Testing for N0016 – Tamoxifen: Averaged**  
 2606 **Experiments**<sup>1,2,3,4,5,6</sup>



2607

2608 Abbreviations: Ral/E2 Reference Standard = concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$  µg/mL  
 2609 17β-estradiol; Flavone = 25 µg/mL flavone control; E2 = 17β-estradiol; DMSO = dimethyl sulfoxide.

2610 <sup>1</sup>Historical mean and standard deviation of the Ral/E2 reference standard.2611 <sup>2</sup>Historical mean and standard deviation of the flavone control2612 <sup>3</sup>Historical mean and standard deviation of the E2 control.

2613 <sup>4</sup>Solid horizontal line represents the historical mean of the E2 control minus three times the standard  
 2614 deviation of the E2 control mean. Values must be below the line without any significant decreases in cell viability in  
 2615 order to be considered positive for antagonism.

2616 <sup>5</sup>Dashed horizontal line represents the historical mean of the DMSO control minus three times the standard  
 2617 deviation of the DMSO mean.

2618 <sup>6</sup>The 25 µg/mL flavone controls are not shown at the concentration at which they were tested. They  
 2619 have been placed on the graph in such a way as to maximize visibility.

2620

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2621 Tamoxifen showed antagonist activity at the majority of concentrations tested. The averaged  $IC_{50}$  (Table  
2622 12-13) value was calculated as the mean of three experiments.

2623

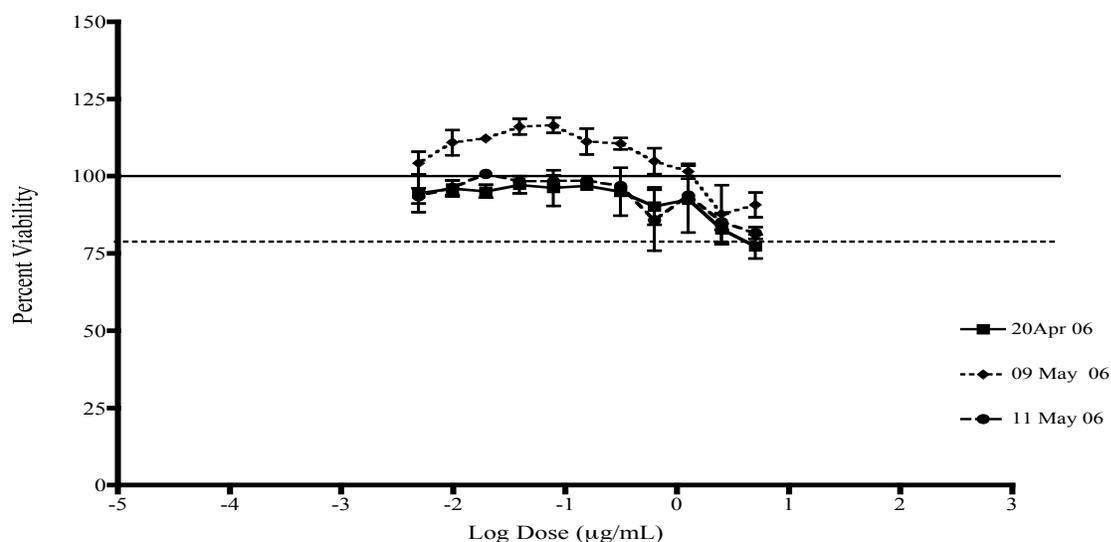
2624 **Table 12-13 Averaged  $IC_{50}$  Value for N0016 – Tamoxifen**

$IC_{50}$ ( $\mu\text{g/mL}$ ) <sup>1,2</sup>	STD DEV	CV
0.16	$4.89 \times 10^{-2}$	31%

2625 Abbreviations: CV = Coefficient of Variation;  $IC_{50}$  = concentration of test  
2626 substance that inhibits the reference estrogen response by 50%;  
2627 STD DEV = Standard Deviation of the Mean;  
2628

2629 Tamoxifen was not cytotoxic at any of the concentrations tested (Figures 12-46, 12-47, and 12-48).

2630

2631 **Figure 12-46 CellTiter-Glo® Viability Assessment for N0016 – Tamoxifen<sup>1,2</sup>**

2632 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

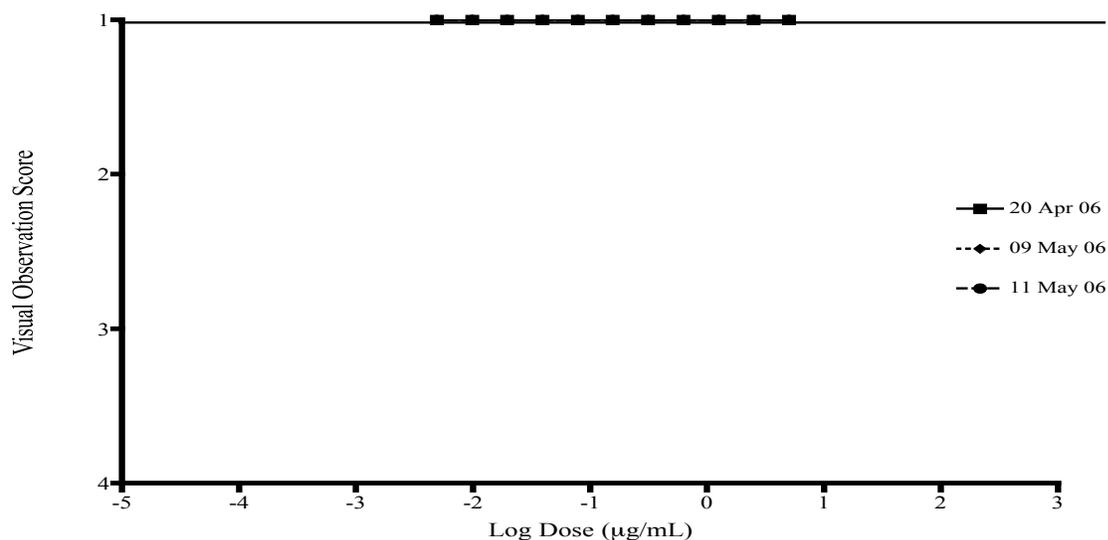
2633  
2634 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability  
2635 below this line are considered cytotoxic and are not included in the evaluation of antagonist activity.

2636

2637

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2637 **Figure 12-47 Visual Observation Viability Assessment for N0016 – Tamoxifen<sup>1</sup>**

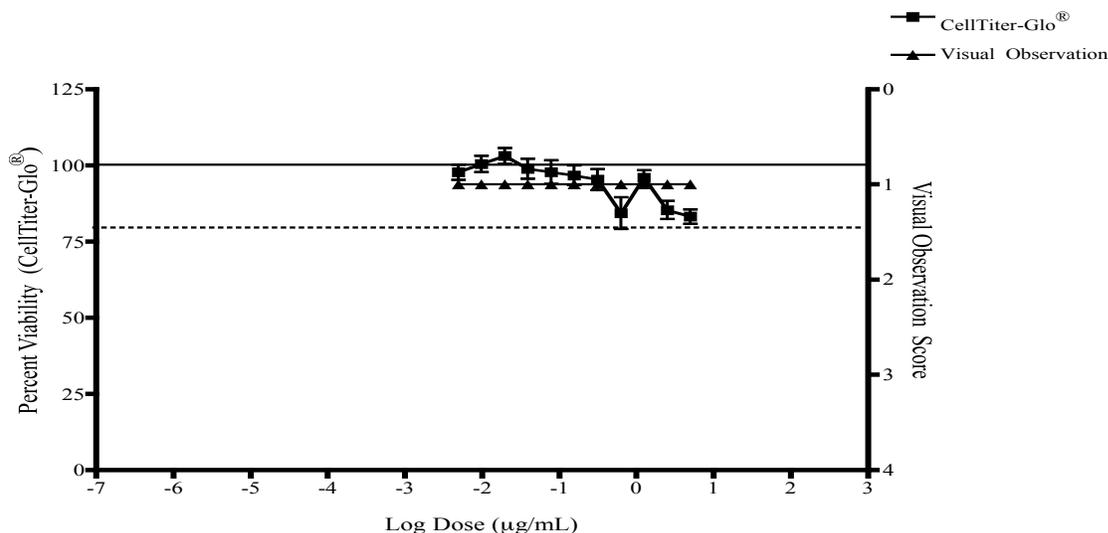


2638

2639 <sup>1</sup>Wells containing cells that exhibit normal morphology and density are given a visual observation  
 2640 score of 1. Wells that are exposed to concentrations resulting in visual observation scores  $\geq 2$  are  
 2641 considered cytotoxic and are not included in the evaluation of antagonist activity.

2642

2643 **Figure 12-48 Combined Qualitative and Quantitative**  
 2644 **Viability Assessments for N0016 – Tamoxifen<sup>1,2</sup>**



2645

2646 <sup>1</sup>Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

2647 <sup>2</sup>Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability  
 2648 below this line are considered cytotoxic and are not included in the evaluation of antagonist activity.

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**2649 13.0 Evaluation of Reference Standard and Control Data**

2650 Agonist and antagonist reference standard, control and induction or reduction data was evaluated to  
2651 determine whether values fell within a range of the historical values. A linear regression was conducted to  
2652 assess the reproducibility of the control data over time.

**2653 13.1 Agonist Reference Standards and Controls**

2654 To determine whether agonist reference standard, control, and induction values changed over time, a  
2655 linear regression analysis was performed with PRISM<sup>®</sup>, using a least squares method. The analysis was  
2656 conducted using averaged reference standard, control, and induction values for all experiments conducted  
2657 on a given day. The slope of the regression line was judged to be statistically significant at  $p < 0.05$  (i.e.,  
2658  $p$  values  $< 0.05$  indicate that values were significantly different over time).

**2659 13.1.1 DMSO Control**

2660 DMSO control values used for tracking of experimental data over time are presented as non-adjusted  
2661 RLUs. Adjusted RLUs are not used because the first step in adjustment is to control for the experimental  
2662 background by subtracting the average experimental DMSO control value from each sample on the  
2663 experimental plate. This gives adjusted DMSO values that are either zero or are extremely small.

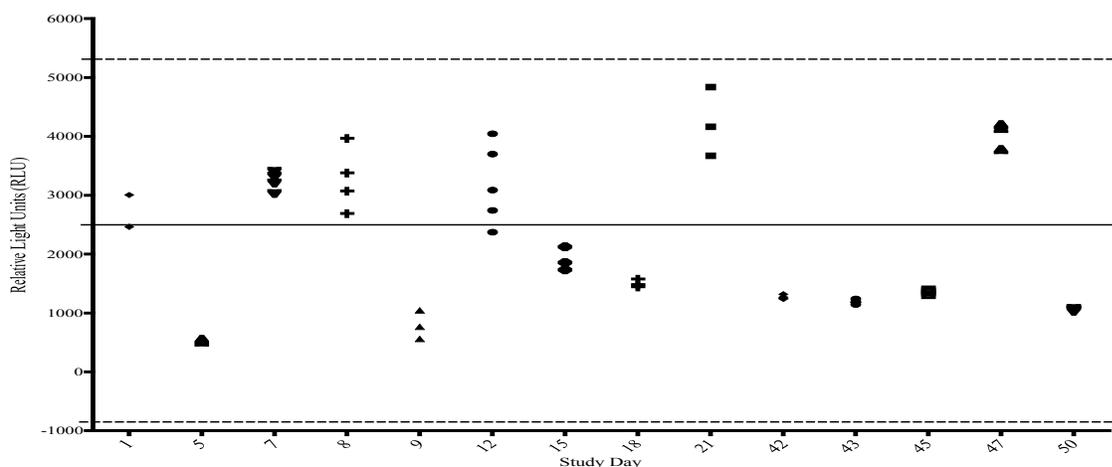
2664 **Figure 13-1** shows the DMSO control values for agonist range finder and comprehensive testing.

2665

2666

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2666 **Figure 13-1 DMSO Control Values for Experiments Conducted during Test Substance Agonist**  
 2667 **Range Finder and Comprehensive Testing<sup>1,2,3,4</sup>**



2668

2669 Abbreviations: DMSO = dimethyl sulfoxide

2670 <sup>1</sup>Values are not adjusted before analysis, and are expressed as relative light units (RLUs).

2671 <sup>2</sup>Each symbol represents the DMSO control value for each experiment performed on a  
 2672 given day.

2673 <sup>3</sup>The solid line across the figure represents the mean of historical DMSO control values across all experiments.

2674 <sup>4</sup>The dashed lines across the figure represent the historical mean plus and minus 2.5 times  
 2675 the standard deviation from that mean.

2676

2677 Each point represents the DMSO control value for each experiment conducted on a given day (the  
 2678 averaged value of the DMSO control wells used on each 96-well plate). The number of experiments per  
 2679 day ranged from one to five. The lines on the figure represent the historical mean, and the mean plus or  
 2680 minus 2.5 times the standard deviation of the DMSO control. All DMSO control values obtained during  
 2681 range finder and comprehensive testing had to fall within these limits for the experiment to be accepted.

2682

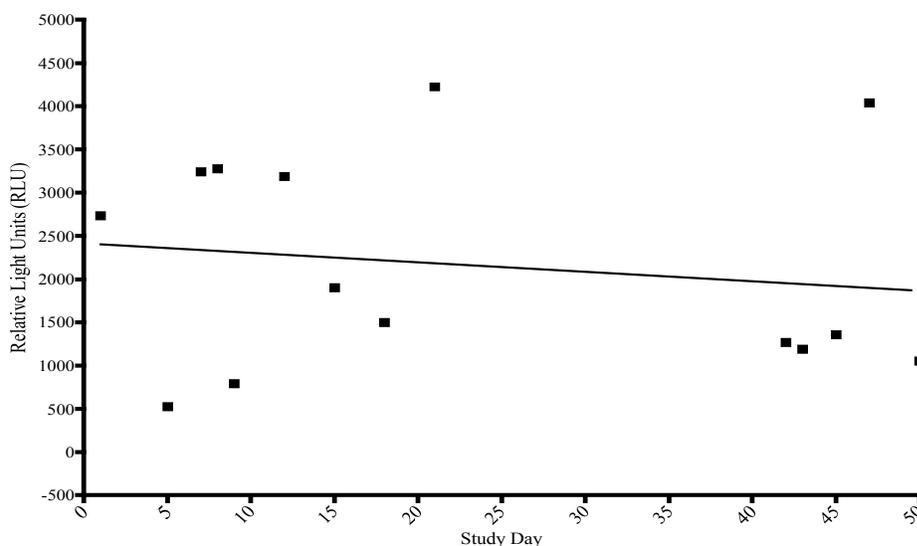
2683 Each point on the regression line represents the averaged DMSO value for all experiments performed on a  
 2684 given day. **Figure 13-2** shows the linear regression of averaged, non-adjusted DMSO control RLU values  
 2685 over time.

2686

2687

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2687 **Figure 13-2 Linear Regression of DMSO Control Values Against Time for**  
2688 **Agonist Experiments<sup>1,2</sup>**



2689

2690 Abbreviations: DMSO = dimethyl sulfoxide

2691 <sup>1</sup>DMSO control values are not adjusted before analysis, and are expressed as relative light units (RLUs).2692 <sup>2</sup>Each symbol represents the mean DMSO control value for experiments performed on a given day.

2693

2694 The slope of the linear regression of the DMSO control was not significantly ( $p=0.58$ ) different from  
2695 zero, showing that the DMSO control values did not vary significantly over time.

2696

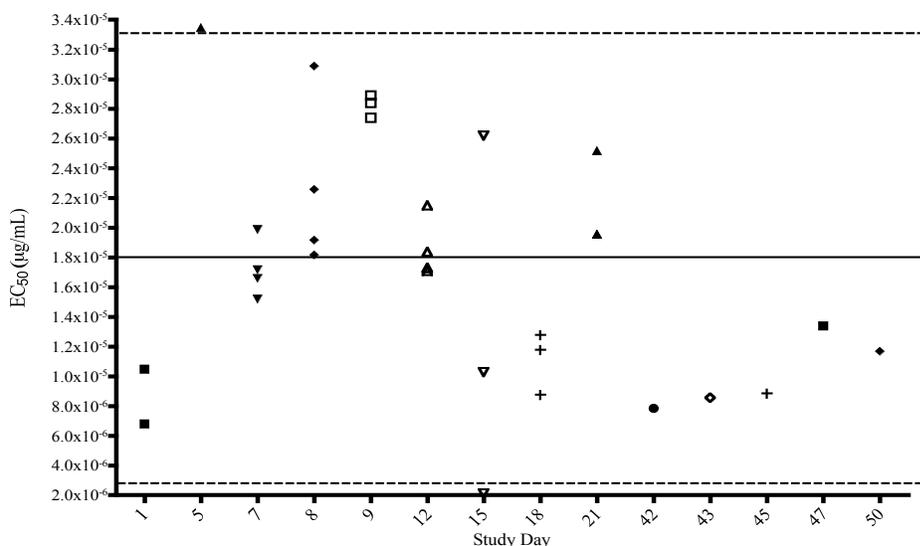
### 2697 13.1.2 EC<sub>50</sub> Value

2698 **Figure 13-3** shows the E2 reference standard EC<sub>50</sub> values ( $\mu\text{g/mL}$ ) for range finder and comprehensive  
2699 testing, which were calculated for each experiment using the Hill function.

2700

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2700 **Figure 13-3 E2 Reference Standard EC<sub>50</sub> Values for Experiments Conducted during**  
 2701 **Test Substance Range Finder and Comprehensive Testing<sup>1,2,3</sup>**



2702

2703 Abbreviations: E2 = 17 $\beta$ -estradiol; EC<sub>50</sub> = half-maximal effect concentration.

2704 <sup>1</sup>Each symbol represents the E2 reference standard EC<sub>50</sub> value for each experiment performed on a given day.

2705 <sup>2</sup>The solid line across the figure represents the mean of historical experiments.

2706 <sup>3</sup>The dashed lines across the figure represent the historical mean plus and minus 2.5 times  
 2707 the Standard deviation from that mean.

2708

2709 Each point represents the E2 reference standard EC<sub>50</sub> value for each experiment conducted on a given  
 2710 day. The number of experiments per day ranged from one to five. The lines on the figure represent the  
 2711 historical mean, and the mean plus or minus 2.5 times the standard deviation of the EC<sub>50</sub> value. All EC<sub>50</sub>  
 2712 values obtained during range finder and comprehensive testing had to fall within these limits for the  
 2713 experiment to be accepted.

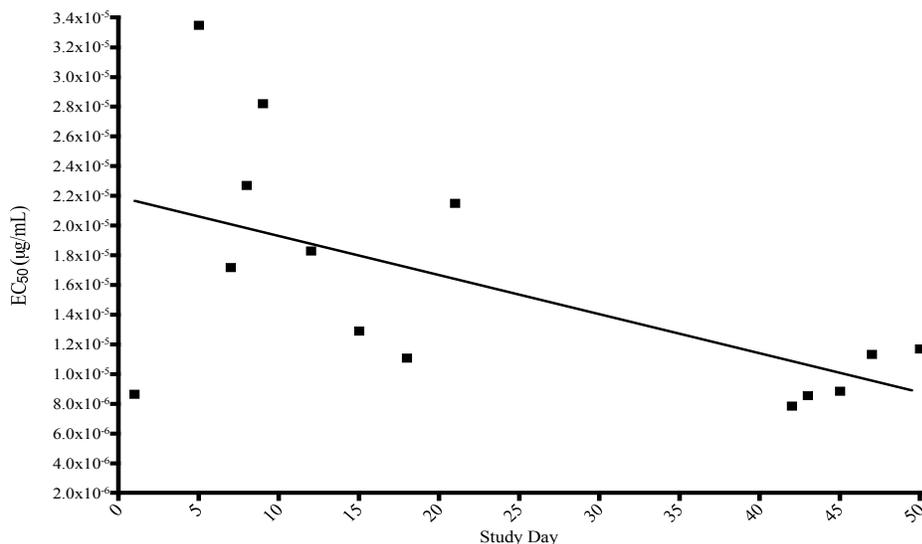
2714

2715 Each point on the regression line represents the averaged E2 reference standard EC<sub>50</sub> value for all  
 2716 experiments performed on a given day. **Figure 13-4** shows the linear regression of EC<sub>50</sub> values over time.

2717

2718

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2718 **Figure 13-4 Linear Regression of E2 Reference Standard EC<sub>50</sub> Values Over Time<sup>1</sup>**

2719

2720 Abbreviations: E2 = 17β-estradiol; EC<sub>50</sub> = half-maximal effect concentration.2721 <sup>1</sup>Each symbol represents the mean E2 reference standard EC<sub>50</sub> value for experiments performed on a given day.

2722

2723 The slope of the linear regression of E2 reference standard EC<sub>50</sub> values was significantly (p=0.03)2724 different from zero, showing that EC<sub>50</sub> values varied significantly over time.

2725

2726 **13.1.3 Induction**

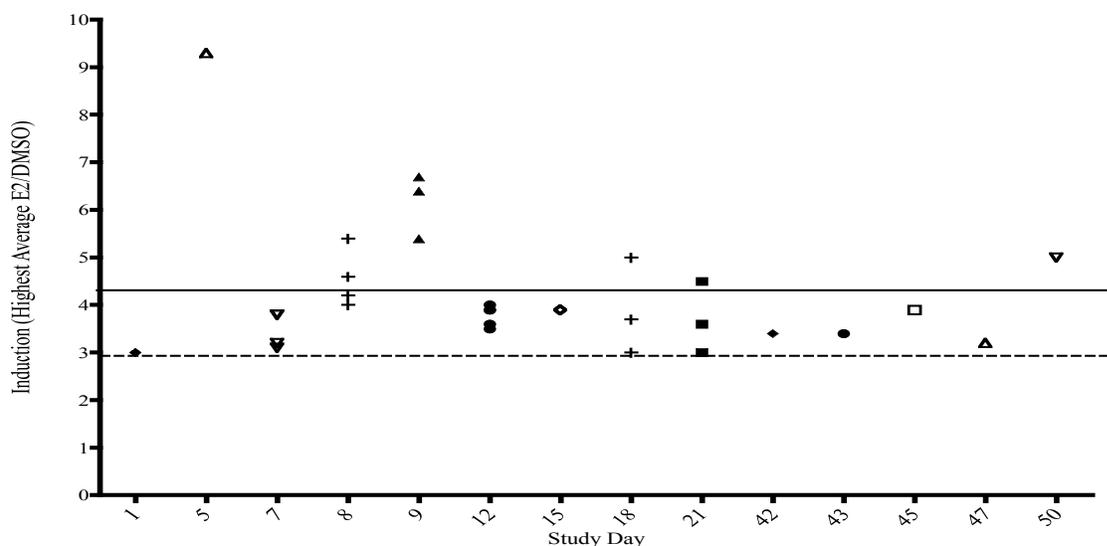
2727 Induction is a measure of the degree of responsiveness of the cells and is calculated by dividing the  
2728 averaged highest non-adjusted E2 reference standard RLU value by the averaged non-adjusted DMSO  
2729 control RLU value. **Figure 13-5** shows the induction values (presented as a ratio) for range finder and  
2730 comprehensive testing.

2731

2732

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2732 **Figure 13-5 Induction Values for Experiments Conducted during Test Substance**  
 2733 **Range Finder and Comprehensive Testing<sup>1,2,3,4</sup>**



2734

2735 <sup>1</sup>Induction values are calculated as the averaged highest non-adjusted 17 $\beta$ -estradiol relative light unit (RLU) value divided  
 2736 by the averaged non-adjusted dimethyl sulfoxide control RLU value for each experiment.

2737 <sup>2</sup>Each symbol represents the induction value for each experiment performed on a given day.

2738 <sup>3</sup>The solid line across the figure represents the mean of historical experiments.

2739 <sup>4</sup>The dashed line represents a value of "3". All values must be at or above this line for an  
 2740 experiment to be included in data analysis.

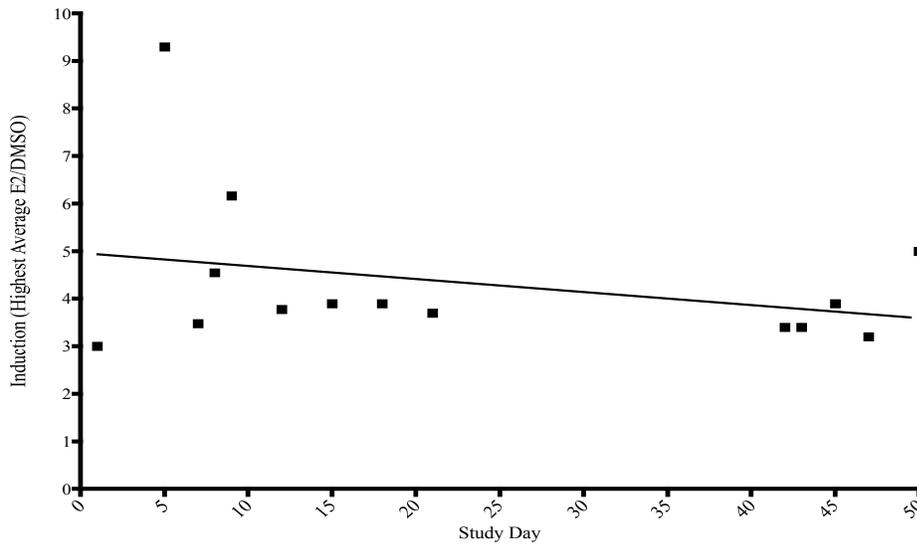
2741

2742 Each point represents the induction value for each experiment conducted on a given day. The number of  
 2743 experiments per day ranged from one to five. The solid line on the figure represents the historical mean of  
 2744 induction values, and the dashed line represents an induction value of three. All induction values obtained  
 2745 during range finder and comprehensive testing had to be greater than or equal to three for the experiment  
 2746 to be accepted.

2747 Each point on the regression line represents the averaged induction value for all experiments performed  
 2748 on a given day. **Figure 13-6** shows the linear regression of induction values over time.

2749

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2749 **Figure 13-6 Linear Regression of Induction Values Against Time**<sup>1,2</sup>

2750

2751 <sup>1</sup>Induction control values are expressed as the averaged highest non-adjusted 17 $\beta$ -estradiol relative light unit (RLU) value divided  
 2752 by the averaged non-adjusted dimethyl sulfoxide control RLU value for each experiment.

2753 <sup>2</sup>Each symbol represents the mean induction value for experiments performed on a given day.

2754

2755 The slope of the linear regression of induction was not significantly ( $p=0.29$ ) different from zero, showing  
 2756 that induction values did not vary significantly over time.

### 2757 13.1.4Methoxychlor Control

2758 Methoxychlor values used for tracking of experimental data over time are presented as adjusted RLUs.

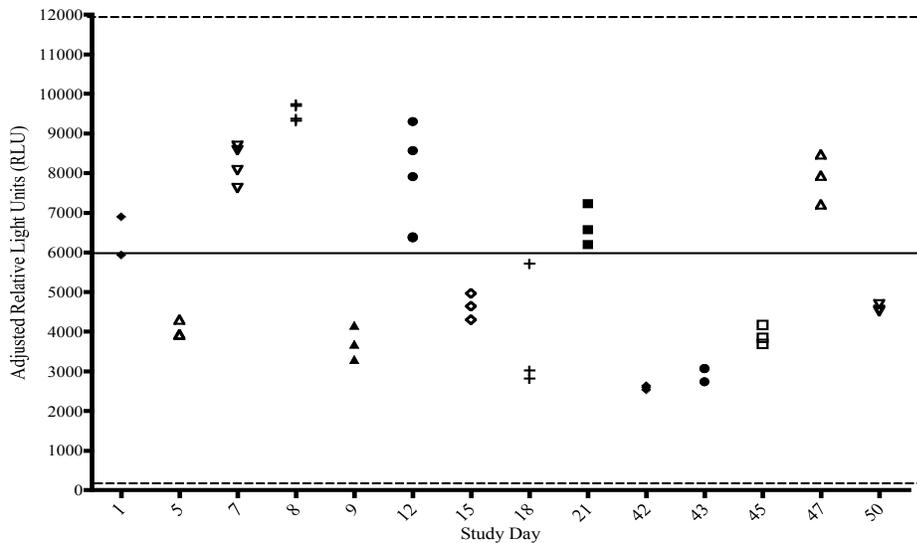
2759 **Figure 13-7** shows the methoxychlor control values for range finder and comprehensive testing.

2760

2761

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2761 **Figure 13-7 Methoxychlor Control Values for Experiments Conducted**  
 2762 **during Range Finder and Comprehensive Testing<sup>1,2,3,4</sup>**



2763

2764 <sup>1</sup>Methoxychlor control values are expressed as adjusted relative light units.

2765 <sup>2</sup>Each symbol represents the methoxychlor value for each experiment performed on a given day.

2766 <sup>3</sup>The solid line across the figure represents the mean of historical experiments.

2767 <sup>4</sup>The dashed lines across the figure represent the historical mean plus and minus 2.5 times  
 2768 the Standard deviation from that mean.

2769

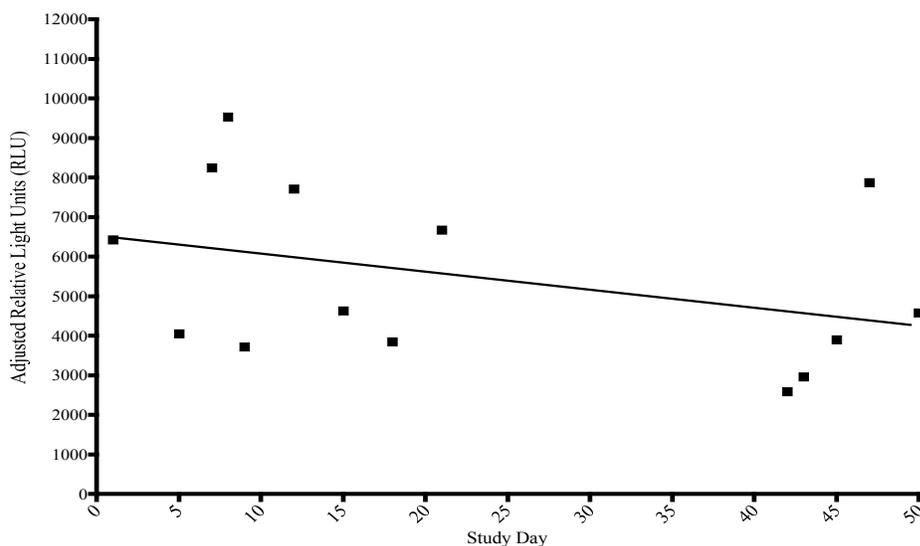
2770 Each point represents the methoxychlor control value for each experiment conducted on a given day. The  
 2771 number of experiments per day ranged from one to five. The lines on the figure represent the historical  
 2772 mean, and the mean plus or minus 2.5 times the standard deviation of the methoxychlor control. All  
 2773 methoxychlor control values obtained during range finder and comprehensive testing had to fall within  
 2774 these limits for the experiment to be accepted.

2775 The linear regression tracks the averaged experimental methoxychlor control values for each day of the  
 2776 study. Each point represents the averaged methoxychlor value for all experiments performed on a given  
 2777 day. **Figure 13-8** shows the linear regression of averaged, methoxychlor control adjusted RLU values  
 2778 over time.

2779

2780

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2780 **Figure 13-8 Linear Regression of Methoxychlor Control Values Against Time<sup>1,2</sup>**

2781

2782 <sup>1</sup>Methoxychlor control values are expressed as adjusted relative light units.2783 <sup>2</sup>Each symbol represents the mean methoxychlor control value for experiments performed on a given day.

2784

2785 The slope of the linear regression of the methoxychlor control was not significantly ( $p=0.19$ ) different  
 2786 from zero, showing that methoxychlor control values did not vary significantly over time.

2787

## 2788 13.2 Antagonist Reference Standards and Controls

2789 To determine whether antagonist reference standard, control, and reduction values changed over time, a  
 2790 linear regression analysis was performed with PRISM<sup>®</sup>, using a least squares method. The analysis was  
 2791 conducted using averaged reference standard, control, and reduction values for all experiments conducted  
 2792 on a given day. The slope of the linear regression was judged to be statistically significant at  $p < 0.05$   
 2793 (i.e.,  $p$  values  $< 0.05$  indicate that values were significantly different over time).

### 2794 13.2.1 DMSO Control

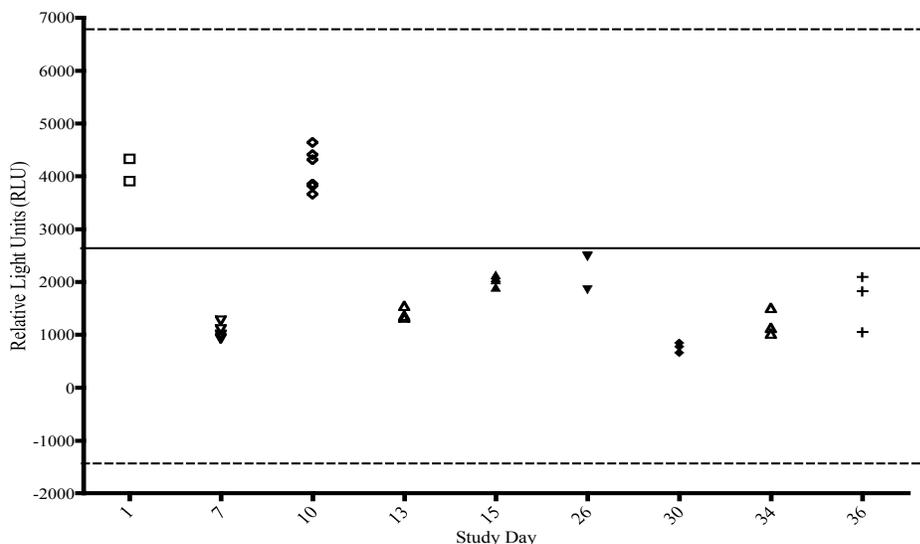
2795 DMSO control values used for tracking of experimental data over time are presented as non-adjusted  
 2796 RLUs. Adjusted RLUs are not used because the first step in adjustment is to control for the experimental  
 2797 background by subtracting the average experimental DMSO control value from each sample on the  
 2798 experimental plate. This practice leads to adjusted DMSO control values that are either zero or are

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2799 extremely small. **Figure 13-9** shows the DMSO control values for range finder and comprehensive  
 2800 testing.

2801

2802 **Figure 13-9 DMSO Control Values for Experiments Conducted during**  
 2803 **Test Substance Antagonist Range Finder and Comprehensive**  
 2804 **Testing<sup>1,2,3,4</sup>**



2805

2806 DMSO = dimethyl sulfoxide

2807 <sup>1</sup>Control values are not adjusted before analysis, and are expressed as relative light units (RLUs).2808 <sup>2</sup>Each symbol represents the DMSO control value for each experiment performed on a given day.2809 <sup>3</sup>The solid line across the figure represents the mean of historical experiments.2810 <sup>4</sup>The dashed lines across the figure represent the historical mean plus and minus 2.5 times  
 2811 the Standard deviation from that mean.

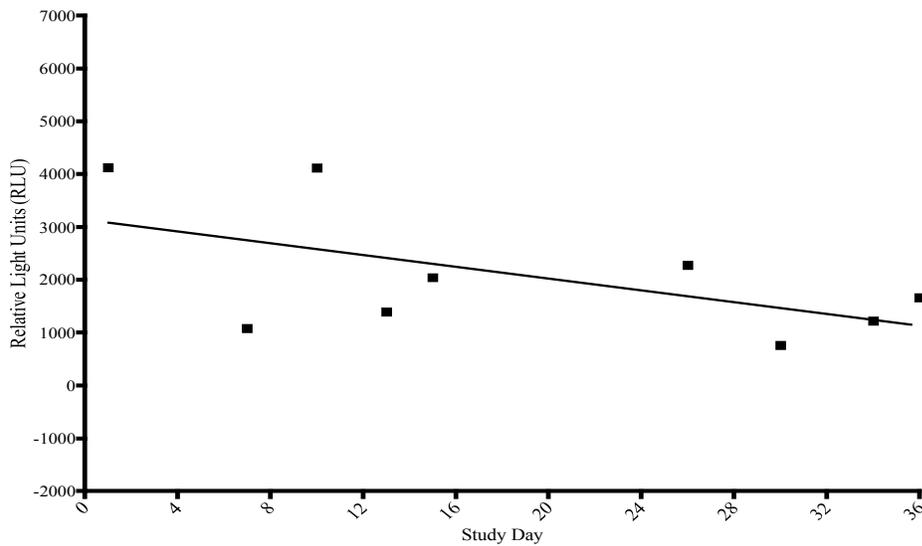
2812

2813 Each point represents the DMSO control value for each experiment conducted on a given day (the  
 2814 averaged value of the DMSO control wells used on each 96-well plate). The number of experiments per  
 2815 day ranged from one to six. The lines on the figure represent the historical mean, and the mean plus and  
 2816 minus 2.5 times the standard deviation of the DMSO control. All DMSO control values obtained during  
 2817 range finder and comprehensive testing had to fall within these limits for the experiment to be accepted.

2818 Each point on the regression line represents the averaged DMSO control value for all experiments  
 2819 performed on a given day. **Figure 13-10** shows the linear regression of averaged, non-adjusted DMSO  
 2820 control RLU values over time.

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2821 **Figure 13-10 Linear Regression of DMSO Control Values Against Time for**  
2822 **Antagonist Experiments<sup>1,2</sup>**



2823

2824 Abbreviations: DMSO = dimethyl sulfoxide

2825 <sup>1</sup>DMSO control values are not adjusted before analysis, and are expressed as relative light units (RLUs).2826 <sup>2</sup>Each symbol represents the mean DMSO control value for experiments performed on a given day.

2827

2828 The slope of the linear regression of the DMSO control was not significantly different from zero ( $p =$   
2829 0.11), showing that DMSO control values did not vary significantly over time.

### 2830 13.2.2E2 Control

2831 E2 control values used for tracking of experimental data over time are presented as adjusted RLUs.

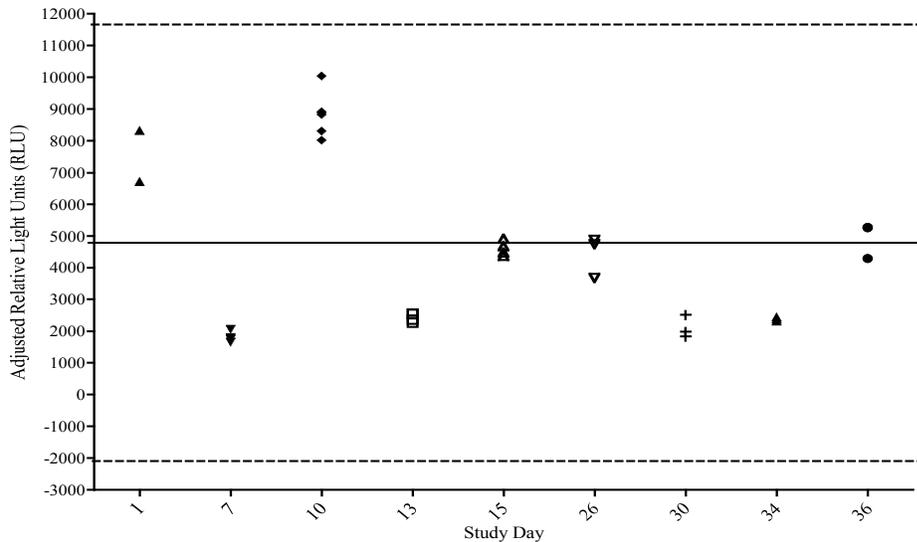
2832 **Figure 13-11** shows the E2 control values for range finder and comprehensive testing.

2833

2834

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2834 **Figure 13-11 E2 Control Values for Experiments Conducted during Range**  
 2835 **Finder and Comprehensive Testing<sup>1,2,3,4</sup>**



2836 Abbreviations: E2 = 17 $\beta$ -estradiol  
 2837

2838 <sup>1</sup>E2 values are expressed as adjusted relative light units.

2839 <sup>2</sup>Each symbol represents the E2 control value for each experiment performed on a given day.

2840 <sup>3</sup>The solid line across the figure represents the mean of historical experiments.

2841 <sup>4</sup>The dashed lines across the figure represent the historical mean plus and minus 2.5 times the standard deviation from that mean.

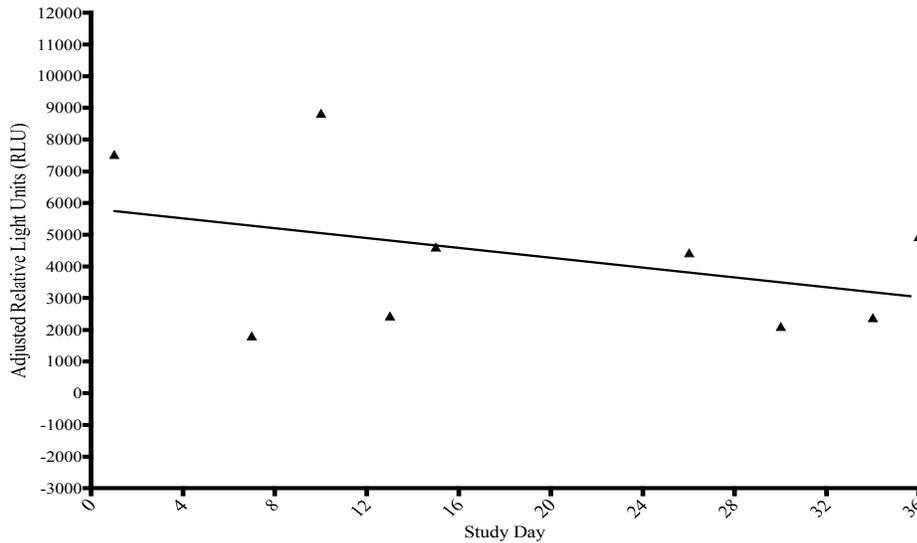
2842

2843 Each point represents the E2 control value for each experiment conducted on a given day. The number of  
 2844 experiments per day ranged from one to six. The lines on the figure represent the historical mean, and the  
 2845 mean plus and minus 2.5 times the standard deviation of the E2 control. All E2 control values obtained  
 2846 during range finder and comprehensive testing had to fall within these limits for the experiment to be  
 2847 accepted.

2848 The linear regression tracks the averaged experimental E2 control values for each day of the study. Each  
 2849 point represents the averaged E2 control value for all experiments performed on a given day. **Figure 13-**  
 2850 **12** shows the linear regression of averaged adjusted E2 control RLU values over time.

2851  
 2852

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2852 **Figure 13-12 Linear Regression of E2 Control Values Against Time<sup>1,2</sup>**

2853

2854 Abbreviations: E2 = 17 $\beta$ -estradiol2855 <sup>1</sup>E2 control values are expressed as adjusted relative light units.2856 <sup>2</sup>Each symbol represents the mean E2 control value for experiments performed on a given day.

2857

2858 The slope of the linear regression of the E2 control was not significantly ( $p=0.29$ ) different from zero,

2859 showing that the E2 control values did not vary significantly over time.

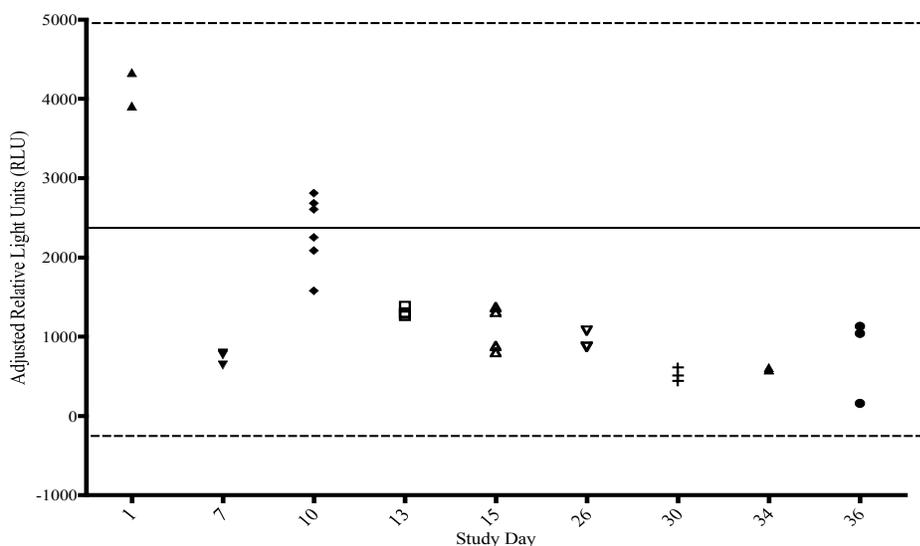
2860 **13.2.3 Flavone Control**2861 Flavone values used for tracking of experimental data over time are presented as adjusted RLUs. **Figure**2862 **13-13** shows the flavone control values for range finder and comprehensive testing.

2863

2864

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2864 **Figure 13-13 Flavone Control Values for Experiments Conducted**  
 2865 **during Range Finder and Comprehensive Testing<sup>1,2,3,4</sup>**



2866 <sup>1</sup>Flavone control values are expressed as adjusted relative light units.

2868 <sup>2</sup>Each symbol represents the flavone control value for each experiment performed on a  
 2869 given day.

2870 <sup>3</sup>The solid line across the figure represents the mean of historical experiments.

2871 <sup>4</sup>The dashed lines across the figure represent the historical mean plus and minus 2.5 times  
 2872 the Standard deviation from that mean.

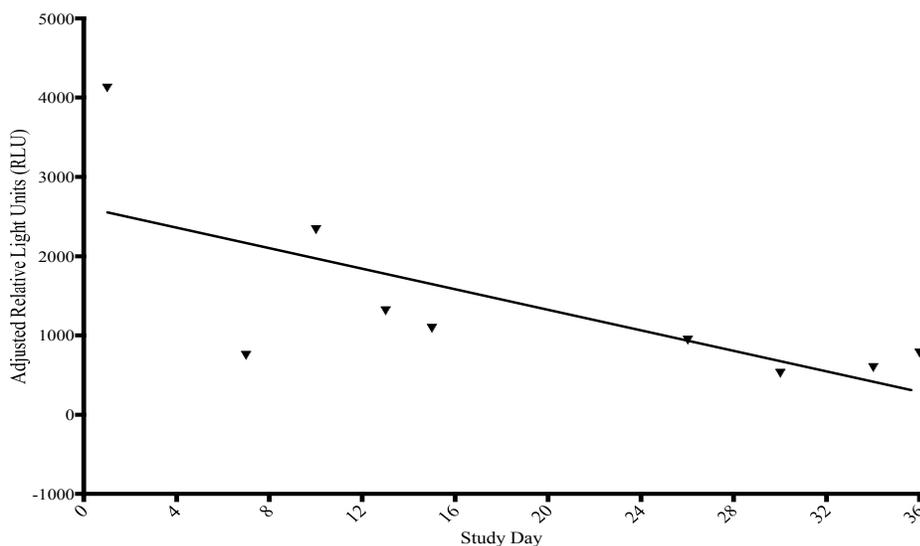
2873

2874 Each point represents the flavone control value for each experiment conducted on a given day. The  
 2875 number of experiments per day ranged from one to six. The lines on the figure represent the historical  
 2876 mean, and the mean plus and minus 2.5 times the standard deviation of the flavone control. All flavone  
 2877 control values obtained during range finder and comprehensive testing had to fall within these limits for  
 2878 the experiment to be accepted.

2879 Each point on the regression line represents the averaged flavone control value for all experiments  
 2880 performed on a given day. **Figure 13-4** shows the linear regression of averaged adjusted flavone control  
 2881 RLU values over time.

2882  
 2883

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2883 **Figure 13-14 Linear Regression of Flavone Control Values Against Time**<sup>1,2</sup>

2884

2885 <sup>1</sup>Flavone control values are expressed as adjusted relative light units.2886 <sup>2</sup>Each symbol represents the mean flavone control value for experiments performed on a given day.

2887

2888 The slope of the linear regression of the flavone control was significantly different from zero ( $p = 0.03$ ),  
2889 showing that flavone control values decreased significantly over time.

2890 **13.2.4IC<sub>50</sub> Value**

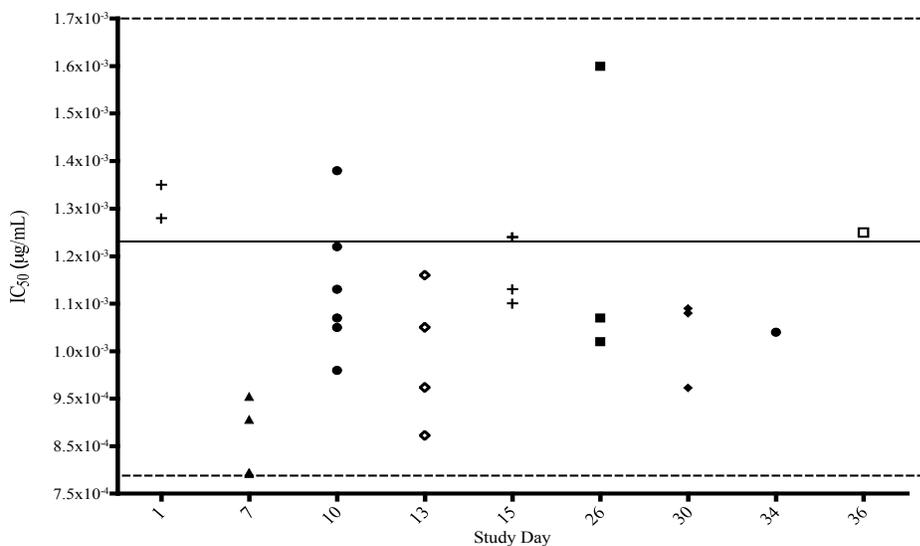
2891 Ral/E2 reference standard IC<sub>50</sub> values are calculated for each experiment using the PRISM<sup>®</sup> Hill function  
2892 and are presented in  $\mu\text{g/mL}$ . **Figure 13-15** shows the Ral/E2 reference standard IC<sub>50</sub> values for range  
2893 finder and comprehensive testing.

2894

2895

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2895 **Figure 13-15 Ral/E2 Reference Standard IC<sub>50</sub> Values for Experiments**  
 2896 **Conducted during Range Finder and Comprehensive**  
 2897 **Testing<sup>1,2,3</sup>**



2898  
 2899  
 2900

Abbreviations: Ral/E2 Reference Standard = concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$  µg/mL; IC<sub>50</sub> = concentration of test substance that inhibits the reference estrogen response by 50%.

2901  
 2902

<sup>1</sup>Each symbol represents the Ral/E2 reference standard IC<sub>50</sub> value for each experiment performed on a given day.

2903

<sup>2</sup>The solid line across the figure represents the mean of historical experiments.

2904  
 2905

<sup>3</sup>The dashed lines across the figure represent the historical mean plus and minus 2.5 times the standard deviation from that mean.

2906

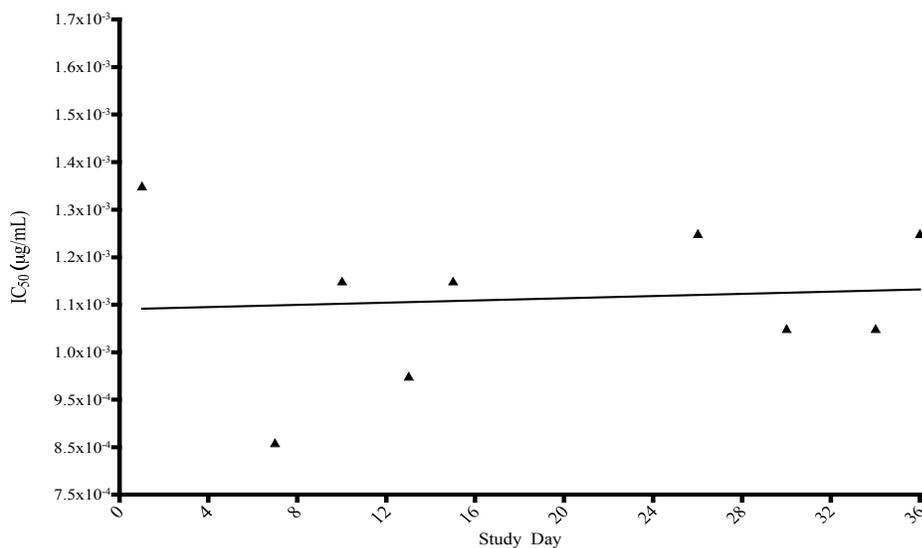
2907 Each point represents the Ral/E2 reference standard IC<sub>50</sub> value for each experiment conducted on a given  
 2908 day. The number of experiments per day ranged from one to six. The lines on the figure represent the  
 2909 historical mean, and the mean plus and minus 2.5 times the standard deviation of the IC<sub>50</sub> control. All IC<sub>50</sub>  
 2910 values obtained during range finder and comprehensive testing had to fall within these limits for the  
 2911 experiment to be accepted.

2912 Each point on the regression line represents the averaged Ral/E2 reference standard IC<sub>50</sub> value for all  
 2913 experiments performed on a given day. **Figure 13-16** shows the linear regression of IC<sub>50</sub> values over time.

2914

2915

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2915 **Figure 13-16 Linear Regression of IC<sub>50</sub> Values Against Time<sup>1</sup>**

2916

2917 IC<sub>50</sub> = concentration of test substance that inhibits the reference estrogen response by 50%2918 <sup>1</sup>Each symbol represents the mean IC<sub>50</sub> value for experiments performed on a given day.

2919

2920 The slope of the linear regression of the IC<sub>50</sub> control was not significantly (p=0.82) different from zero,2921 showing that the IC<sub>50</sub> control data did not vary significantly over time.2922 **13.2.5 Reduction**

2923 Reduction is a measure of the degree of responsiveness of the cells and is calculated by dividing the

2924 averaged highest non-adjusted Ral/E2 reference standard value by the averaged lowest non-adjusted

2925 Ral/E2 control value. **Figure 13-17** shows the reduction values (presented as a ratio) for range finder and

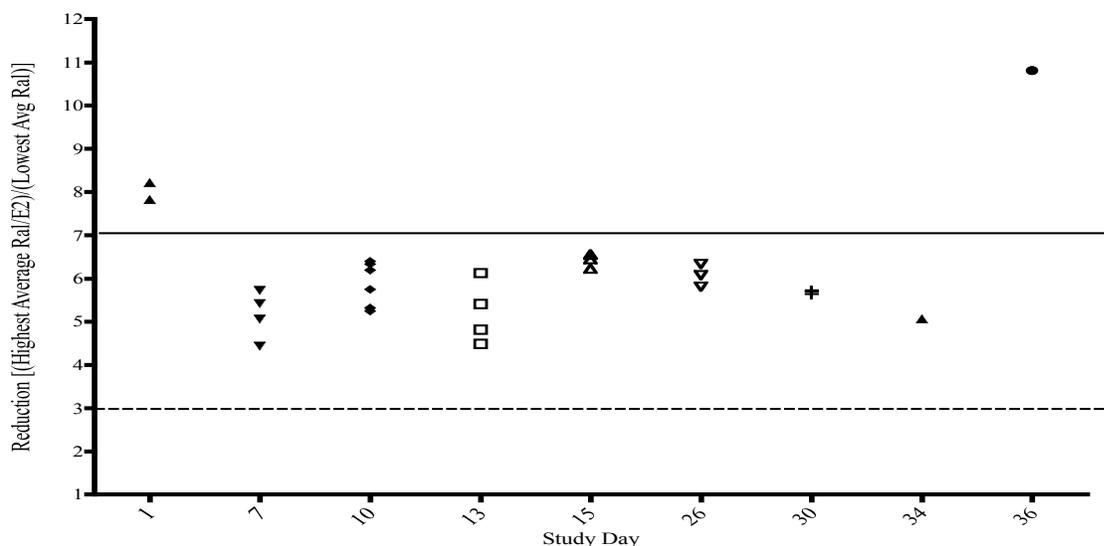
2926 comprehensive testing.

2927

2928

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2928 **Figure 13-17 Reduction Values for Experiments Conducted during**  
 2929 **Range Finder and Comprehensive Testing<sup>1,2,3,4</sup>**



2930  
 2931 <sup>1</sup>Reduction values are calculated as the averaged highest non-adjusted Ral/E2 (concentrations of raloxifene with a fixed concentration  
 2932 of  $2.5 \times 10^{-5}$   $\mu\text{g/mL}$ ) values divided by the averaged non-adjusted Ral/E2 values for each experiment.

2933 <sup>2</sup>Each symbol represents the reduction value for each experiment performed on a given day.

2934 <sup>3</sup>The solid line across the figure represents the mean of historical experiments.

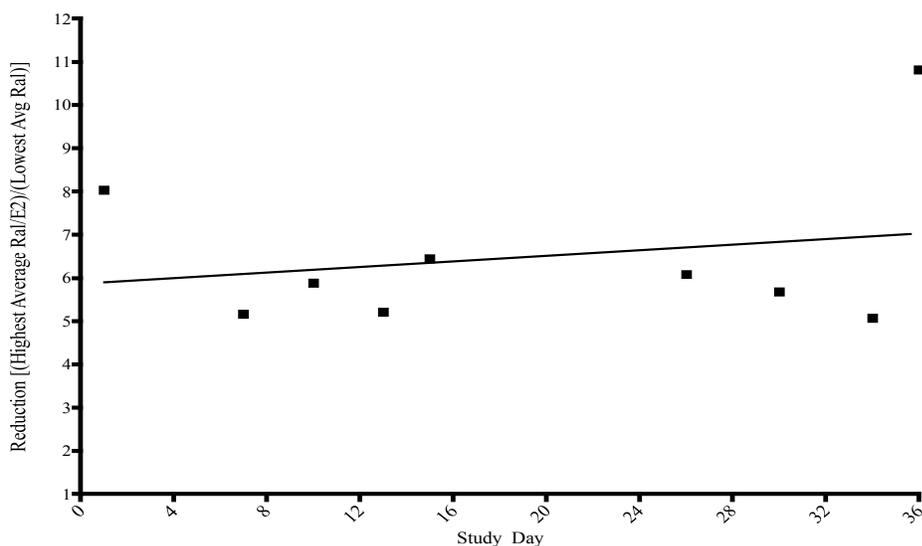
2935 <sup>4</sup>The dashed lines across the figure represent the historical mean plus and minus 2.5 times  
 2936 the standard deviation from that mean.

2937  
 2938 Each point represents the reduction value for each experiment conducted on a given day. The number of  
 2939 experiments per day ranged from one to six. The line on the figure represents the historical mean of  
 2940 reduction values, and the dashed line represents a reduction value of three. All reduction values obtained  
 2941 during range finder and comprehensive testing had to be greater than or equal to three for the experiment  
 2942 to be accepted.

2943 Each point on the regression line represents the averaged reduction value for all experiments performed  
 2944 on a given day. **Figure 13-18** shows the linear regression of reduction values over time.

2945  
 2946

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2946 **Figure 13-18 Linear Regression of Reduction Values Against Time<sup>1,2</sup>**

2947  
2948 <sup>1</sup>Reduction control values are expressed as the averaged highest non-adjusted Ral/E2 (concentrations of raloxifene with a fixed concentration of  
2949  $2.5 \times 10^{-5}$   $\mu\text{g/mL}$ ) values divided by the averaged non-adjusted Ral/E2 values for each experiment.

2950 <sup>2</sup>Each symbol represents the mean reduction value for experiments performed on a given day.

2951  
2952 The slope of the linear regression of reduction was not significantly ( $p=0.56$ ) different from zero, showing  
2953 that the reduction values did not vary significantly over time.

2954 **13.3 Summary of Results for Agonist and Antagonist Reference Standards and Controls**

2955 Agonist and antagonist results for reference standards, controls, induction or reduction for range finder  
2956 and comprehensive testing are summarized in **Table 13-1**.

2957

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2957 **Table 13-1 Mean Values for Agonist and Antagonist Reference Standards and Controls**

	Control	Mean	Standard Deviation	N <sup>1</sup>
<b>Agonist Controls</b>	DMSO Control <sup>2</sup>	2386	1213	33
	EC <sub>50</sub> Value <sup>3</sup>	1.74 x 10 <sup>-5</sup>	7.87 x 10 <sup>-6</sup>	33
	Induction <sup>4</sup>	4.2	1.3	33
	Methoxychlor Control <sup>5</sup>	6218	2306	33
<b>Antagonist Controls</b>	DMSO Control <sup>2</sup>	2252	1304	28
	E2 Control <sup>6</sup>	4664	2751	28
	Flavone Control <sup>6</sup>	1149	808	28
	IC <sub>50</sub> Value <sup>3</sup>	1.14 x 10 <sup>-3</sup>	2.25 x 10 <sup>-4</sup>	28
	Reduction <sup>6</sup>	6.06	1.36	28

2958 Abbreviations: DMSO = dimethyl sulfoxide; E2 = 17β-estradiol; EC<sub>50</sub> = half-maximal effect concentration;  
 2959 IC<sub>50</sub> = concentration of test substance that inhibits the reference estrogen response by 50%.

2960 <sup>1</sup>N = Number of experiments. Fewer experiments were conducted for antagonist testing than for agonist testing.

2961 <sup>2</sup>Values are expressed as unadjusted relative light units.

2962 <sup>3</sup>Values are expressed as μM.

2963 <sup>4</sup>Induction is expressed as the ratio of the averaged highest unadjusted RLU value for the E2 reference standard in each experiment over the  
 2964 averaged DMSO control value.

2965 <sup>5</sup>Values are expressed as adjusted relative light units.

2966 <sup>6</sup>Reduction is expressed as the ratio of the averaged highest unadjusted RLU value for the Ral/E2 reference standard in each experiment over the  
 2967 averaged lowest unadjusted RLU value for the Ral/E2 reference standard.

2968  
 2969 Agonist and antagonist linear regression results for reference standards, controls, induction or reduction  
 2970 for range finder and comprehensive testing are summarized in **Table 13-2**.

2971

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2971 **Table 13-2 Linear Regression Analysis of Agonist and Antagonist Reference Standards and**  
 2972 **Controls<sup>1</sup>**

	Control	Slope	P-value (Slope)	r <sup>2</sup>	y-intercept
<b>Agonist Controls</b>	DMSO Control	-11.09	0.58*	0.03	2420
	EC <sub>50</sub> Value	$-2.63 \times 10^{-2}$	0.03	0.35	$2.2 \times 10^{-5}$
	Induction	-0.03	0.29*	0.09	4.97
	Methoxychlor Control	-45.70	0.19*	0.14	6539
<b>Antagonist Controls</b>	DMSO Control	-56.02	0.11*	0.32	3147
	E2 Control	-77.90	0.29*	0.16	5837
	Flavone Control	-64.89	0.03	0.50	2625
	IC <sub>50</sub> Value	$1.18 \times 10^{-6}$	0.82*	0.01	$1.14 \times 10^{-3}$
	Reduction	0.03	0.56*	0.05	5.86

2973 Abbreviations: DMSO = dimethyl sulfoxide; E2 = 17 $\beta$ -estradiol; EC<sub>50</sub> = half-maximal effect concentration; IC<sub>50</sub> = concentration of test substance  
 2974 that inhibits the reference estrogen response by 50%.

2975 <sup>1</sup>Each experiment was assigned a number based on the order in which testing occurred,  
 2976 without respect to the time lapsing between tests.

2977 \*The slope of the linear regression across experiments is not statistically different from zero.

2978 **14.0 Comparison of CellTiter-Glo<sup>®</sup> versus visual observation Methods of**  
 2979 **assessing cell viability**

2980 As part of the BG1Luc ER TA protocol standardization study, XDS evaluated the use of the CellTiter-  
 2981 Glo<sup>®</sup> (Promega Corporation) quantitative cell viability assay. Cell viability is measured by a luminescent  
 2982 signal that is proportional to the amount of adenosine triphosphate (ATP) in viable cells. Separate plates  
 2983 must be used for CellTiter-Glo<sup>®</sup> and BG1Luc ER TA as both assays use luminescence platforms.

2984 CellTiter-Glo<sup>®</sup> assays were conducted for all agonist and antagonist experiments during the BG1Luc ER  
 2985 TA protocol standardization study. A qualitative method using visual observation to assess cell viability  
 2986 was also conducted for all agonist and antagonist experiments during the protocol standardization study.  
 2987 Criteria for assessing and scoring cell viability using XDS's visual observation method are provided in  
 2988 **Table 7-1.**

2989 A comparison of E2 reference standard data from the LUMI-CELL<sup>®</sup> and CellTiter-Glo<sup>®</sup> assays indicated  
 2990 that no decrease in response in the BG1Luc ER TA occurred when cell viability was at least 80% in the  
 2991 CellTiter-Glo<sup>®</sup> assay. In addition, CellTiter-Glo<sup>®</sup> values of 80% or above corresponded with a score of 1  
 2992

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2993 in the visual observation method. Therefore, concentrations of test substance that caused a reduction in  
 2994 cell viability below 80% using CellTiter-Glo<sup>®</sup> or that had viability scores of 2 or more in the visual  
 2995 observation method were classified as cytotoxic and these data were not used to assess ER activity in the  
 2996 BG1LUC ER TA protocol standardization study.

2997 A critical consideration in standardizing BG1Luc ER TA protocols is the efficacy of limiting the  
 2998 assessment of cell viability to visual observation. This would greatly reduce the effort and cost of cell  
 2999 viability assessment by eliminating the need for running concurrent parallel plates required when using  
 3000 the CellTiter-Glo<sup>®</sup> method. In the protocol standardization study, CellTiter-Glo<sup>®</sup> results from the testing  
 3001 of substances (eight for agonism and eight for antagonism) were compared to results from the XDS visual  
 3002 observation method.

### 3003 14.1 Agonist Range Finder Testing

3004 Cytotoxicity was only observed at the highest concentration tested (100 µg/mL) for coded test substances  
 3005 during agonist range finder testing. All substances tested were classified as cytotoxic at this concentration  
 3006 except for atrazine (N0001). Classification of cell viability agreed between the two methods (i.e.,  
 3007 CellTiter-Glo<sup>®</sup> and visual observation) for all substances except for corticosterone (N0004), which was  
 3008 classified as “not cytotoxic” using the CellTiter-Glo<sup>®</sup> method (80% viability) but as “cytotoxic” using the  
 3009 visual observation method (score of “2”) (see . **Table 14-2** and **Figure 14-1**).

3010

3011 **Table 14-2 Cell Viability for Agonist Range Finder Testing at 100 µg/mL**

Code	Substance Name	% Cell Viability <sup>1</sup>	Visual Observation <sup>2</sup>
N0001	Atrazine	93%	1
N0002	Bisphenol A	6%	4
N0003	Bisphenol B	6%	4
N0004	Corticosterone	80%	2
N0005	<i>o,p'</i> -DDT	12%	4
N0006	Diethylstilbestrol	6%	4
N0007	17 $\alpha$ -ethinyl estradiol	30%	3
N0008	Flavone	12%	4

3012 Abbreviation: *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane

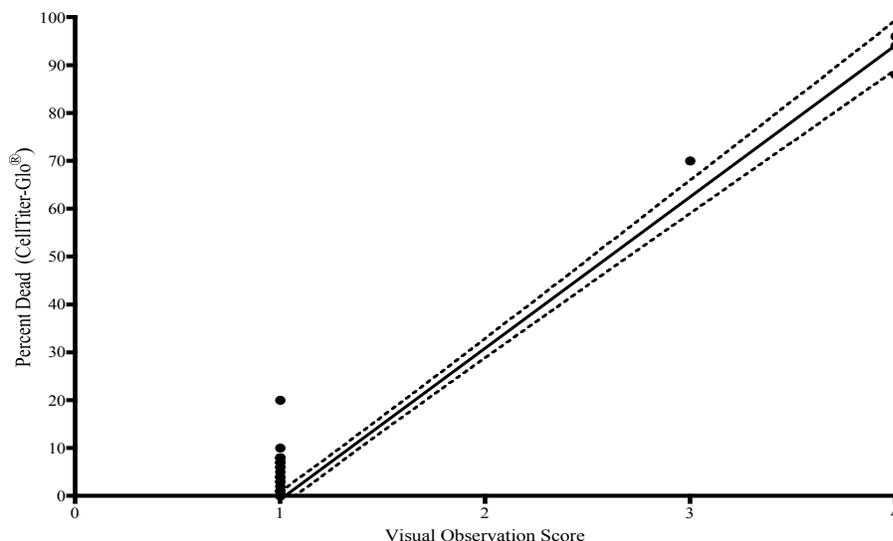
3013 <sup>1</sup>Cell viability as measured by CellTiter-Glo<sup>®</sup>.

3014 <sup>2</sup>See **Table 7-1** for a description of the visual observation scores.

3015

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3015 **Figure 14-1 CellTiter-Glo<sup>®</sup> versus Visual Observation Scores for Agonist Range Finder**  
3016 **Testing<sup>1,2,3</sup>**



3017

3018 <sup>1</sup>Each point on the figure represents a single replicate well for a single test substance.

3019 <sup>2</sup>The solid line represents the linear regression as calculated by PRISM<sup>®</sup>.

3020 <sup>3</sup>The dashed lines represent the 95% confidence limits of the linear regression as calculated by PRISM<sup>®</sup>.

### 3021 14.2 Agonist Comprehensive Testing

3022 Cytotoxicity was not observed at any of the concentrations used in the comprehensive testing for  
3023 agonism. Classification of cell viability agreed between the two methods with all concentrations tested  
3024 scoring at 87% or above using the CellTiter-Glo<sup>®</sup> method and at “1” using the visual observation method.

### 3025 14.3 Antagonist Range Finder Testing

3026 Cytotoxicity was only observed at the highest concentration tested (50 µg/mL) for five of the eight coded  
3027 test substances during agonist range finder testing. The remaining three substances, BBP (N0009), DBA  
3028 (N0010) and genistein (N0011), were not classified as cytotoxic at any concentration tested.

3029 Classification of cell viability agreed between the two methods and results are presented in **Table 14-3**  
3030 and **Figure 14-2**.

3031

3032

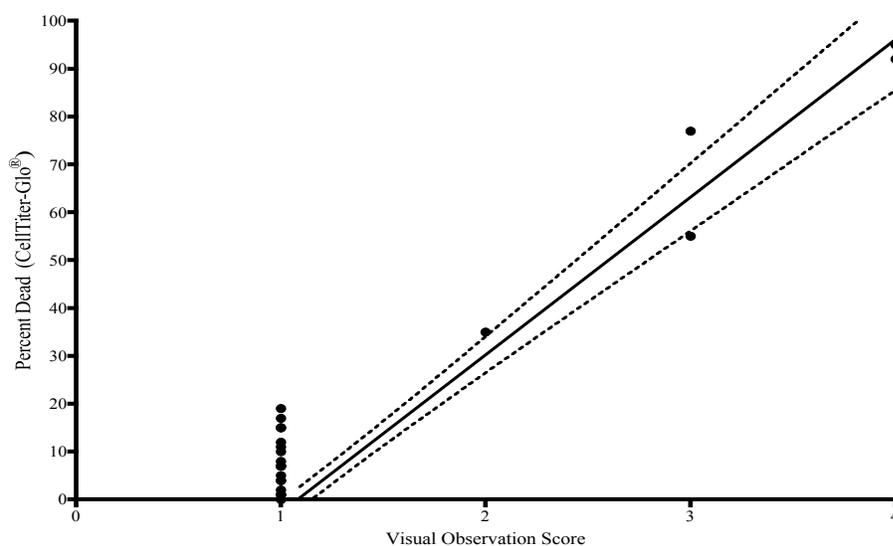
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3032 **Table 14-3 Cell Viability for Antagonist Range Finder Testing at 50 µg/mL**

Code	Substance Name	% Cell Viability <sup>1</sup>	Visual Observation <sup>2</sup>
N0009	Butylbenzyl phthalate	111%	1
N0010	Dibenzo[ <i>a,h</i> ]anthracene	103%	1
N0011	Genistein	85%	1
N0012	Flavone	65%	2
N0013	<i>p</i> -n-nonylphenol	8%	4
N0014	Progesterone	45%	3
N0015	<i>o,p'</i> -DDT	23%	3
N0016	Tamoxifen	5%	4

3033 Abbreviations: *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane3034 <sup>1</sup>Cell viability as measured by CellTiter-Glo®.3035 <sup>2</sup>See Table 7-1 for a description of the visual observation scores.

3036

3037 **Figure 14-2 CellTiter-Glo® versus Visual Observation Scores for Antagonist Range Finder**  
3038 **Testing<sup>1,2,3</sup>**

3039

3040 <sup>1</sup>Each point on the figure represents a single replicate well for a single test substance.3041 <sup>2</sup>The solid line represents the linear regression as calculated by PRISM®.3042 <sup>3</sup>The dashed lines represent the 95% confidence limits of the linear regression as calculated by PRISM®.

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**3043 14.4 Antagonist Comprehensive Testing**

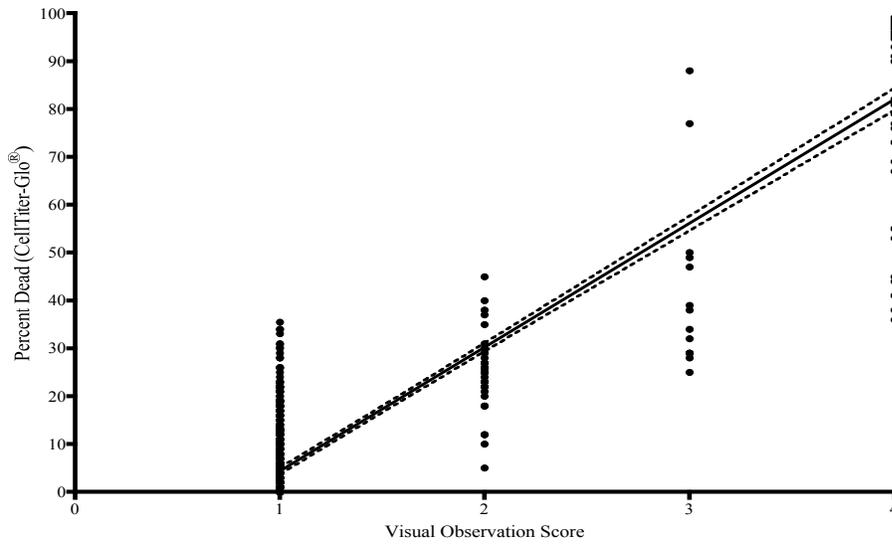
3044 Cytotoxicity was observed at various concentrations used in the comprehensive testing of five of the eight  
3045 coded antagonist test substances. The remaining three substances, DBA (N0010), genistein (N0011), and  
3046 tamoxifen (N0016), were not classified as cytotoxic at any concentration tested.

3047 An assessment of cell viability is especially important when testing for antagonism in order to determine  
3048 whether reduction of luminescence is based on cytotoxicity or reduced ER mediated transcriptional  
3049 activity. Therefore, BG1Luc ER TA results must be considered when comparing methods for assessing  
3050 cell viability. For this comparison, BG1Luc ER TA results are expressed as percent reduction of E2 and is  
3051 defined as the ability of a given concentration of test substance to reduce the ER TA activity induced by  
3052 the E2 control ( $2.5 \times 10^{-5}$  µg/mL, a concentration of E2 that induces 80-90% of maximum ER TA in the  
3053 test system). BG1Luc ER TA results for the five substances that showed cytotoxicity are compared to  
3054 scores from CellTiter-Glo<sup>®</sup> and visual observation methods in **Sections 14.4.1** through **14.4.6** below. The  
3055 data presented and discussed does not include all concentrations tested in each experiment but is limited  
3056 to those concentrations that were classified as cytotoxic in one or more experiments and focuses on visual  
3057 observation scores that did not correspond with CellTiter-Glo<sup>®</sup> cell viability values (i.e., cell viability of  
3058 80% or above should correspond to a visual observation score of “1”). Comparison of CellTiter-Glo<sup>®</sup> data  
3059 and visual observation scores for all concentrations of substances tested for antagonism are presented  
3060 graphically in **Figure 14-3**.

3061

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3061 **Figure 14-3 CellTiter-Glo<sup>®</sup> versus Visual Observation Scores for Antagonist Comprehensive**  
3062 **Testing<sup>1,2,3</sup>**



3063

3064 <sup>1</sup>Each point on the figure represents a single replicate well for a single test substance.

3065 <sup>2</sup>The solid line represents the linear regression as calculated by PRISM<sup>®</sup>.

3066 <sup>3</sup>The dashed lines represent the 95% confidence limits of the linear regression as calculated by PRISM<sup>®</sup>.

3067

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3067

3068 **14.4.1N0009 - BBP**

3069 Selected BG1Luc ER TA, CellTiter-Glo<sup>®</sup>, and visual observation results for BBP are provided in **Table**  
 3070 **14-4**.

3071 **Table 14-4 Comparison of Selected Results for N0009 - BBP**

Date	Concentration (µg/mL)	% Reduction of E2 <sup>1</sup>	% Cell Viability <sup>2</sup>	Visual Observation
12 April 06	50	67	76	2
	25	24	74	2
15 April 06	50	83	84	1
	25	68	82	1
	12.5	24	83	1
18 April 06	50	44	75	2
	25	35	70	1
	12.5	8	74	1

3072 Abbreviations: BBP = butylbenzyl phthalate; E2 = 17β-estradiol

3073 <sup>1</sup>Percent reduction of E2 is calculated as the relative light unit (RLU) value for the test substance at a given concentration divided by the averaged  
 3074 E2 control RLU value times 100.

3075 <sup>2</sup>Cell viability as measured by CellTiter-Glo<sup>®</sup>.

3076

3077 A comparison of the results indicated the following:

- 3078 • 12 April 06 experiment:
- 3079 —CellTiter-Glo<sup>®</sup> values and visual observation scores agreed.
- 3080 —Concentrations which reduce E2 activity were classified as cytotoxic, and were not used to  
 3081 assess ER activity.
- 3082 —Test substance was classified as negative for ER antagonist activity.
- 3083 • 15 April /06 experiment:
- 3084 —CellTiter-Glo<sup>®</sup> values and visual observation scores agreed.
- 3085 —None of the concentrations reducing E2 activity were classified as cytotoxic by either  
 3086 CellTiter-Glo<sup>®</sup> or visual observation, so they were used to assess ER activity.
- 3087 —Test substance was classified as positive for ER antagonist activity.
- 3088 • 18 April 06 experiment:

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3089 —CellTiter-Glo<sup>®</sup> values and visual observation scores did not correspond at the 25 and 12.5  
3090 µg/mL concentrations.

3091 —The concentration (25 µg/mL) which reduced E2 activity was classified as cytotoxic by  
3092 CellTiter-Glo<sup>®</sup> and was not used to assess ER activity.

3093 —The 25 µg/mL concentration would have been classified as positive using visual  
3094 observations.

3095 Butylbenzyl phthalate was classified as negative for ER antagonist activity when cell viability was  
3096 measured using CellTiter-Glo<sup>®</sup>, but it would have been classified as positive if using visual observations.

#### 3097 14.4.2N0012 - Flavone

3098 Selected BG1Luc ER TA, CellTiter-Glo<sup>®</sup>, and visual observation results for flavone are provided in  
3099 **Table 14-5**.

3100 **Table 14-5 Comparison of Selected Results for N0012 - Flavone**

Date	Concentration (µg/mL)	% Reduction of E2 <sup>1</sup>	% Cell Viability <sup>2</sup>	Visual Observation
12 April 06	50	93	83	2
	25	72	78	1
	12.5	38	78	1
	6.25	9	85	1
15 April 06	50	99	91	2
	25	90	86	1
	12.5	37	85	1
	6.25	0	86	1
18 April 06	50	77	74	2
	25	66	75	1
	12.5	16	79	1

3101 Abbreviations: E2 = 17β-estradiol

3102 <sup>1</sup>Percent reduction of E2 is calculated as the relative light unit (RLU) value for the test substance at a given concentration divided by the averaged  
3103 E2 control RLU value times 100.

3104 <sup>2</sup>Cell viability as measured by CellTiter-Glo<sup>®</sup>. Concentrations of test substances that cause a decrease in cell viability to below 80% are  
3105 considered to be cytotoxic and are not included in data analyses.

3106  
3107 A comparison of the results indicated the following:

- 3108 • 12 April 06 experiment:

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- 3109 —CellTiter-Glo<sup>®</sup> values and visual observation scores did not agree.
- 3110 —Two of the concentrations which reduced E2 activity (25 and 12.5 µg/mL) were classified
- 3111 as cytotoxic by CellTiter-Glo<sup>®</sup> and were not used to assess ER activity.
- 3112 —These concentrations would have been classified as positive if using visual observations.
- 3113 • 15 April 06 experiment:
- 3114 —CellTiter-Glo<sup>®</sup> values and visual observation scores did not agree at 50 µg/mL.
- 3115 —Using CellTiter-Glo<sup>®</sup>, the two concentrations (50 and 25 µg/mL) causing a reduction in E2
- 3116 activity were not cytotoxic and were used to assess ER activity.
- 3117 —Using visual observations, the 50 µg/mL concentration would have been classified as
- 3118 cytotoxic.
- 3119 —Flavone would have been classified as positive for ER antagonist activity using CellTiter-
- 3120 Glo<sup>®</sup> and negative using visual observations.
- 3121 • 18 April 06 experiment:
- 3122 —CellTiter-Glo<sup>®</sup> values and visual observation scores did not correspond at the 25 and 12.5
- 3123 µg/mL concentrations.
- 3124 —Two of the concentrations reducing E2 activity (25 and 12.5 µg/mL) were classified as
- 3125 cytotoxic by CellTiter-Glo<sup>®</sup> and were not used to assess ER activity.
- 3126 —These concentrations would have been classified as positive for ER antagonist activity
- 3127 using visual observations.
- 3128 Flavone was classified as positive for ER antagonist activity when cell viability was measured using
- 3129 CellTiter-Glo<sup>®</sup>, but it would have been classified as negative if using visual observations only.
- 3130

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3130 **14.4.3N0013 - Nonylphenol**

3131 Selected BG1Luc ER TA, CellTiter-Glo<sup>®</sup>, and visual observation results for nonylphenol are provided in  
 3132 **Table 14-6.**

3133 **Table 14-6 Comparison of Selected Results for N0013 - Nonylphenol**

Date	Concentration (µg/mL)	% Reduction of E2 <sup>1</sup>	% Cell Viability <sup>2</sup>	Visual Observation
15 April 06	12.5	99	29	4
	6.25	44	82	1
20 April 06	12.5	99	29	3
	6.25	61	75	2
1 May 06	12.5	99	64	3
	6.25	34	84	1

3134 Abbreviations: E2 = 17β-estradiol

3135 <sup>1</sup>Percent reduction of E2 is calculated as the relative light unit (RLU) value for the test substance at a given concentration divided by the averaged  
 3136 E2 control RLU value times 100.

3137 <sup>2</sup>Cell viability as measured by CellTiter-Glo<sup>®</sup>.

3138

3139 A comparison of the results indicated the following:

3140 • 15 April 06 experiment:

3141 —CellTiter-Glo<sup>®</sup> values and visual observation scores agreed.

3142 —The 12.5 µg/mL concentration, which reduced E2 activity, was classified as cytotoxic and  
 3143 was not used to assess ER activity.

3144 —Substance was classified as positive for ER antagonist activity at 6.25 µg/mL using both  
 3145 CellTiter-Glo<sup>®</sup> and visual observations.

3146 • 20 April 06 experiment:

3147 —CellTiter-Glo<sup>®</sup> values and visual observation scores agreed.

3148 —Both concentrations reducing E2 activity were classified as cytotoxic and were not used to  
 3149 assess ER activity.

3150 —Substance was classified as negative for ER antagonist activity using both CellTiter-Glo<sup>®</sup>  
 3151 and visual observations.

3152 • 1 May 06 experiment:

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3153 —CellTiter-Glo<sup>®</sup> values and visual observation scores agreed.  
 3154 —The 12.5 µg/mL concentration, which reduced E2 activity, was classified as cytotoxic and  
 3155 was not used to assess ER activity.

3156 Nonylphenol was classified as positive for ER antagonist activity at 6.25 µg/mL using both CellTiter-  
 3157 Glo<sup>®</sup> and visual observations.

3158

#### 3159 14.4.4N0014 - Progesterone

3160 Selected BG1Luc ER TA, CellTiter-Glo<sup>®</sup>, and visual observation results for progesterone are provided in  
 3161 **Table 14-7**.

3162

3163 **Table 14-7 Comparison of Selected Results for N0014 - Progesterone**

Date	Concentration (µg/mL)	% Reduction of E2 <sup>1</sup>	% Cell Viability <sup>2</sup>	Visual Observation
15 April 06	12.5	73	86	1
	6.25	39	92	1
20 April 06	25	99	62	2
	12.5	61	72	1
	6.25	20	93	1
1 May 06	25	87	62	3
	12.5	49	69	3

3164 Abbreviations: E2 = 17β-estradiol

3165 <sup>1</sup>Percent reduction of E2 is calculated as the relative light unit (RLU) value for the test substance at a given concentration divided by the averaged  
 3166 E2 control RLU value times 100.

3167 <sup>2</sup>Cell viability as measured by CellTiter-Glo<sup>®</sup>.

3168

3169 A comparison of results indicated the following:

- 3170 • 15 April 06 experiment:
  - 3171 —CellTiter-Glo<sup>®</sup> values and visual observation scores agreed.
  - 3172 —Neither concentration reducing E2 activity (12.5 and 6.25 µg/mL) was classified as
  - 3173 cytotoxic with either CellTiter-Glo<sup>®</sup> or visual observations, so both concentrations were
  - 3174 used to assess ER activity.
  - 3175 —Progesterone was classified as positive for ER antagonist activity using both CellTiter-  
 3176 Glo<sup>®</sup> and visual observations.

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- 3177 • 20 April 06 experiment:
- 3178 —CellTiter-Glo<sup>®</sup> values and visual observation scores did not correspond at 12.5 µg/mL.
- 3179 —The concentrations reducing E2 activity (25 and 12.5 µg/mL) were classified as cytotoxic
- 3180 using CellTiter-Glo<sup>®</sup>, so they were not used to assess ER activity.
- 3181 —12.5 µg/mL would have been considered positive for antagonism using visual
- 3182 observations.
- 3183 • 1 May 06 experiment:
- 3184 —CellTiter-Glo<sup>®</sup> values and visual observation scores agreed.
- 3185 —Concentrations reducing E2 activity were classified as cytotoxic using both CellTiter-Glo<sup>®</sup>
- 3186 and visual observations, so they were not used to assess ER activity.

3187 Progesterone was classified as negative for ER antagonist activity.

3188

#### 3189 14.4.5N0015 - *o,p'*-DDT

3190 Selected BG1Luc ER TA, CellTiter-Glo<sup>®</sup>, and visual observation results for *o,p'*-DDT are provided in

3191 **Table 14-8.**

3192

3193 **Table 14-8 Comparison of Selected Results for N0015 - *o,p'*-DDT**

Date	Concentration (µg/mL)	% Reduction of E2 <sup>1</sup>	% Cell Viability <sup>2</sup>	Visual Observation
20 April 06	50	99	19	4
	25	99	45	4
	12.5	40	75	2
1 May 06	50	99	26	4
	25	99	59	4
	12.5	22	74	2
5 May 06	50	99	20	4
	25	87	60	3
	12.5	29	82	2

3194 Abbreviation: *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane; E2 = 17β-estradiol

3195 <sup>1</sup>Percent reduction of E2 is calculated as the relative light unit (RLU) value for the test substance at a given concentration divided by the averaged  
3196 E2 control RLU value times 100.

3197 <sup>2</sup>Cell viability as measured by CellTiter-Glo<sup>®</sup>.

3198

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3199 A comparison of the results indicated the following:

3200 • 20 April 06 experiment:

3201 —CellTiter-Glo<sup>®</sup> values and visual observation scores agreed.

3202 —Concentrations reducing E2 activity were classified as cytotoxic using both CellTiter-Glo<sup>®</sup>  
3203 and visual observations, so they were not used to assess ER activity.

3204 —*o,p'*-DDT was classified as negative for ER antagonist activity using both CellTiter-Glo<sup>®</sup>  
3205 and visual observations.

3206 • 1 May 06 experiment:  
3207

3208 —CellTiter-Glo<sup>®</sup> values and visual observation scores agreed.

3209 —Concentrations reducing E2 activity were classified as cytotoxic using both CellTiter-Glo<sup>®</sup>  
3210 and visual observations, so they were not used to assess ER activity.

3211 —*o,p'*-DDT was classified as negative for ER antagonist activity using both CellTiter-Glo<sup>®</sup>  
3212 and visual observations.

3213 • 5 May 06 experiment:

3214 —CellTiter-Glo<sup>®</sup> values and visual observation scores did not correspond at 12.5 µg/mL.

3215 —Concentrations reducing E2 activity were classified as cytotoxic by visual observation but  
3216 not at 12.5 µg/mL with CellTiter-Glo<sup>®</sup>.

3217 *o,p'*-DDT was classified as negative for ER antagonist activity when using visual observations, but would  
3218 have been classified positive for antagonism at 12.5 µg/mL with CellTiter-Glo<sup>®</sup>.

3219

## 3220 **15.0 Problems Encountered during the Protocol Standardization Study**

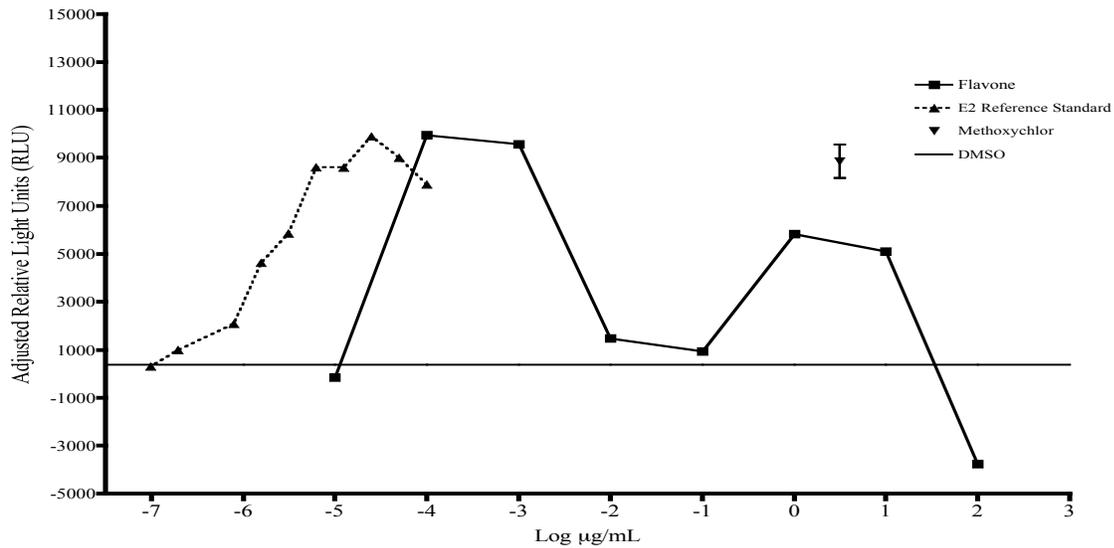
### 3221 **15.1 Aberrant Range Finder Concentration-Response Curve for N0008 - Flavone**

3222 During protocol standardization, flavone yielded a biphasic concentration-response curve (**Figure 15-1**).

3223

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3223 **Figure 15-1 Initial Agonist Range Finder for N0008 – Flavone<sup>1</sup>**



3224

3225 Abbreviations: E2 = 17β-estradiol; methoxychlor = 3.13 µg/mL methoxychlor control; DMSO = dimethyl sulfoxide.

3226 <sup>1</sup>Line represents the mean of four DMSO replicates plus three times the standard

3227 deviation of the DMSO mean.

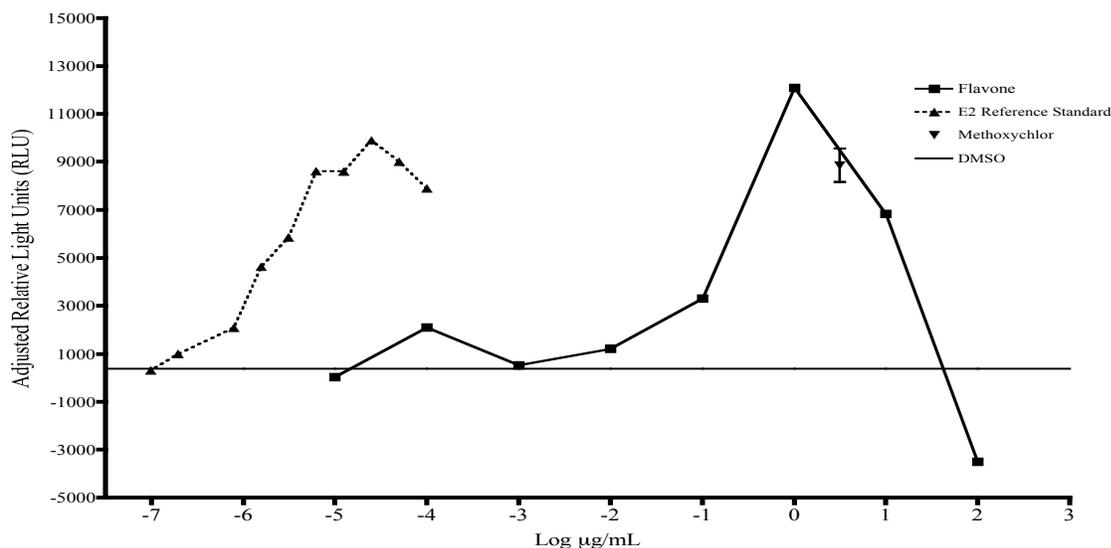
3228

3229 Due to concerns about possible experimental error, range finder testing for this substance was repeated  
 3230 three additional times (**Figure 15-2**).

3231

3232

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3232 **Figure 15-2 Repeated Agonist Range Finder for N0008 – Flavone<sup>1</sup>**

3233

3234 Abbreviations: E2 = 17β-estradiol; methoxychlor = 3.13 µg/mL methoxychlor control; DMSO = dimethyl sulfoxide.

3235 <sup>1</sup>Line represents the mean of four DMSO replicates plus three times the standard deviation of the  
3236 DMSO mean.

3237

3238 Repeat range finder testing of flavone showed that the peak occurring at the lowest concentrations of  
3239 flavone did not appear consistently. It is possible that contamination was present during the creation of  
3240 two of the four serial dilutions used to produce the four flavone finder concentration-response  
3241 curves, either in the batch of flavone sent to the laboratory or in the test tubes used to make the dilution.

3242

3243 **15.2 Errors in Serial Dilutions**

3244 During protocol standardization, each serial dilution used on an experimental plate was independently  
3245 diluted, such that serial dilution A is different from serial dilutions B and C. Serial dilutions were  
3246 performed in this manner in order to minimize the loss of experimental data caused by experimenter error.

3247

3248 **15.2.1N0001 – Atrazine**

3249 The atrazine agonist experiment conducted on 04/04/06 had a portion of replicate serial dilution B  
3250 omitted due to experimenter error. In dilution B, adjusted RLU values for atrazine (**Table 15-1**) were  
3251 significantly higher than those obtained in either the range finder testing or the other experiments using  
3252 atrazine. These values were excluded from analysis.

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3253 **Table 15-1 Adjusted RLU Values for the Atrazine Experiment Conducted on**  
 3254 **4 April 06**

Atrazine Concentration ( $\mu\text{g/mL}$ )	Serial Dilution A <sup>1</sup>	Serial Dilution B <sup>1</sup>	Serial Dilution C <sup>1</sup>
$1.00 \times 10^{-2}$	<b><i>2444</i></b> <sup>2</sup>	867	634
$5.00 \times 10^{-3}$	298	441	901
$2.50 \times 10^{-3}$	428	467	513
$1.25 \times 10^{-3}$	251	128	500
$6.25 \times 10^{-4}$	62	224	488
$3.13 \times 10^{-4}$	826	<b><i>7061</i></b>	275
$1.56 \times 10^{-4}$	147	<b><i>7544</i></b>	475
$7.81 \times 10^{-5}$	79	<b><i>7302</i></b>	516
$3.91 \times 10^{-5}$	-97	<b><i>5416</i></b>	210
$1.95 \times 10^{-5}$	24	<b><i>3844</i></b>	440
$9.77 \times 10^{-6}$	<b><i>5505</i></b>	<b><i>2498</i></b>	328

3255 Abbreviations: RLU = relative light unit

3256 <sup>1</sup>Values are presented as adjusted RLUs.

3257 <sup>2</sup>Values presented in bolded and italicized text did not pass the Q test for outliers (Zar 1984).

3258

### 3259 **15.2.2N0004 – Corticosterone**

3260 The corticosterone agonist experiment conducted on 8 April 06 had a portion of replicate serial dilution B  
 3261 omitted due to experimenter error. In dilution B, adjusted RLU values for corticosterone (**Table 15-2**)  
 3262 were significantly higher than those obtained in either the range finder testing or the other experiments  
 3263 using corticosterone. These values were excluded from analysis.

3264

3265

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3265 **Table 15-2 Adjusted RLU Values for the Corticosterone Experiment**  
 3266 **Conducted on 8 April 06**

Corticosterone Concentration ( $\mu\text{g/mL}$ )	Serial Dilution A <sup>1</sup>	Serial Dilution B <sup>1</sup>	Serial Dilution C <sup>1</sup>
$5.00 \times 10^{-1}$	-832	<b><i>11202</i></b> <sup>2</sup>	191
$2.50 \times 10^{-1}$	-86	800	2519
$1.25 \times 10^{-1}$	-331	-148	2347
$6.25 \times 10^{-2}$	338	185	1340
$3.13 \times 10^{-2}$	<b><i>13577</i></b>	835	1139
$1.56 \times 10^{-2}$	<b><i>9268</i></b>	-147	276
$7.81 \times 10^{-3}$	<b><i>8044</i></b>	434	327
$3.91 \times 10^{-3}$	<b><i>5894</i></b>	-646	-1222
$1.95 \times 10^{-3}$	<b><i>2162</i></b>	-788	-754
$9.77 \times 10^{-4}$	544	-823	-422
$6.10 \times 10^{-4}$	150	-495	<b><i>2788</i></b>

3267 RLU = relative light unit

3268 <sup>1</sup>Values are presented as adjusted RLUs.

3269 <sup>2</sup>Values presented in bolded and italicized text did not pass the Q test for outliers (Zar 1984).

3270

3271

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3271 **15.2.3N0006 – Diethylstilbestrol**

3272 The diethylstilbestrol agonist experiment conducted on 17 April 06 had a portion of replicate serial  
 3273 dilution A omitted due to experimenter error. In this serial dilution, adjusted RLU values for  
 3274 diethylstilbestrol (**Table 15-3**), which had been decreasing with decreasing concentration of  
 3275 diethylstilbestrol suddenly increased. Since diethylstilbestrol did not exhibit a biphasic response in any  
 3276 other experiment, these values were excluded from analysis.

3277 **Table 15-3 Adjusted RLU Values for the Diethylstilbestrol Experiment**  
 3278 **Conducted on 17 April 06**

Diethylstilbestrol Concentration ( $\mu\text{g/mL}$ )	Serial Dilution A <sup>1</sup>	Serial Dilution B <sup>1</sup>	Serial Dilution C <sup>1</sup>
$1.00 \times 10^{-4}$	9654	9640	9854
$5.00 \times 10^{-5}$	10667	10105	7880
$2.50 \times 10^{-5}$	8847	6428	7728
$1.25 \times 10^{-5}$	4382	6239	7649
$6.25 \times 10^{-6}$	3112	2964	3032
$3.13 \times 10^{-6}$	<b>8626<sup>2</sup></b>	2103	1710
$1.56 \times 10^{-6}$	<b>9220</b>	557	2064
$7.81 \times 10^{-7}$	<b>8530</b>	-370	467
$3.91 \times 10^{-7}$	<b>7499</b>	570	708
$1.95 \times 10^{-7}$	<b>5442</b>	-161	1083
$9.77 \times 10^{-8}$	<b>5196</b>	-323	91

3279 RLU = relative light unit

3280 <sup>1</sup>Values are presented as adjusted RLUs.

3281 <sup>2</sup>Values presented in bolded and italicized text did not pass the Q test for outliers (Zar 1984).

3282

3283

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3283 **15.2.4N0007 – EE**

3284 The EE agonist experiment conducted on 17 April 06 had the entirety of replicate serial dilution B  
 3285 omitted due to experimenter error. In this serial dilution, adjusted RLU values for EE (**Table 15-4**),  
 3286 remain stable throughout the entire concentration-response curve.

3287

3288 **Table 15-4 Adjusted RLU Values for the EE Experiment Conducted on**3289 **17 April 06**

<b>EE Concentration (<math>\mu\text{g/mL}</math>)</b>	<b>Serial Dilution A<sup>1</sup></b>	<b>Serial Dilution B<sup>1</sup></b>	<b>Serial Dilution C<sup>1</sup></b>
1.00 x 10 <sup>-4</sup>	8305	<b>9011<sup>3</sup></b>	9148
5.00 x 10 <sup>-5</sup>	9516	<b>8772</b>	7682
2.50 x 10 <sup>-5</sup>	10280	<b>8154</b>	7573
1.25 x 10 <sup>-5</sup>	7081	<b>8643</b>	7984
6.25 x 10 <sup>-6</sup>	8773	<b>8955</b>	6288
3.13 x 10 <sup>-6</sup>	7161	<b>9431</b>	4655
1.56 x 10 <sup>-6</sup>	6064	<b>8689</b>	2577
7.81 x 10 <sup>-7</sup>	4047	<b>8145</b>	1212
3.91 x 10 <sup>-7</sup>	3590	<b>8840</b>	2223
1.95 x 10 <sup>-7</sup>	2248	<b>9345</b>	2885
9.77 x 10 <sup>-8</sup>	2406	<b>9015</b>	521

3290 Abbreviations: RLU = relative light unit; EE = 17 $\alpha$ -ethinyl estradiol3291 <sup>1</sup>Values are presented as adjusted RLUs.3292 <sup>3</sup>Values presented in bolded text were discarded because they did not exhibit a concentration-responsive decrease in adjusted RLUs.

3293

3294 **16.0 Deviations from protocol.**3295 **16.1 Alteration of Testing Concentrations during Agonist Comprehensive Testing**

3296 Concentrations for comprehensive testing were selected during evaluation of range finder results. These  
 3297 concentrations were selected to optimize the possibility of detecting a positive result during  
 3298 comprehensive testing. For agonism, the starting concentration for serial dilution was selected as the  
 3299 concentration that was a log-dilution higher than that giving the highest adjusted RLU value during range  
 3300 finder testing. Selection of this concentration allowed for saturation to be reached at the highest

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3301 concentrations tested. After the first experiment, two substances, bisphenol B (N0003), and flavone  
3302 (N0008) did not reach saturation, so their starting concentrations were adjusted to start at a double serial  
3303 dilution higher in order to generate concentration-response curves that reached saturation.

### 3304 **16.2 Alteration of Highest Testing Concentration for Antagonist Testing**

3305 According to the protocol, the highest antagonist concentration used for range finder testing should have  
3306 been 100 µg/mL, a concentration that had previously been determined to be the limit of solubility. Upon  
3307 receipt of the final report, it was noted that the highest concentration used in antagonist testing was 50  
3308 µg/mL, which contradicted the concentrations reported by XDS during antagonist range finding. Queries  
3309 to XDS determined that the 50 µg/mL starting concentrations were correct and had previously been  
3310 incorrectly reported because the experimenter had failed to account for the additional dilution caused by  
3311 the 1:1 dilution resulting from the addition of  $2.5 \times 10^{-5}$  µg/mL E2 to the mixing tubes. This deviation was  
3312 not relevant for BBP, flavone, nonylphenol, progesterone, and *o,p'*-DDT, because although these  
3313 compounds were not tested to the limit concentration or the limit of solubility, they were tested at  
3314 concentrations high enough to cause cytotoxicity. The two substances that were not cytotoxic at 50  
3315 µg/mL, DBA and genistein, were not soluble at 100 µg/mL.

3316

### 3317 **16.3 Alteration of Testing Concentrations for Tamoxifen Comprehensive Testing**

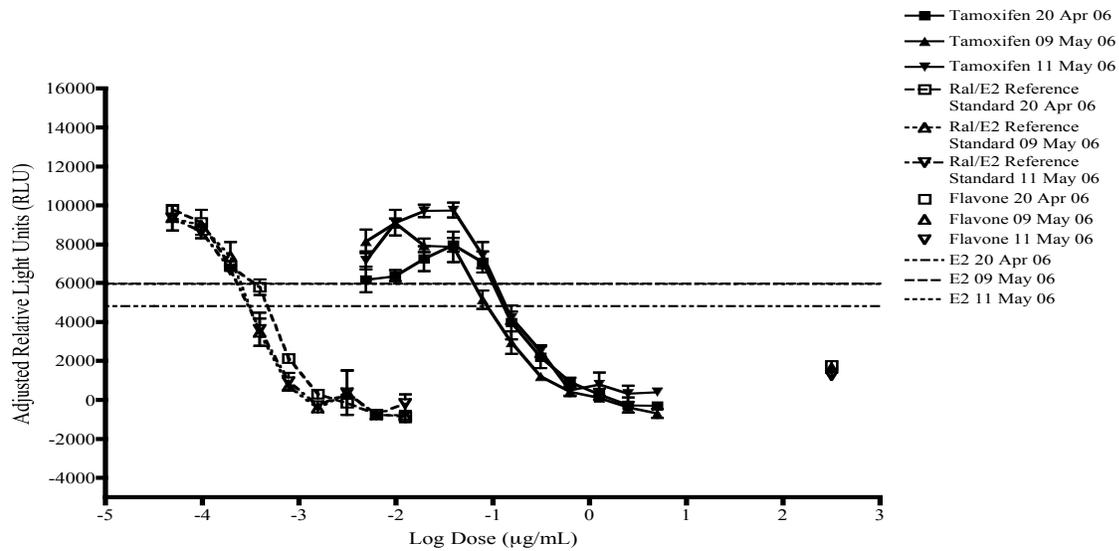
3318 A starting concentration for tamoxifen comprehensive testing of 5 µg/mL was selected as the result of  
3319 range finder testing (**Figure 16-1**). No cytotoxicity was observed at any concentration during this  
3320 experiment (**Figure 16-2**). After the first comprehensive experiment, the starting concentration of  
3321 tamoxifen was changed to 50 µg/mL to better define the high end of the concentration-response curve.  
3322 However, this shift resulted in a concentration-response curve that did not reach saturation at the highest  
3323 concentrations tested (**Figure 16-3**) because of excessive cytotoxicity (**Figure 16-4**). Two additional  
3324 experiments (**Figure 16-1**) were performed with the original starting concentration of tamoxifen with no  
3325 observable cytotoxicity at any concentration (**Figure 16-2**).

3326

3327

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3327 **Figure 16-1 Tamoxifen Concentration-Response Curve when the Starting**  
 3328 **Concentration is 5 µg/mL<sup>1,2</sup>**



3329  
 3330 Abbreviations: Ral/E2 Reference Standard = concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$  µg/mL  
 3331  $17\beta$ -estradiol; Flavone = 25 µg/mL flavone control; E2 =  $17\beta$ -estradiol.

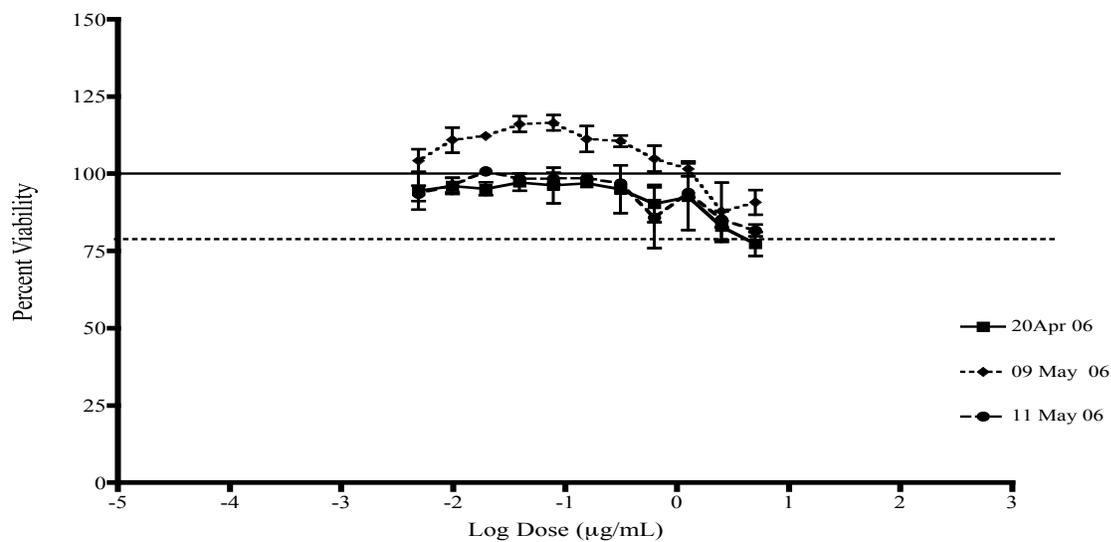
3332 <sup>1</sup>Line represents the mean of three E2 replicates minus three times the standard deviation of the E2 mean.

3333 <sup>2</sup>The 25 µg/mL flavone controls are not shown at the concentration at which they were tested. They have been placed  
 3334 on the graph in such a way as to maximize visibility.

3335

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3335 **Figure 16-2 CellTiter-Glo® Viability Data for Tamoxifen Experiments when the Starting**  
3336 **Concentration is 5  $\mu\text{g}/\text{mL}$ <sup>1,2</sup>**



3337

3338 <sup>1</sup>Solid line drawn across the graph at 100 percent viability indicates 100% viability

3339 as measured in DMSO solvent control.

3340 <sup>2</sup>Dashed line drawn across the graph at 80% viability indicates the viability limit for this assay.

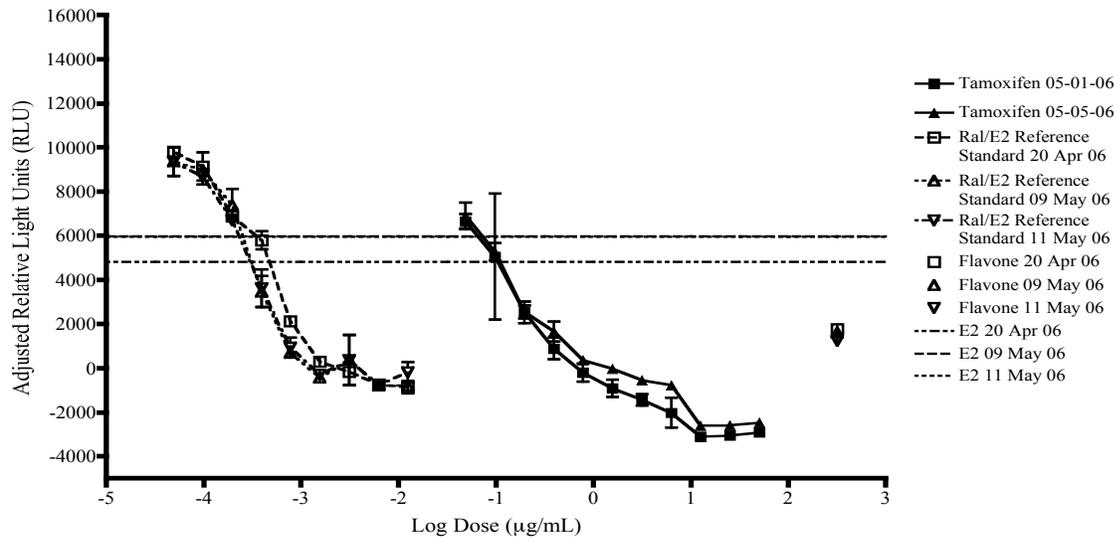
3341 Points that fall below this line are not included in data analyses.

3342

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3342

3343 **Figure 16-3 Tamoxifen Concentration-Response Curve when the**  
 3344 **Starting Concentration is 50 µg/mL<sup>1,2</sup>**



3345

3346 Abbreviations: Ral/E2 Reference Standard = concentrations of raloxifene with a fixed concentration of  $2.5 \times 10^{-5}$  µg/mL  
 3347 17β-estradiol; Flavone = 25 µg/mL flavone control; E2 = 17β-estradiol.

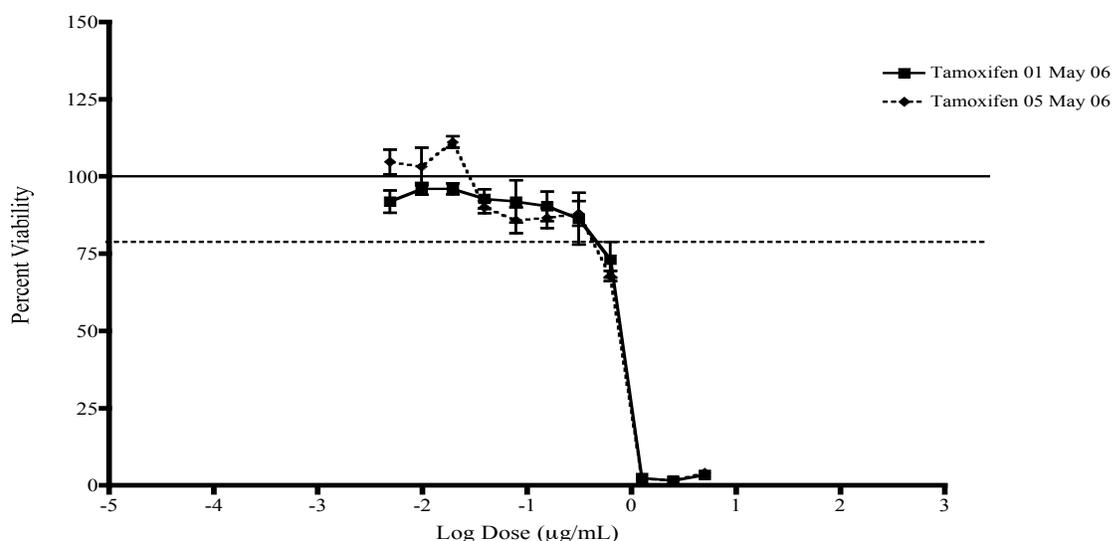
3348 <sup>1</sup>Line represents the mean of three E2 replicates minus three times the standard deviation of the E2 mean.

3349 <sup>2</sup>The 25 µg/mL flavone controls are not shown at the concentration at which they were tested. They have been placed  
 3350 on the graph in such a way as to maximize visibility.

3351

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3351 **Figure 16-4 CellTiter-Glo® Viability Data for Tamoxifen Experiments**  
 3352 **when the Starting Concentration is 50 µg/mL<sup>1,2</sup>**



3353

3354 <sup>1</sup>Solid line drawn across the graph at 100 percent viability indicates 100% viability

3355 as measured in DMSO solvent control.

3356 <sup>2</sup>Dashed line drawn across the graph at 80% viability indicates the viability limit for this assay.

3357 Points that fall below this line are not included in data analyses.

## 3358 17.0 Cell Culture Failures

3359 During protocol standardization, there were several instances where cells that were being cultured for use  
 3360 in the BG1Luc ER TA did not perform to previously established historical norms, or exhibited decreased  
 3361 viability.

3362

## 3363 17.1 Cytotoxicity Due to G418

3364 On 9 November 05, a new lot (#30234193) of G418 was added to media used on the cells in growth  
 3365 flasks to select cells containing the luciferase reporter gene. Twenty-four hours later, cell viability was  
 3366 reduced by more than 50%. A new aliquot of frozen cells was thawed and subcultured and a different lot  
 3367 (#30234198) of G418 was added to a single flask containing the new subculture. No signs of cytotoxicity  
 3368 were observed in the new subculture indicating that the previous lot (#30234193) of G418 was the likely  
 3369 cause of the cytotoxicity. Based on this information, the BG1Luc ER TA cell culture Standard Operating  
 3370 Procedure (SOP) was modified to test the performance of new lots of G418 for the cell selection process.

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## 3371 17.2 Decreased Viability and Diminished Response

3372 During the period from 27 December 05 to 27 February 06, cells exhibited abnormal morphology, poor  
3373 growth, and decreased viability at higher concentrations of the E2 reference standard. During the same  
3374 period, experiments showed a shift in reference standard EC<sub>50</sub> and IC<sub>50</sub> values. A common feature for  
3375 both agonist and antagonist assays was the cell culture media; therefore, various components of the media  
3376 were investigated as the cause of the abnormal results. Several potential causes were investigated and are  
3377 discussed below.

3378

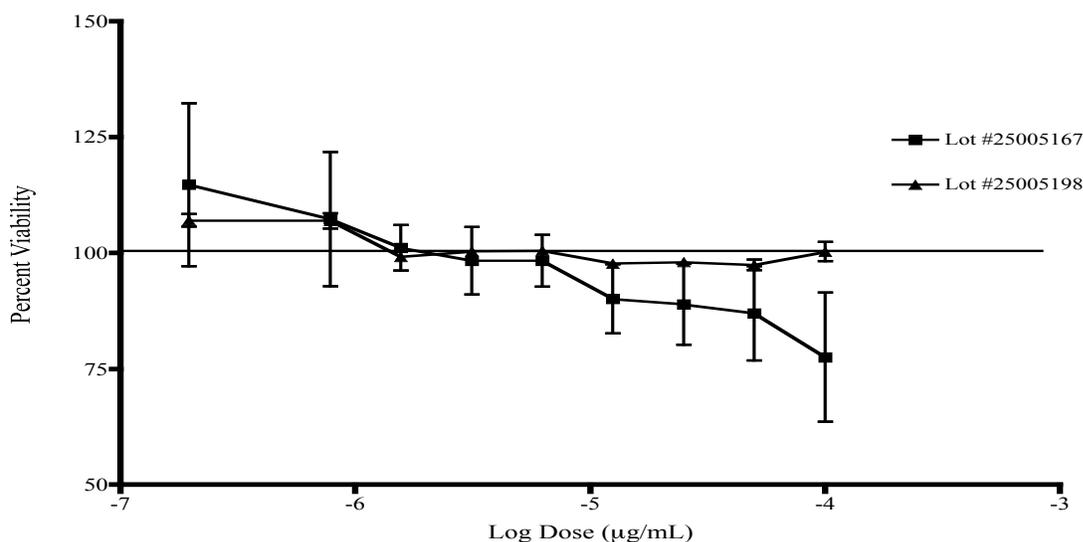
### 3379 17.2.1 L-Glutamine

3380 A new lot of L-glutamine (#25005167) had been in use since 27 December 05. A different lot  
3381 (#25005198) was tested, and cells exhibited improved morphology and viability (**Figure 17-1**), indicating  
3382 that the previous lot (#25005167) of L-glutamine may have contributed to the decreases in viability.

3383 Based on this information, the BG1Luc ER TA cell culture SOP was modified to test the performance of  
3384 new lots of L-glutamine used for cell culture.

3385

3386 **Figure 17-1 Increased Toxicity of E2 in the Presence of L-Glutamine Lot #25005167<sup>1</sup>**



3387

3388 Abbreviations: E2 = 17β-estradiol

3389 <sup>1</sup>Horizontal line indicates 100% viability as measured in DMSO solvent control.

3390

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**3391 17.2.2 Stripped FBS**

3392 On 5 January 06, a new bottle of FBS was used for ongoing cell culture. Cells from this culture exhibited  
3393 increased background luminescence in solvent controls and decreased induction for E2 reference standard  
3394 in the BG1Luc ER TA. Background and induction improved when a different bottle of FBS was used to  
3395 culture cells, indicating possible estrogenic contamination of the bottle used on 5 January 06. Based on  
3396 this information, the BG1Luc ER TA cell culture SOP was modified to test the performance of new  
3397 bottles of FBS used for cell culture.

3398

**3399 17.2.3 Tissue culture flasks.**

3400 On 27 December 05, a new lot of tissue culture flasks was used for ongoing cell culture. New flasks were  
3401 purchased from a different manufacturer and cells from ongoing cultures were transferred to the new  
3402 flasks. Cell morphology and viability improved, and reference standard EC<sub>50</sub> and IC<sub>50</sub> values return to  
3403 historical norms. Based on this information, the BG1Luc ER TA cell culture SOP was specifically  
3404 modified to test the performance of new lots of cell culture flasks.

3405

**3406 18.0 Substance Concentrations Tested and the ICCVAM Recommended Limit  
3407 Concentration**

3408 The ICCVAM Guidelines (ICCVAM 2003, 2006) recommend that both agonist and antagonist assays test  
3409 up to a limit concentration of 1mM, within the limits of test substance solubility and toxicity. Validation  
3410 studies were conducted in a blinded manner requiring test substances to be coded. Therefore,  
3411 concentrations to be tested were specified on a µg/mL basis, with the limit concentration being 1000  
3412 µg/mL, in the absence of solubility or cytotoxicity constraints. However, none of the test substances could  
3413 be tested to the intended limit concentration of 1 mg/mL because none were soluble at this concentration  
3414 in cell culture media containing 1% DMSO, so the limit concentration for protocol standardization was  
3415 set to 100 µg/mL, one log concentration lower than the recommended limit concentration.

3416 Upon completion of testing and data analysis, molar concentrations of test substances were calculated and  
3417 are presented in the sections below.

3418

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3419 **18.1 Agonist Concentrations Tested**

3420 The limit concentration for test substance used during agonist range finder testing was 100 µg/mL. This  
 3421 limit concentration correlated with a range of molar concentrations ranging from 282 to 463 µM (**Table**  
 3422 **18-1**).

3423

3424 **Table 18-1 Maximum Concentration of Test Substances Tested in the BG1Luc ER TA Agonist**  
 3425 **Protocol**

Maximum Concentration Used in Agonist Testing				
Substance	Concentration in µg/mL		Concentration in µM <sup>1</sup>	
	Range Finder	Comprehensive Testing	Range Finder	Comprehensive Testing
Atrazine	100	0.01	463	0.046
Bisphenol A	100	10	438	43.8
Bisphenol B	100	1.25	412	5.16
Corticosterone	100	1	288	2.89
<i>o,p'</i> -DDT	100	10	282	28.2
Diethylstilbestrol	100	1.00 x 10 <sup>-4</sup>	372	3.73 x 10 <sup>-4</sup>
EE	100	1.00 x 10 <sup>-4</sup>	337	3.37 x 10 <sup>-4</sup>
Flavone	100	50	450	22.5

3426

Abbreviations: EE = 17α-ethinyl estradiol; *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane

3427

<sup>1</sup>Formula weights used to calculate molarity were taken from MSDS sheets provided to NICEATM by the National Toxicology Program

3428

Substances Inventory

3429

3430 The highest concentrations used during comprehensive testing ranged from 1.00 x 10<sup>-6</sup> to 50 µg/mL,  
 3431 corresponding to 3.37 x 10<sup>-4</sup> to 43.8 µM.

3432

3433 **18.2 Antagonist Concentrations Tested**

3434 Because of solubility considerations, the highest concentration of test substance used during antagonist  
 3435 range finder testing was 50 µg/mL, which corresponded to a range of molar concentrations ranging from  
 3436 135 to 227 µM (**Table 18-2**).

3437

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3438 **Table 18-2 Maximum Concentration of Test Substances Tested in the BG1Luc ER TA**  
 3439 **Antagonist Protocol**

Maximum Concentration Used in Antagonist Testing				
Substance	Concentration in $\mu\text{g/mL}$		Concentration in $\mu\text{M}^1$	
	Range Finder	Comprehensive Testing	Range Finder	Comprehensive Testing
BBP	50	50	160	160
DBA	50	5	179	18
Genistein	50	50	185	185
Flavone	50	50	225	225
Nonylphenol	50	50	227	227
Progesterone	50	50	159	159
<i>o,p'</i> -DDT	50	50	141	141
Tamoxifen	50	5	135	13

3440 Abbreviations: BBP = butylbenzyl phthalate; DBA = dibenzo[*a,h*]anthracene; *o,p'*-DDT = 1,1,1-Trichloro-2-  
 3441 (*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane  
 3442 <sup>1</sup>Formula weights used to calculate molarity were taken from MSDS sheets provided to NICEATM by the National Toxicology Program  
 3443 Substances Inventory

3444

3445 The highest concentrations used during comprehensive testing ranged from 5 to 50  $\mu\text{g/mL}$ , correlating to  
 3446 13 to 227  $\mu\text{M}$ .

3447

3448 **19.0 Overview of Results from the BG1Luc ER TA Protocol Standardization**  
 3449 **Study**

3450 **19.1 Agonist Results**

3451 Of the eight test substances evaluated during agonist testing, six were positive (bisphenol A, bisphenol B,  
 3452 *o,p'*-DDT, diethylstilbestrol, EE, and flavone), and two were negative (atrazine and corticosterone) for  
 3453 agonist activity.  $\text{EC}_{50}$  values were calculated for all positive test substances and are presented in **Table**  
 3454 **19-1**.  $\text{EC}_{50}$  values are presented as both  $\mu\text{g/mL}$  and  $\mu\text{M}$  values. A range of concentrations (in both  $\mu\text{g/mL}$   
 3455 and  $\mu\text{M}$  values) at which each substance was active is also presented.

3456

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3456 **Table 19-1 EC<sub>50</sub> Values Obtained in the BG1Luc ER TA Agonist Protocol**

Substance	Data Presented as µg/mL		Data Presented as µM <sup>1</sup>	
	EC <sub>50</sub>	Activity Range	EC <sub>50</sub>	Activity Range
Atrazine	Negative	Negative	Negative	Negative
Bisphenol A	0.09	0.08 to 10	0.38	0.34 to 43.8
Bisphenol B	0.05	0.02 to 1.25	0.21	0.08 to 5.16
Corticosterone	Negative	Negative	Negative	Negative
<i>o,p'</i> -DDT	0.38	0.16 to 10	1.08	0.44 to 28.2
Diethylstilbestrol	1.26 x 10 <sup>-5</sup>	3.13 x 10 <sup>-6</sup> to 1.00 x 10 <sup>-4</sup>	4.69 x 10 <sup>-5</sup>	1.17 x 10 <sup>-5</sup> to 3.73 x 10 <sup>-4</sup>
EE	3.87 x 10 <sup>-6</sup>	7.81 x 10 <sup>-7</sup> to 1.00 x 10 <sup>-4</sup>	1.31 x 10 <sup>-5</sup>	2.64 x 10 <sup>-6</sup> to 3.37 x 10 <sup>-4</sup>
Flavone	6.88	0.31 to 5	31	14.1 to 22.5

3457 Abbreviations: EC<sub>50</sub> = half-maximal effect concentration; EE = 17α-ethinyl estradiol;  
 3458 *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane

3459 <sup>1</sup>Formula weights used to calculate molarity were taken from MSDS sheets provided to NICEATM by the National Toxicology Program  
 3460 Substances Inventory.

3461

3462 **19.2 Comparison of Agonist Results with ICCVAM Meta Data**

3463 **Table 19-2** compares EC<sub>50</sub> values obtained during protocol standardization to the *in vitro* ER TA results  
 3464 compiled and published in the ICCVAM Guidelines (ICCVAM meta data) presented in the ICCVAM  
 3465 Guidelines (ICCVAM 2003, 2006).

3466

3467 **Table 19-2 EC<sub>50</sub> Values Obtained in BG1LUC ER TA Agonist Testing Compared to Published**  
 3468 **ICCVAM Meta Data**

Substance	EC <sub>50</sub> *	ICCVAM EC <sub>50</sub> *
Atrazine	Negative	Negative
Bisphenol A	0.38	0.40
Bisphenol B	0.21	NR
Corticosterone	Negative	Negative
<i>o,p'</i> -DDT	1.08	0.66
Diethylstilbestrol	4.69 x 10 <sup>-5</sup>	1.9 x 10 <sup>-5</sup>
EE	3.87 x 10 <sup>-6</sup>	1.1 x 10 <sup>-5</sup>

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Substance	EC <sub>50</sub> *	ICCVAM EC <sub>50</sub> *
Flavone	31.0	NR

3469 Abbreviations: EC<sub>50</sub> = half-maximal effect concentration; EE = 17 $\alpha$ -ethinyl estradiol; *o,p'*-DDT = 1,1,1-  
3470 Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane; NR = Not Reported

3471 \*Values are reported in  $\mu$ M

3472 The EC<sub>50</sub> values obtained during protocol standardization were similar to those reported in the ICCVAM  
3473 Guidelines (ICCVAM 2003, 2006), with the largest difference (one order of magnitude) between EC<sub>50</sub>  
3474 values for EE.

3475

### 3476 19.3 Antagonist Results

3477 Of the eight test substances evaluated during antagonist testing, four were positive (DBA, genistein,  
3478 flavone, and tamoxifen), and four were negative (BBP, nonylphenol, progesterone, and *o,p'*-DDT) for  
3479 agonist activity. IC<sub>50</sub> values were calculated for all positive test substances and are presented in **Table 19-**  
3480 **3**. IC<sub>50</sub> values are presented as both  $\mu$ g/mL and  $\mu$ M values. A range of concentrations (in both  $\mu$ g/mL and  
3481  $\mu$ M values) at which each substance was active is also presented.

3482

3483 **Table 19-3 IC<sub>50</sub> Values Obtained in the BG1Luc ER TA Antagonist Protocol**

Substance	Data Presented as $\mu$ g/mL		Data Presented as $\mu$ M	
	IC <sub>50</sub>	Activity Range	IC <sub>50</sub>	Activity Range
BBP	Negative	Negative	Negative	Negative
DBA	NC	0.31 to 1.25	NC	1.12 to 4.49
Genistein	NC	50	NC	185.0
Flavone	NC	12.5 to 50.0	NC	56.3 to 225.0
Nonylphenol	Negative	Negative	Negative	Negative
Progesterone	Negative	Negative	Negative	Negative
<i>o,p'</i> -DDT	Negative	Negative	Negative	Negative
Tamoxifen	0.16	0.16 to 5.00	0.43	0.42 to 13.5

3484 Abbreviations: IC<sub>50</sub> = concentration of test substance that inhibits the reference estrogen response by 50%; BBP = butylbenzyl phthalate; DBA =  
3485 dibenzo[*a,h*]anthracene; *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane; NC = Not Calculated

3486

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3487 **19.4 Comparison of Antagonist Results with ICCVAM Meta Data**

3488 The ICCVAM Guidelines (ICCVAM 2003, 2006) did not have IC<sub>50</sub> values reported for any of test  
 3489 substances evaluated during protocol standardization. **Table 19-4** lists the IC<sub>50</sub> values obtained during  
 3490 protocol standardization with the ICCVAM meta data presented in the ICCVAM Guidelines (ICCVAM  
 3491 2003, 2006).

3492 **Table 19-4 IC<sub>50</sub> Values Obtained in BG1LUC ER TA Antagonist**  
 3493 **Testing Compared to Published ICCVAM Meta Data**

Substance	IC <sub>50</sub> *	ICCVAM IC <sub>50</sub> *
BBP	Negative	NR
DBA	NC	NR
Genistein	NC	NR
Flavone	NC	NR
Nonylphenol	Negative	NR
Progesterone	Negative	Negative
<i>o,p'</i> -DDT	Negative	NR
Tamoxifen	0.43	NR

3494 Abbreviations: IC<sub>50</sub> = concentration of test substance that inhibits the reference estrogen response by 50%;  
 3495 BBP = butylbenzyl phthalate; DBA = dibenzo[*a,h*]anthracene; *o,p'*-DDT = 1,1,1-Trichloro-2-  
 3496 (*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane; NC = Not Calculated; NR = Not Reported

3497 \*Values are reported in μM

3498

3499 **20.0 The Accuracy of the BG1Luc ER TA**

3500 There is no established “gold standard” animal or human data set to serve as a reference for determining  
 3501 the accuracy of *in vitro* test methods for identifying substances with estrogen activity *in vivo*. For this  
 3502 study, ICCVAM meta data was compared with the BG1Luc ER TA protocol standardization study  
 3503 results. One difficulty in using the ICCVAM meta data compilation as a reference database is the lack of  
 3504 agreement among published studies regarding the positive or negative responses of a number of the  
 3505 substances recommended by ICCVAM for *in vitro* ER TA validation studies. This lack of agreement  
 3506 among laboratories is largely due to the diversity of test methods and the varied decision criteria  
 3507 developed by different investigators to evaluate ER TA activity. Another concern with using the list of  
 3508 ICCVAM recommended validation substances is that the classification of some substances is based on a  
 3509 single test in a single laboratory using a system that may not have been well-defined or was based on  
 3510 theory.

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3511

3512 **20.1 Evaluation of Agonist Concordance**

3513 Using the data obtained during standardization of the agonist protocol, the accuracy statistics (i.e.,  
 3514 concordance, sensitivity, specificity, positive and negative predictivity, and false negative and false  
 3515 positive rates) for the agonist protocol of the BG1Luc ER TA were calculated (see **Table 20-1**).

- 3516 • Positive in LUMI-CELL<sup>®</sup> ER and ICCVAM Positive 6 substances
- 3517 • Negative in BG1LUC ER TA and ICCVAM Positive 0 substances
- 3518 • Negative in BG1LUC ER TA and ICCVAM Negative 2 substances
- 3519 • Positive in BG1LUC ER TA and ICCVAM Negative 0 substances

3520

3521 **Table 20-1 Concordance Analysis between BG1LUC ER TA**  
 3522 **Assay Agonist Protocol and ICCVAM Agonist Meta Data.**

<b>BG1LUC ER TA Classification</b>	<b>ICCVAM Agonist Classification</b>			
		Positive	Negative	Total
	Positive	6	0	6
	Negative	0	2	2
Total	6	2	8	

3523

3524 Concordance = 100% (8/8)

3525 Sensitivity = 100% (6/6)

False Negative Rate = 0% (0/6)

3526 Specificity = 100% (2/2)

False Positive Rate = 0% (0/2)

3527 Positive Predictivity = 100 % (6/6)

Negative Predictivity = 100% (2/2)

3528

3529 The classification of substances as either positive or negative for agonism using results from the BG1Luc  
 3530 ER TA protocol standardization study are in complete agreement with the ICCVAM meta data  
 3531 classification for those substances.

3532

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3533 **20.2 Evaluation of Antagonist Concordance**

3534 Using the data obtained during the standardization of the antagonist protocol, the accuracy statistics (i.e.,  
 3535 concordance, sensitivity, specificity, positive and negative predictivity, and false negative and false  
 3536 positive rates) for the antagonist protocol of the BG1Luc ER TA were calculated (see **Table 20-2**).

- 3537 • Positive in LUMI-CELL<sup>®</sup> ER and ICCVAM Positive 4 substances
- 3538 • Negative in BG1LUC ER TA and ICCVAM Positive 2 substances<sup>5</sup>
- 3539 • Negative in BG1LUC ER TA and ICCVAM Negative 2 substances
- 3540 • Positive in BG1LUC ER TA and ICCVAM Negative 0 substances

3541

3542 **Table 20-2 Concordance Analysis between BG1Luc ER TA**3543 **Antagonist Protocol and ICCVAM Antagonist Meta-Data**

	ICCVAM Antagonist Classification			
		Positive	Negative	Total
BG1LUC ER TA Classification	Positive	4	0	4
	Negative	2	2	4
	Total	6	2	8

3544

3545 Concordance = 75% (6/8)

3546 Sensitivity = 100% (4/4)

False Negative Rate = 0% (0/4)

3547 Specificity = 50% (2/4)

False Positive Rate = 50% (2/4)

3548 Positive Predictivity = 67% (4/6)

Negative Predictivity = 100% (2/2)

3549

3550 The classification of substances as either positive or negative for antagonism using results from the  
 3551 BG1Luc ER TA protocol standardization study are in agreement with the ICCVAM meta data  
 3552 classification except for two substances classified as antagonists by the ICCVAM meta data but as  
 3553 negative in BG1Luc ER TA. However, as mentioned above, classifications of substances in the ICCVAM

<sup>5</sup> Data from which ICCVAM meta data for antagonism is derived often does not account for cytotoxicity of potential antagonist substances. The two substances (nonylphenol and *o,p'*-DDT) classified as negative for antagonism in the LUMI-CELL<sup>®</sup> ER assay but positive in the ICCVAM meta data are considered negative because they caused a decrease in cell viability to below the 80% limit.

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3554 meta data are sometimes based on a single test in a single laboratory using a system that may not have  
3555 been well-defined or were based on theory rather than experimentally obtained data. It is not known if the  
3556 two substances classified as negative by the BG1Luc ER TA but positive in the ICCVAM meta data had  
3557 previously been evaluated for cytotoxicity, which, unless specifically controlled for, could result in a  
3558 mistaken classification as antagonists in ER TA test methods.

## 3559 **21.0 Further considerations**

### 3560 **21.1 Considerations on the Need for Cell Viability Evaluations during Agonist Testing.**

3561 In range finder testing, the six substances that were positive for agonism all exhibited significant  
3562 decreases in cell viability to below the 80% limit at the highest concentration tested (100 µg/mL). This  
3563 correlated with the visual observations, which scored this concentration as having moderate to high levels  
3564 of cytotoxicity. In all cases, this coincided with a decreased response in the BG1Luc ER TA.

3565 None of the substances tested showed significant decreases in cell viability (80% limit) during  
3566 comprehensive testing. This agrees with the visual observations, which scored all concentrations tested as  
3567 having normal cell morphology.

3568

### 3569 **21.2 Considerations on the Need for Cell Viability Evaluations during Antagonist Testing.**

3570 In range finder testing, seven of the eight substances exhibited decreased ER TA activity at the highest  
3571 concentration tested (50 µg/mL). Six of these substances also exhibited significant decreases in cell  
3572 viability below the 80% limit at the same concentration. This agreed with the visual observations, which  
3573 scored this concentration as having low to high levels of cell toxicity.

3574 In comprehensive testing, seven of the eight substances exhibited decreased ER TA activity at the highest  
3575 concentrations tested. Four of these substances also exhibited significant decreases in cell viability.  
3576 Therefore, these concentrations were considered cytotoxic rather than antagonistic. Cell viability for the  
3577 remaining three substances exhibiting decreased ER TA activity did not fall below the 80% limit and  
3578 where therefore considered antagonists. There was a high degree of correlation between visual  
3579 observation scores and CellTiter-Glo® values for all substances with the exception of flavone, which did  
3580 not fall below the 80% limit, but had a visual observation score of 2 (low levels of cell toxicity) at the  
3581 highest concentration tested (50 µg/mL).

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3582 **21.3 Choosing Concentrations for Comprehensive Testing when Range Finder Exhibits**  
3583 **Biphasic Response**

3584 One substance tested during agonist range finding, flavone, exhibited a biphasic concentration-response.  
3585 Concentrations for comprehensive testing were selected from the higher concentrations showing activity  
3586 in the range finder. In comprehensive testing flavone was positive for agonism at these concentrations.  
3587 However, no evaluation of activity was conducted for those concentrations showing activity at the lowest  
3588 concentrations tested in the range finder. It is recommended that in cases where range finding indicates  
3589 activity in a biphasic manner, comprehensive testing should be conducted at concentrations that would  
3590 allow evaluation of both phases.

3591 **21.4 Considerations for Reporting Activity Levels for Substances which are Active, but for**  
3592 **which an EC<sub>50</sub> or IC<sub>50</sub> Value Cannot be Calculated.**

3593 One of the limitations of the use of the Hill equation to calculate EC<sub>50</sub> and IC<sub>50</sub> values is that it requires  
3594 that full concentration-response curves be generated for an EC<sub>50</sub> or IC<sub>50</sub> value to be obtained. Of the four  
3595 substances which tested positive for antagonism (DBA, genistein, flavone, and tamoxifen), an IC<sub>50</sub> value  
3596 could only be calculated for tamoxifen.

3597 **22.0 SUMMARY**

3598 NICEATM has conducted an agonist and antagonist protocol standardization study for the *in vitro*  
3599 BG1Luc ER TA developed by XDS. Protocol standardization procedures were based on  
3600 recommendations made in the ICCVAM Guidelines (ICCVAM 2003, 2006). Specific goals of the study  
3601 were to standardize procedures and develop two GLP-compliant protocols for using the BG1Luc ER TA  
3602 to identify ER agonists and antagonists, quantify cell viability, and develop historical databases for  
3603 reference standards and controls for these protocols.

3604 Reference standards and controls selected and standardized for the agonist assay were a 10-point dilution  
3605 of E2 as reference standard, 1% DMSO as solvent control, and 3.13 µg/mL methoxychlor as the positive  
3606 control. Reference standards and controls selected and standardized for the antagonist assay were a nine-  
3607 point dilution of raloxifene with a fixed concentration of 2.5 x 10<sup>-5</sup> µg/mL E2 as reference standard, 1%  
3608 DMSO as solvent control, 2.5 x 10<sup>-5</sup> µg/mL E2 as E2 control, and 25 µg/mL flavone with 2.5 x 10<sup>-5</sup>  
3609 µg/mL E2 as positive control.

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3610 CellTiter-Glo® (Promega Inc.) was selected and standardized for use with LUMI-CELL® ER assay  
3611 protocols. Assessment of cell viability was also conducted qualitatively using a method developed by  
3612 XDS based on visual observations of cellular morphology.

3613 The agonist historical database was established by conducting 10 independent experiments using the 10-  
3614 point E2 reference standard run in duplicate, solvent control run in quadruplicate, and the methoxychlor  
3615 positive control run in triplicate in each 96-well plate.

3616 The antagonist historical database was established by conducting 10 independent experiments using the a  
3617 nine-point Ral/E2 reference standard run in duplicate, solvent control run in triplicate, and the E2 control  
3618 and flavone control run in triplicate in each 96-well plate.

3619 The adequacy of the standardized protocols was demonstrated using eight substances covering a range of  
3620 ER agonist and antagonist activities, respectively. The substances selected for agonist testing were  
3621 atrazine, bisphenol A, bisphenol B, corticosterone, *o,p'*-DDT, diethylstilbestrol, EE, and flavone. These  
3622 substances were selected from the subset of minimum substances recommended for validation of *in vitro*  
3623 ER assays in the ICCVAM Guidelines. They were selected for their estrogen receptor agonist activity  
3624 classification, including those that are negative for agonism, and for properties that might make them  
3625 problematic, including limited solubility or potential cell viability.

3626 Results obtained for estrogenic activity for each substance tested using the standardized agonist protocol  
3627 exhibited 100% concordance with ICCVAM meta data. There was a high degree of correlation between  
3628 visual observation scores and CellTiter-Glo® values for all substances.

3629 The substances selected for antagonist testing were BBP, DBA, flavone, genistein, nonylphenol,  
3630 progesterone, *o,p'*-DDT, and tamoxifen. These substances were selected from the subset of minimum  
3631 substances recommended for validation of *in vitro* ER assays in the ICCVAM Guidelines (ICCVAM  
3632 2003, 2006). They were selected for their estrogen receptor antagonist activity classification, including  
3633 those that are negative for antagonism, and for properties that might make them problematic, including  
3634 limited solubility or potential cytotoxicity.

3635 Results obtained for anti-estrogenic activity for each substance tested using the standardized antagonist  
3636 protocol exhibited 75% concordance with ICCVAM meta data. Data from which ICCVAM meta data for  
3637 antagonism is derived often does not account for cytotoxicity of potential antagonist substances. The two  
3638 substances (nonylphenol and *o,p'*-DDT) classified as negative for antagonism in the BG1Luc ER TA but  
3639 positive in the ICCVAM meta data are considered negative because they caused a significant decrease in  
3640 cell viability. There was also a high degree of correlation between the visual observation and CellTiter-  
3641 Glo® methods of assessing cell viability for all substances tested.



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## 3704 **24.0glossary**<sup>6</sup>

3705 **Acceptance Criteria**<sup>7</sup> Minimum standards for the performance of experimental controls and  
3706 reference standards. All acceptance criteria must be met for an experiment to be considered  
3707 valid.

3708  
3709 **Accuracy**<sup>7</sup> (a) The closeness of agreement between a test method result and an accepted  
3710 reference value. (b) The proportion of correct outcomes of a test method. It is a measure of test  
3711 method performance.

3712  
3713 **Adenosine Triphosphate** A nucleotide involved in energy metabolism and required for RNA  
3714 synthesis; it occurs in all cells and is used to store energy in the form of high-energy phosphate  
3715 bonds.

3716  
3717 **Agonist** A substance that produces a response, e.g., transcription, when it binds to a specific  
3718 receptor.

3719  
3720 **Androgen** A class of steroid hormone, which includes testosterone and 5 $\alpha$ -  
3721 dihydrotestosterone, responsible for the development and maintenance of the male reproductive  
3722 system.

3723  
3724 **Androgen Receptor** The receptor to which androgens bind.

3725  
3726 **Antagonist** A substance that inhibits a response, e.g., transcription, when it binds to a  
3727 specific receptor.

3728  
3729 **Cell Density** The density of cells growing in a monolayer in a single well of a tissue culture  
3730 plate.

3731  
3732 **Cell Morphology** The shape and appearance of cells grown in a monolayer in a single well  
3733 of a tissue culture plate. Cells that are dying often exhibit abnormal cellular morphology.

3734  
3735 **Culture Medium** An aqueous solution containing vitamins, minerals and growth factors to  
3736 support the growth of cells in culture.

3737  
3738 **Coded Test Substances** Substances labeled by code rather than name so that they can be  
3739 tested and evaluated without knowledge of their identity or anticipation of test results. Coded test  
3740 substances are used to avoid intentional or unintentional bias when evaluating laboratory or test  
3741 method performance.

3742  
3743 **Coefficient of Variation** A statistical representation of the precision of a test. It is expressed  
3744 as a percentage and is calculated as follows:

---

<sup>6</sup> The definitions in this Glossary are restricted to their uses with respect to endocrine mechanisms and actions.

<sup>7</sup> Definition used by the Interagency Coordinating Committee on the Validation of Alternative Methods.

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3745 
$$\left( \frac{\textit{standard deviation}}{\textit{mean}} \right) \times 100$$

3746

3747 **Comprehensive Test** The test performed for determination of an EC- or IC<sub>50</sub> value.  
 3748 Compared to the range finder test the comprehensive test uses a smaller dilution factor for the  
 3749 concentrations tested.

3750

3751 **Concordance**<sup>7</sup> The proportion of all substances tested that are correctly classified as positive  
 3752 or negative. It is a measure of test method performance and it is often used interchangeably with  
 3753 “accuracy”.

3754

3755 **Control** Substances selected for use during the research, development, protocol  
 3756 standardization, and validation of a proposed test method having a known response. Controls are  
 3757 used to evaluate the ongoing performance of a test method. All experimental controls must fall  
 3758 within established historical norms for an experiment to pass “acceptance criteria” and be  
 3759 considered valid.

3760

3761 **Cytotoxicity** The adverse effects resulting from interference with structures and/or processes  
 3762 essential for cell survival, proliferation, and/or function. For most substances, toxicity is a  
 3763 consequence of non-specific alternations in “basal cell functions” (i.e., via mitochondria, plasma  
 3764 membrane integrity, etc.).

3765

3766

3767 **Dilution**3768 **EC<sub>50</sub>**3769 **Endocrine**3770 **Endocrine Disruptor**3771 **Fluorescence**3772 **Hill Function**3773 **Historical Database**3774 **IC<sub>50</sub>**3775 ***In Vitro***3776 **Luciferase**3777 **Luminescence**3778 **Plasmid**3779 **Precipitation**3780 **Protocol**3781 **Protocol Standardization**3782 **Q Test**

3783

3784 **Receptor** A protein or protein complex, which binds to specific molecules or the purpose of  
 3785 transporting them elsewhere in the cell, or for producing a chemical signal.

3786

3787 **Receptor Binding Assay** An assay to measure the ability of a substances to bind to a hormone  
 3788 receptor protein, which is typically performed by measuring the ability of the substances to  
 3789 displace the bound natural hormone.

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3790

3791 **Reference Substance**

3792 **Serial Dilution**

3793 **Transfection**

3794 **Transcription**

3795 **Transcriptional Activation**

3796

3797 **Validated Test Method** An accepted test method for which validation studies have been  
3798 completed to determine the accuracy and reliability of the method for a specific proposed use.

3799

3800 **Validation** The process by which the reliability and accuracy of a procedure are established  
3801 for a specific purpose.

3802 **Xenobiotic**

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3810 **Appendix A**

3811 **Experiments Conducted during the BG1LUC ER TA Protocol Standardization Effort**

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3866 **Table A-1 Experiments Conducted during Agonist Plate Design**

Experiment I.D. <sup>1</sup>	Substance Code <sup>2</sup>	Date	Plate Induction <sup>3</sup>	EC <sub>50</sub> (µg/mL) <sup>4</sup>	Rationale for Unacceptability <sup>5</sup>
Temp1	E2	09/16/05	not calculated	not calculated	Acceptable
Temp1a	E2	09/16/05	not calculated	not calculated	Acceptable
Temp2	E2	09/16/05	not calculated	not calculated	Acceptable
ICCVAM New Plate	E2	09/23/05	not calculated	not calculated	Acceptable
ICCVAM Old Plate	E2	09/23/05	12.6	not calculated	Acceptable
ICCVAM Template QC Test	E2	09/29/05	7.4	not calculated	Acceptable
Temp CM	E2	09/30/05	8.6	not calculated	Acceptable
new compound test	E2	10/06/05	6.5	not calculated	Acceptable

3867 <sup>1</sup>Experiment I.D. is the name assigned by the laboratory to each test plate (1 test plate = 1 experiment)3868 <sup>2</sup>The substance code is used by the laboratory to track the main substance being tested during the experiment (controls are not reflected in the  
3869 substance code)3870 <sup>3</sup>Plate induction was not calculated during early plate design experiments3871 <sup>4</sup>EC<sub>50</sub> values for the E2 reference standard were not calculated during the plate design phase.3872 <sup>5</sup>Acceptability of plate design experiments was determined by comparison of the shape of the E2 reference standard curve to historical laboratory  
3873 curves

3874

3875 **Table A-2 Experiments Conducted during Compilation of the Agonist Historical Database**

Experiment I.D. <sup>1</sup>	Substance Code <sup>2</sup>	Date	Plate Induction <sup>3</sup>	EC <sub>50</sub> (µg/mL)	Rationale for Unacceptability
AG 1	E2	10/12/05	8.9	2.58 x 10 <sup>-5</sup>	Acceptable
AG 2	E2	10/12/05	9.9	2.90 x 10 <sup>-5</sup>	Acceptable
AG 3	E2	10/12/05	8.2	2.80 x 10 <sup>-5</sup>	Acceptable
AG 4	E2	10/15/05	10.8	2.53 x 10 <sup>-5</sup>	Acceptable
AG 5	E2	10/15/05	11.7	2.26 x 10 <sup>-5</sup>	Acceptable
AG 6	E2	10/19/05	9.3	2.27 x 10 <sup>-5</sup>	Acceptable
AG 7	E2	10/19/05	8.0	2.09 x 10 <sup>-5</sup>	Acceptable
AG 8	E2	10/21/05	10.5	2.58 x 10 <sup>-5</sup>	Acceptable
AG 9	E2	10/21/05	7.1	2.09 x 10 <sup>-5</sup>	Acceptable
AG 10	E2	10/24/05	7.0	2.14 x 10 <sup>-5</sup>	Acceptable

3876 <sup>1</sup>Experiment I.D. is the name assigned by the laboratory to each test plate (1 test plate = 1 experiment)3877 <sup>2</sup>The substance code is used by the laboratory to track the main substance being tested during the experiment (controls are not reflected in the  
3878 substance code)3879 <sup>3</sup>Plate induction values are calculated as the averaged highest non-adjusted E2 values divided by the averaged non-adjusted DMSO values for  
3880 each experiment.

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3881 **Table A-3 Agonist Range Finder Experiments**

Experiment I.D.	Substance Code	Date	Plate Induction	EC <sub>50</sub> (µg/mL) <sup>4</sup>	Rationale for Unacceptability
N0001 - N0004	N0001 - N0004	02/14/06	3.3	not calculated	Abnormal Morphology
N0005 - N0008	N0005 - N0008	02/14/06	2.9	not calculated	Low Induction; Abnormal Morphology
N0001 - N0004	N0001 - N0004	02/27/06	2.7	not calculated	Low Induction; Abnormal Morphology
N0005 - N0008	N0005 - N0008	02/27/06	3.0	not calculated	Abnormal Morphology
N1-N4 Range	N0001 - N0004	03/28/06	3.4	6.82 x 10 <sup>-6</sup>	Acceptable
N5-N8 Range	N0005 - N0008	03/28/06	3.4	1.05 x 10 <sup>-5</sup>	Acceptable
N0008 Range trip <sup>5</sup>	N0008	05/09/06	3.4	8.57 x 10 <sup>-6</sup>	Acceptable

3882 <sup>1</sup>Experiment I.D. is the name assigned by the laboratory to each test plate (1 test plate = 1 experiment)3883 <sup>2</sup>The substance code is used by the laboratory to track the main substance being tested during the experiment (controls are not reflected in the  
3884 substance code)3885 <sup>3</sup>Plate induction values are calculated as the averaged highest non-adjusted E2 values divided by the averaged non-adjusted DMSO values for  
3886 each experiment.3887 <sup>4</sup>EC<sub>50</sub> values for the E2 reference standard were not calculated for plates that did not meet acceptance criteria3888 <sup>5</sup>A separate range finder experiment was conducted for N0008 – flavone, to determine whether the biphasic concentration-response curve  
3889 observed during initial  
3890 range finder testing was repeatable.

3891

3892 **Table A-4 Agonist Comprehensive Testing Experiments**

Experiment I.D. <sup>1</sup>	Substance Code <sup>2</sup>	Date	Plate Induction <sup>3</sup>	EC <sub>50</sub> (µg/mL)	Rationale for Unacceptability
N0002-ag1	N0002	04/01/06	9.3	3.35 x 10 <sup>-5</sup>	Acceptable
N0001-ag1	N0001	04/03/06	3.8	1.66 x 10 <sup>-5</sup>	Acceptable
N0002-ag1	N0002	04/03/06	3.8	1.52 x 10 <sup>-5</sup>	Acceptable
N0003-ag1	N0003	04/03/06	3.2	1.99 x 10 <sup>-5</sup>	Acceptable
N0004-ag1	N0004	04/03/06	3.1	1.72 x 10 <sup>-5</sup>	Acceptable
N0001 ag2	N0001	04/04/06	5.4	1.92 x 10 <sup>-5</sup>	Acceptable
N0002 ag2	N0002	04/04/06	4.0	2.26 x 10 <sup>-5</sup>	Acceptable
N0003 ag2	N0003	04/04/06	4.2	3.09 x 10 <sup>-5</sup>	Acceptable
N0004 ag2	N0004	04/04/06	4.6	1.82 x 10 <sup>-5</sup>	Acceptable
N0001 ag3	N0001	04/05/06	6.4	2.84 x 10 <sup>-5</sup>	Acceptable
N0002 ag3	N0002	04/05/06	6.7	2.74 x 10 <sup>-5</sup>	Acceptable
N0003 ag3	N0003	04/05/06	5.4	2.89 x 10 <sup>-5</sup>	Acceptable
N0004 ag3	N0004	04/08/06	3.6	1.71 x 10 <sup>-5</sup>	Acceptable
N0005 ag1	N0005	04/08/06	3.9	1.73 x 10 <sup>-5</sup>	Acceptable
N0006 ag1	N0006	04/08/06	3.9	1.84 x 10 <sup>-5</sup>	Acceptable

3893 <sup>1</sup>Experiment I.D. is the name assigned by the laboratory to each test plate (1 test plate = 1 experiment)3894 <sup>2</sup>The substance code is used by the laboratory to track the main substance being tested during the experiment (controls are not reflected in the  
3895 substance code)3896 <sup>3</sup>Plate induction values are calculated as the averaged highest non-adjusted E2 values divided by the averaged non-adjusted DMSO values for  
3897 each experiment.

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3899 **Table A-4 (Continued) Agonist Comprehensive Testing Experiments**

Experiment I.D. <sup>1</sup>	Substance Code <sup>2</sup>	Date	Plate Induction <sup>3</sup>	EC <sub>50</sub> (µg/mL)	Rationale for Unacceptability
N0007 ag1	N0007	04/08/06	4.0	1.73 x 10 <sup>-5</sup>	Acceptable
N0008 ag1	N0008	04/08/06	3.5	2.15 x 10 <sup>-5</sup>	Acceptable
N0006 ag1	N0006	04/11/06	3.9	1.03 x 10 <sup>-5</sup>	Acceptable
N0007 ag1	N0007	04/11/06	3.9	2.12 x 10 <sup>-6</sup>	Acceptable
N0008 ag1	N0008	04/11/06	3.9	2.62 x 10 <sup>-5</sup>	Acceptable
N0005 ag2	N0005	04/14/06	3.7	1.18 x 10 <sup>-5</sup>	Acceptable
N0006 ag2	N0006	04/14/06	3.0	1.28 x 10 <sup>-5</sup>	Acceptable
N0007 ag2	N0007	04/14/06	5.0	8.77 x 10 <sup>-6</sup>	Acceptable
N0005 ag3	N0005	04/17/06	3.6	1.96 x 10 <sup>-5</sup>	Acceptable
N0006 ag3	N0006	04/17/06	3.0	2.52 x 10 <sup>-5</sup>	Acceptable
N0007 ag3	N0007	04/17/06	4.5	1.96 x 10 <sup>-5</sup>	Acceptable
N0007 ag3	N0007	05/08/06	3.4	7.86 x 10 <sup>-6</sup>	Acceptable
N0008 ag3	N0008	05/11/06	3.9	8.86 x 10 <sup>-6</sup>	Acceptable
N0008 ag4	N0008	05/13/06	3.2	1.34 x 10 <sup>-5</sup>	Acceptable
N0008 ag5	N0008	05/16/06	5.0	1.17 x 10 <sup>-5</sup>	Acceptable

<sup>1</sup>Experiment I.D. is the name assigned by the laboratory to each test plate (1 test plate = 1 experiment)

<sup>2</sup>The substance code is used by the laboratory to track the main substance being tested during the experiment (controls are not reflected in the substance code)

<sup>3</sup>Plate induction values are calculated as the averaged highest non-adjusted E2 values divided by the averaged non-adjusted DMSO values for each experiment.

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**Table A-5 Experiments Conducted during Antagonist Plate Design**

Experiment I.D. <sup>1</sup>	Substance Code <sup>2</sup>	Date	Plate Reduction <sup>3</sup>	IC <sub>50</sub> (µg/mL) <sup>4</sup>	Rationale for Unacceptability <sup>5</sup>
Ral x3	Raloxifene	10/06/05	5.6	not calculated	Acceptable
Antagonist Test	Raloxifene	10/07/05	3.7	not calculated	Acceptable
Ral x3	Raloxifene	10/10/05	7.6	not calculated	Acceptable
Ral x3	Raloxifene	10/13/05	3.4	not calculated	Acceptable
AN 1	Raloxifene	10/22/05	10.2	not calculated	Acceptable
AN 2	Raloxifene	10/22/05	9.0	not calculated	Acceptable
AN 3	Raloxifene	10/22/05	6.8	not calculated	Acceptable
AN6r	Raloxifene	12/02/05	7.2	not calculated	Acceptable
AN7r	Raloxifene	12/02/05	5.8	not calculated	Acceptable
AN8r	Raloxifene	12/04/05	4.7	not calculated	Acceptable
AN9	Raloxifene	12/06/05	6.1	not calculated	Acceptable
AN10	Raloxifene	12/06/05	6.2	not calculated	Acceptable

<sup>1</sup>Experiment I.D. is the name assigned by the laboratory to each test plate (1 test plate = 1 experiment)

<sup>2</sup>The substance code is used by the laboratory to track the main substance being tested during the experiment (controls are not reflected in the substance code)

<sup>3</sup>Plate reduction was calculated as the averaged highest non-adjusted Ral/E2 values divided by the averaged non-adjusted Ral/E2 values for each experiment.

<sup>4</sup>IC<sub>50</sub> values for the ral/E2 reference standard were not calculated during the plate design phase.

<sup>5</sup>Acceptability of antagonist plate design experiments was determined by calculation of reduction, and by observation of the shape of the ral/E2 reference standard curve.

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3916 **Table A-5 (Continued) Experiments Conducted during Antagonist Plate Design**

Experiment I.D. <sup>1</sup>	Substance Code <sup>2</sup>	Date	Plate Reduction <sup>3</sup>	IC <sub>50</sub> (µg/mL) <sup>4</sup>	Rationale for Unacceptability <sup>5</sup>
AN12	Raloxifene	12/06/05	6.2	not calculated	Acceptable
AN13	Raloxifene	12/09/05	6.0	not calculated	Acceptable
AN14	Raloxifene	12/09/05	5.8	not calculated	Acceptable
New Curve Design	Raloxifene	12/09/05	6.8	not calculated	Acceptable
AN15	Raloxifene	12/12/05	5.6	not calculated	Acceptable
New Curve Design - 4 A	Raloxifene	12/14/05	8.1	not calculated	Acceptable
New Curve Design - 4 B	Raloxifene	12/14/05	9.0	not calculated	Acceptable
AN New 3 - BE - 1	Raloxifene	12/27/05	11.1	not calculated	Acceptable
AN New 3 - BE - 2	Raloxifene	12/27/05	9.5	not calculated	Acceptable
AN New 3 - BE - 4	Raloxifene	12/29/05	11.2	not calculated	Acceptable
AN New 3 - BE - 5	Raloxifene	12/29/05	10.6	not calculated	Acceptable
AN New 3 - BE - 1b	Raloxifene	12/30/05	10.6	not calculated	Acceptable
AN New 3 - BE - 2b	Raloxifene	12/30/05	11.3	not calculated	Acceptable
AN New 3 - BE - 3b	Raloxifene	12/30/05	10.3	not calculated	Acceptable
AN New 3 - BE - 4b	Raloxifene	12/30/05	9.5	not calculated	Acceptable
AN New 3 - BE - 5b	Raloxifene	12/30/05	10.3	not calculated	Acceptable
New 3-1b, Estradiol Test	Raloxifene	01/03/06	5.1	not calculated	Acceptable

3917 <sup>1</sup>Experiment I.D. is the name assigned by the laboratory to each test plate (1 test plate = 1 experiment)3918 <sup>2</sup>The substance code is used by the laboratory to track the main substance being tested during the experiment (controls are not reflected in the  
3919 substance code)3920 <sup>3</sup>Plate reduction was calculated as the averaged highest non-adjusted Ral/E2 values divided by the averaged non-adjusted Ral/E2 values for each  
3921 experiment.3922 <sup>4</sup>IC<sub>50</sub> values for the ral/E2 reference standard were not calculated during the plate design phase.3923 <sup>5</sup>Acceptability of antagonist plate design experiments was determined by calculation of reduction, and by observation of the shape of the ral/E2  
3924 reference standard  
3925 curve.

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3927 **Table A-6 Experiments Conducted during Compilation of the Antagonist Historical Database**

Experiment I.D. <sup>1</sup>	Substance Code <sup>2</sup>	Date	Plate Reduction <sup>3</sup>	IC <sub>50</sub> (µg/mL)	Rationale for Unacceptability
New 3A	Raloxifene	12/17/05	6.4	8.20 x 10 <sup>-4</sup>	Acceptable
New 3B	Raloxifene	12/17/05	5.5	1.50 x 10 <sup>-3</sup>	Acceptable
New 3C	Raloxifene	12/20/05	9.0	1.10 x 10 <sup>-3</sup>	Acceptable
New 3D	Raloxifene	12/20/05	6.1	7.20 x 10 <sup>-4</sup>	Acceptable
New 3E	Raloxifene	12/20/05	8.0	1.30 x 10 <sup>-3</sup>	Acceptable
New 3F	Raloxifene	12/23/05	8.6	9.50 x 10 <sup>-4</sup>	Acceptable
New 3G	Raloxifene	12/23/05	7.1	1.70 x 10 <sup>-3</sup>	Acceptable
New 3H	Raloxifene	12/27/05	7.2	6.50 x 10 <sup>-4</sup>	Acceptable
New 3I	Raloxifene	12/27/05	7.1	1.70 x 10 <sup>-3</sup>	Acceptable
New 3J	Raloxifene	12/27/05	6.9	1.40 x 10 <sup>-3</sup>	Acceptable

3928 <sup>1</sup>Experiment I.D. is the name assigned by the laboratory to each test plate (1 test plate = 1 experiment)3929 <sup>2</sup>The substance code is used by the laboratory to track the main substance being tested during the experiment (controls are not reflected in the  
3930 substance code)3931 <sup>3</sup>Plate reduction was calculated as the averaged highest non-adjusted Ral/E2 values divided by the averaged non-adjusted Ral/E2 values for each  
3932 experiment.

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3934 **Table A-7 Experiments Conducted during Redesign of the Ral/E2 Reference Standard**

Experiment I.D. <sup>1</sup>	Substance Code <sup>2</sup>	Date	Plate Reduction <sup>3</sup>	IC <sub>50</sub> (µg/mL)	Rationale for Unacceptability
Ral serial test	Raloxifene	04/01/06	6.9	not calculated	Acceptable
Ral 1-2A	Raloxifene	04/04/06	9.8	1.51 x 10 <sup>-3</sup>	Acceptable
Ral 1-2B	Raloxifene	04/04/06	9.1	1.49 x 10 <sup>-3</sup>	Acceptable
Ral 1-2C	Raloxifene	04/04/06	9.7	1.53 x 10 <sup>-3</sup>	Acceptable
Ral 1-2D	Raloxifene	04/04/06	9.4	1.50 x 10 <sup>-3</sup>	Acceptable

3935 <sup>1</sup>Experiment I.D. is the name assigned by the laboratory to each test plate (1 test plate = 1 experiment)3936 <sup>2</sup>The substance code is used by the laboratory to track the main substance being tested during the experiment (controls are not reflected in the  
3937 substance code)3938 <sup>3</sup>Plate reduction was calculated as the averaged highest non-adjusted Ral/E2 values divided by the averaged non-adjusted Ral/E2 values for each  
3939 experiment.

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3941 **Table A-8 Antagonist Range Finder Experiments**

Experiment I.D. <sup>1</sup>	Substance Code <sup>2</sup>	Date	Plate Reduction <sup>3</sup>	IC <sub>50</sub> (µg/mL)	Rationale for Unacceptability
N9 - N12	N0009 - N0012	04/06/06	8.2	1.33 x 10 <sup>-3</sup>	Acceptable
N13 - N16	N0013 - N0016	04/06/06	7.8	1.40 x 10 <sup>-3</sup>	Acceptable

3942 <sup>1</sup>Experiment I.D. is the name assigned by the laboratory to each test plate (1 test plate = 1 experiment)3943 <sup>2</sup>The substance code is used by the laboratory to track the main substance being tested during the experiment (controls are not reflected in the  
3944 substance code)3945 <sup>3</sup>Plate reduction was calculated as the averaged highest non-adjusted Ral/E2 values divided by the averaged non-adjusted Ral/E2 values for each  
3946 experiment.

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3948 **Table A-9 Antagonist Comprehensive Testing Experiments**

Experiment I.D. <sup>1</sup>	Substance Code <sup>2</sup>	Date	Plate Reduction <sup>3</sup>	IC <sub>50</sub> (µg/mL)	Rationale for Unacceptability
N0009 ant1	N0009	04/12/06	4.4	9.55 x 10 <sup>-4</sup>	Acceptable
N0010 ant1	N0010	04/12/06	5.1	7.93 x 10 <sup>-4</sup>	Acceptable
N0011 ant1	N0011	04/12/06	5.4	7.96 x 10 <sup>-4</sup>	Acceptable
N0012 ant1	N0012	04/12/06	5.7	9.07 x 10 <sup>-4</sup>	Acceptable
N0009 ant2	N0009	04/15/06	6.4	1.18 x 10 <sup>-3</sup>	Acceptable
N0010 ant2	N0010	04/15/06	6.2	1.01 x 10 <sup>-3</sup>	Acceptable
N0011 ant2	N0010	04/15/06	6.3	1.10 x 10 <sup>-3</sup>	Acceptable
N0012 ant2	N0012	04/15/06	5.8	1.12 x 10 <sup>-3</sup>	Acceptable
N0013 ant1	N0013	04/15/06	5.3	1.27 x 10 <sup>-3</sup>	Acceptable
N0014 ant1	N0014	04/15/06	5.3	1.43 x 10 <sup>-3</sup>	Acceptable
N0009ant3	N0009	04/18/06	5.4	9.74 x 10 <sup>-4</sup>	Acceptable
N0010 ant3	N0010	04/18/06	4.8	1.21 x 10 <sup>-3</sup>	Acceptable
N0011 ant3	N0011	04/18/06	6.1	8.73 x 10 <sup>-4</sup>	Acceptable
N0012 ant3	N0012	04/18/06	4.5	1.10 x 10 <sup>-3</sup>	Acceptable
N0013 ant2	N0013	04/20/06	6.6	1.29 x 10 <sup>-3</sup>	Acceptable
N0014 ant2	N0014	04/20/06	6.5	1.18 x 10 <sup>-3</sup>	Acceptable
N0015 ant1	N0015	04/20/06	6.2	1.15 x 10 <sup>-3</sup>	Acceptable
N0016 ant1	N0016	04/20/06	6.4	1.29 x 10 <sup>-3</sup>	Acceptable
N0013 ant3	N0013	05/01/06	6.3	1.12 x 10 <sup>-3</sup>	Acceptable
N0015 ant2	N0015	05/01/06	5.8	1.07 x 10 <sup>-3</sup>	Acceptable
N0016 ant2	N0016	05/01/06	6.1	1.65 x 10 <sup>-3</sup>	Acceptable
N0014 ant3	N0014	05/05/06	5.7	9.73 x 10 <sup>-4</sup>	Acceptable
N0015 ant3	N0015	05/05/06	5.6	1.14 x 10 <sup>-3</sup>	Acceptable
N0016 ant3	N0016	05/05/06	5.7	1.13 x 10 <sup>-3</sup>	Acceptable
N0016 ant4	N0016	05/09/06	5.1	1.09 x 10 <sup>-3</sup>	Acceptable
N0016 ant5	N0016	05/11/06	10.8	1.30 x 10 <sup>-3</sup>	Acceptable

3949 <sup>1</sup>Experiment I.D. is the name assigned by the laboratory to each test plate (1 test plate = 1 experiment)3950 <sup>2</sup>The substance code is used by the laboratory to track the main substance being tested during the experiment (controls are not reflected in the  
3951 substance code)3952 <sup>3</sup>Plate reduction was calculated as the averaged highest non-adjusted Ral/E2 values divided by the averaged non-adjusted Ral/E2 values for each  
3953 experiment.

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3954 **Table A-10 CellTiter-Blue<sup>™</sup> Experiments Conducted during Protocol Standardization**

Experiment I.D. <sup>1</sup>	Substance Code <sup>2</sup>	Date	Rationale for Unacceptability
Via Test 5 Blue	Raloxifene	12-8-05	No color developed
Via Test 7 Blue	Raloxifene	12-14-05	No color developed

3955 <sup>1</sup>Experiment I.D. is the name assigned by the laboratory to each test plate (1 test plate = 1 experiment)3956 <sup>2</sup>The substance code is used by the laboratory to track the main substance being tested during the experiment  
3957 (controls are not reflected in the substance code)

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3959 **Table A-11 CellTiter-Glo<sup>®</sup> Experiments Conducted during Protocol Standardization**

Experiment I.D. <sup>1</sup>	Substance Code <sup>2</sup>	Date	Rationale for Unacceptability
Via b	Raloxifene and E2	10/28/05	Acceptable
Via Test 2	Raloxifene and E2	12/04/05	Acceptable
Via Test 3	E2	12/08/05	Acceptable
Via Test 4 Glo	Raloxifene and E2	12/08/05	Acceptable
Via Test 6 Glo	Raloxifene and E2	12/14/05	Acceptable
Ag Via 1	E2	12/28/05	Acceptable
Ag Via 2	E2	12/28/05	Acceptable
AG Via 3	E2	01/03/06	Acceptable
AG Via 4	E2	01/03/06	Acceptable
Flav VIABILITY	Flavone	01/31/06	Acceptable
N1 - N4 Via	N1 - N4	02/14/06	Acceptable
N5 - N8 Via	N5 - N8	02/14/06	Acceptable
N0001 - N0004 Via b	N1 - N4	02/27/06	Acceptable
N0005 - N0008 Via b	N5 - N8	02/27/06	Acceptable
Agonist N0001 - N0004 costar plate Via	N1 - N4	03/06/06	Acceptable
Agonist N0001 - N0004 new plate Via	N1 - N4	03/06/06	Acceptable
Agonist N0005 - N0008 Costar sealed plate via	N5 - N8	03/06/06	Acceptable
Agonist N0001 - N0004 Viability	N1 - N4	03/08/06	Acceptable
Agonist N0005 - N0008 Viability	N5 - N8	03/08/06	Acceptable

3960 <sup>1</sup>Experiment I.D. is the name assigned by the laboratory to each test plate (1 test plate = 1 experiment)3961 <sup>2</sup>The substance code is used by the laboratory to track the main substance being tested during the experiment (controls are not reflected in the  
3962 substance code)

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3963 **Table A-11 (Continued) CellTiter-Glo<sup>®</sup> Experiments Conducted during Protocol Standardization**

Experiment I.D. <sup>1</sup>	Substance Code <sup>2</sup>	Date	Rationale for Unacceptability
Agonist N0001 - N0004 Viability	N1 - N4	03/09/06	Acceptable
bg1, 11-12-03, G418 QC via	G418	03/12/06	Acceptable
bg1, 11-12-03, G418 treated cell via	G418	03/12/06	Acceptable
111203 agonist, antagonist corning flasks via	E2	03/12/06	Acceptable
111203 agonist, antagonist falcon flasks via	E2	03/12/06	Acceptable
bg1-DB agonist, antagonist, via QC	E2	03/12/06	Acceptable
N0001 Via	N0001	03/18/06	Acceptable
N0002 Via	N0002	03/18/06	Acceptable
bg1 3801, Denisons DMEM&FBS(A19)	E2	03/19/06	Acceptable
bg1 3801, Denisons DMEM&FBS(A20)	E2	03/19/06	Acceptable
N0001 Via	N0001	03/20/06	Acceptable
N0002 Via	N0002	03/20/06	Acceptable
N0002-ag1 via	N0002	04/01/06	Acceptable
N0001-ag1 via	N0001	04/03/06	Acceptable
N0002-ag1 via	N0002	04/03/06	Acceptable
N0003-ag1 via	N0003	04/03/06	Acceptable
N0004-ag1 via	N0004	04/03/06	Acceptable
N0001 ag2 via	N0001	04/04/06	Acceptable
N0002 ag2 via	N0002	04/04/06	Acceptable
N0003 ag2 via	N0003	04/04/06	Acceptable
N0004 ag2 via	N0004	04/04/06	Acceptable
N0001 ag3 via	N0001	04/05/06	Acceptable
N0002 ag3 via	N0002	04/05/06	Acceptable
N0003 ag3 via	N0003	04/05/06	Acceptable
N0009 - N00012 via	N0009 - N00012	04/06/06	Acceptable
N0013 - N0016 via	N0013 - N0016	04/06/06	Acceptable

3964 <sup>1</sup>Experiment I.D. is the name assigned by the laboratory to each test plate (1 test plate = 1 experiment)3965 <sup>2</sup>The substance code is used by the laboratory to track the main substance being tested during the experiment (controls are not reflected in the  
3966 substance code)

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3967 **Table A-11 (Continued) CellTiter-Glo<sup>®</sup> Experiments Conducted during Protocol Standardization**

Experiment I.D. <sup>1</sup>	Substance Code <sup>2</sup>	Date	Rationale for Unacceptability
N0004 ag3 via	N0004	04/08/06	Acceptable
N0005 ag1 via	N0005	04/08/06	Acceptable
N0006 ag1 via	N0006	04/08/06	Acceptable
N0007 ag1 via	N0007	04/08/06	Acceptable
N0008 ag1 via	N0008	04/08/06	Acceptable
N0006 ag1 via	N0006	04/11/06	Acceptable
N0007 ag1 via	N0007	04/11/06	Acceptable
N0008 ag1 via	N0008	04/11/06	Acceptable
N0005 ag2 via	N0005	04/14/06	Acceptable
N0006 ag2 via	N0006	04/14/06	Acceptable
N0007 ag2 via	N0007	04/14/06	Acceptable
N0005 ag3 via	N0005	04/17/06	Acceptable
N0006 ag3 via	N0006	04/17/06	Acceptable
N0007 ag3 via	N0007	04/17/06	Acceptable
N0009 ant1 via	N0009	04/12/06	Acceptable
N0010 ant1 via	N0010	04/12/06	Acceptable
N0011 ant1 via	N0011	04/12/06	Acceptable
N0012 ant1 via	N0012	04/12/06	Acceptable
N0009 ant2 via	N0009	04/15/06	Acceptable
N0010 ant2 via	N0010	04/15/06	Acceptable
N0011 ant2 via	N0011	04/15/06	Acceptable
N0012 ant2 via	N0012	04/15/06	Acceptable
N0013 ant1 via	N0013	04/15/06	Acceptable
N0014 ant1 via	N0014	04/15/06	Acceptable
N0009 ant3 via	N0009	04/18/06	Acceptable
N0010 ant3 via	N0010	04/18/06	Acceptable
N0011 ant3 via	N0011	04/18/06	Acceptable
N0012 ant3 via	N0012	04/18/06	Acceptable
N0013 ant2 via	N0013	04/20/06	Acceptable
N0014 ant2 via	N0014	04/20/06	Acceptable
N0015 ant1 via	N0015	04/20/06	Acceptable
N0016 ant1 via	N0016	04/20/06	Acceptable
N0013 ant3 via	N0013	05/01/06	Acceptable
N0015 ant2 via	N0015	05/01/06	Acceptable
N0014 ant3 via	N0014	05/05/06	Acceptable
N0015 ant3 via	N0015	05/05/06	Acceptable

<sup>1</sup>Experiment I.D. is the name assigned by the laboratory to each test plate (1 test plate = 1 experiment)<sup>2</sup>The substance code is used by the laboratory to track the main substance being tested during the experiment (controls are not reflected in the substance code)

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3971 **Table A-11 (Continued) CellTiter-Glo<sup>®</sup> Experiments Conducted during Protocol Standardization**

Experiment I.D. <sup>1</sup>	Substance Code <sup>2</sup>	Date	Rationale for Unacceptability
N0016 ant2 via	N0016	05/01/06	Acceptable
N0016 ant3 via	N0016	05/05/06	Acceptable
N0007 ag3 via	N0007	05/08/06	Acceptable
N0016 ant4 via	N0016	05/09/06	Acceptable
N0008 Range Trip via	N0008	05/09/06	Acceptable
N0016 ant5 via	N0016	05/11/06	Acceptable
N0008 ag3 via	N0008	05/11/06	Acceptable
N0008 ag4 via	N0008	05/13/06	Acceptable
N0008 ag5 via	N0008	05/16/06	Acceptable

3972 <sup>1</sup>Experiment I.D. is the name assigned by the laboratory to each test plate (1 test plate = 1 experiment)3973 <sup>2</sup>The substance code is used by the laboratory to track the main substance being tested during the experiment (controls are not reflected in the  
3974 substance code)

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3976 **Table A-12 Experiments Conducted to Evaluate Abnormal Responses and/or Decreased**  
3977 **Viability**

Quality Testing of Materials							
Experiment I.D. <sup>1</sup>	Substance Code <sup>2</sup>	Date	Plate Induction <sup>3</sup>	EC <sub>50</sub> (µg/mL) <sup>4</sup>	Plate Reduction <sup>5</sup>	IC <sub>50</sub> (µg/mL) <sup>6</sup>	Rationale for Unacceptability
BE Test Mick BE, John BE	E2	01/05/06	2.6	NC	N/A	N/A	Low Induction; Abnormal Morphology
Time Course DMSO	Ral	01/21/06	N/A	N/A	6.1	NC	Abnormal Morphology
Time Course EtOH	Ral	01/21/06	N/A	N/A	4.3	NC	Abnormal Morphology
Time Course Mix 1 and 2	Ral	01/21/06	N/A	N/A	11.1	NC	Abnormal Morphology

3978 Abbreviations: N/A = Not Applicable; NC = Not Calculated; Ral = Raloxifene

3979 <sup>1</sup>Experiment I.D. is the name assigned by the laboratory to each test plate (1 test plate = 1 experiment)3980 <sup>2</sup>The substance code is used by the laboratory to track the main substance being tested during the experiment (controls are not reflected in the  
3981 substance code)3982 <sup>3</sup>Plate induction values are calculated as the averaged highest non-adjusted E2 values divided by the averaged non-adjusted DMSO values for  
3983 each experiment.3984 <sup>4</sup>EC<sub>50</sub> values for the E2 reference standard were not calculated for plates that did not meet acceptance criteria3985 <sup>5</sup>Plate reduction was calculated as the averaged highest non-adjusted ral/E2 values divided by the averaged non-adjusted ral/E2 values for each  
3986 experiment.3987 <sup>6</sup>IC<sub>50</sub> values for the ral/E2 reference standard were not calculated for plates that did not meet acceptance criteria

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DO NOT CITE, QUOTE, OR DISTRIBUTE

3988 **Table A-12 (Continued) Experiments Conducted to Evaluate Abnormal Responses and/or**  
 3989 **Decreased Viability**

Quality Testing of Materials							
Experiment I.D. <sup>1</sup>	Substance Code <sup>2</sup>	Date	Plate Induction <sup>3</sup>	EC <sub>50</sub> (µg/mL) <sup>4</sup>	Plate Reduction <sup>5</sup>	IC <sub>50</sub> (µg/mL) <sup>6</sup>	Rationale for Unacceptability
DMSO Test	E2	01/31/06	2.9	NC	N/A	N/A	Low Induction; Abnormal Morphology
Rinsed vs non Rinsed	E2	01/31/06	2.0	NC	N/A	N/A	Low Induction; Abnormal Morphology
Old Flasks	E2	02/03/06	4.9	NC	N/A	N/A	Abnormal Morphology
berthold vs lucy1	E2	02/16/06	3.3	NC	N/A	N/A	Abnormal Morphology
NOB-F - Testing Stripped FBS and DMSO	E2	02/22/06	4.7	NC	N/A	N/A	Abnormal Morphology
NOB-R - Testing Stripped FBS and DMSO	E2	02/22/06	6.4	NC	N/A	N/A	Abnormal Morphology
OB-F - Testing Stripped FBS and DMSO	E2	02/22/06	4.1	NC	N/A	N/A	Abnormal Morphology

3990 Abbreviations: N/A = Not Applicable; NC = Not Calculated; Ral = Raloxifene

3991 <sup>1</sup>Experiment I.D. is the name assigned by the laboratory to each test plate (1 test plate = 1 experiment)

3992 <sup>2</sup>The substance code is used by the laboratory to track the main substance being tested during the experiment (controls are not reflected in the  
 3993 substance code)

3994 <sup>3</sup>Plate induction values are calculated as the averaged highest non-adjusted E2 values divided by the averaged non-adjusted DMSO values for  
 3995 each experiment.

3996 <sup>4</sup>EC<sub>50</sub> values for the E2 reference standard were not calculated for plates that did not meet acceptance criteria

3997 <sup>5</sup>Plate reduction was calculated as the averaged highest non-adjusted ral/E2 values divided by the averaged non-adjusted ral/E2 values for each  
 3998 experiment.

3999 <sup>6</sup>IC<sub>50</sub> values for the ral/E2 reference standard were not calculated for plates that did not meet acceptance criteria

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4000 **Table A-12 (Continued) Experiments Conducted to Evaluate Abnormal Responses and/or**  
 4001 **Decreased Viability**

Quality Testing of Materials							
Experiment I.D. <sup>1</sup>	Substance Code <sup>2</sup>	Date	Plate Induction <sup>3</sup>	EC <sub>50</sub> (µg/mL) <sup>4</sup>	Plate Reduction <sup>5</sup>	IC <sub>50</sub> (µg/mL) <sup>6</sup>	Rationale for Unacceptability
OB-R - Testing Stripped FBS and DMSO	E2	02/22/06	4.7	NC	N/A	N/A	Abnormal Morphology
no G418, RPMI, lot	E2	02/23/06	4.2	NC	N/A	N/A	Abnormal Morphology
NO G418 vs G418, DMEM	E2	02/25/06	4.3	NC	N/A	N/A	Abnormal Morphology
bg1, 110ul G418 RPMI, DMEM new vs bad	E2	03/01/06	3.0	NC	N/A	N/A	Abnormal Morphology
bg1, 220ul G418 RPMI, DMEM new vs bad	E2	03/01/06	3.4	NC	N/A	N/A	Abnormal Morphology
bg1, new plate, rinsed vs non-rinsed tubes	E2	03/01/06	6.5	NC	N/A	N/A	Abnormal Morphology

4002 Abbreviations: N/A = Not Applicable; NC = Not Calculated; Ral = Raloxifene

4003 <sup>1</sup>Experiment I.D. is the name assigned by the laboratory to each test plate (1 test plate = 1 experiment)

4004 <sup>2</sup>The substance code is used by the laboratory to track the main substance being tested during the experiment (controls are not reflected in the  
 4005 substance code)

4006 <sup>3</sup>Plate induction values are calculated as the averaged highest non-adjusted E2 values divided by the averaged non-adjusted DMSO values for  
 4007 each experiment.

4008 <sup>4</sup>EC<sub>50</sub> values for the E2 reference standard were not calculated for plates that did not meet acceptance criteria

4009 <sup>5</sup>Plate reduction was calculated as the averaged highest non-adjusted ral/E2 values divided by the averaged non-adjusted ral/E2 values for each  
 4010 experiment.

4011 <sup>6</sup>IC<sub>50</sub> values for the ral/E2 reference standard were not calculated for plates that did not meet acceptance criteria

4012

DO NOT CITE, QUOTE, OR DISTRIBUTE

4012 **Table A-12 (Continued) Experiments Conducted to Evaluate Abnormal Responses and/or**  
 4013 **Decreased Viability**

Quality Testing of Materials							
Experiment I.D. <sup>1</sup>	Substance Code <sup>2</sup>	Date	Plate Induction <sup>3</sup>	EC <sub>50</sub> (µg/mL) <sup>4</sup>	Plate Reduction <sup>5</sup>	IC <sub>50</sub> (µg/mL) <sup>6</sup>	Rationale for Unacceptability
Agonist N0005 - N0008 derws cells new plate	N0005 - N0008	03/05/06	4.4	NC	N/A	N/A	Abnormal Morphology
Agonist N0001 - N0004 Costar plate	N0001 - N0004	03/05/06	3.4	NC	N/A	N/A	Abnormal Morphology
Agonist N0001 - N0004 new plate	N0001 - N0004	03/06/06	1.3	NC	N/A	N/A	Low Induction; Abnormal Morphology
bg1, agonist, antagonist QC	E2	03/07/06	4.9	NC	10.9	N/A	Abnormal Morphology
Agonist N0001 - N0004	N0001 - N0004	03/08/06	1.9	NC	N/A	N/A	Low Induction; Abnormal Morphology
Agonist N0005 - N0008	N0005 - N0008	03/08/06	1.9	NC	N/A	N/A	Low Induction; Abnormal Morphology
Agonist N0001 - N0004	N0001 - N0004	03/09/06	1.4	NC	N/A	N/A	Low Induction; Abnormal Morphology
Beta tests	E2	03/09/06	1.4	NC	N/A	N/A	Low Induction; Abnormal Morphology

4014 Abbreviations: N/A = Not Applicable; NC = Not Calculated; Ral = Raloxifene

4015 <sup>1</sup>Experiment I.D. is the name assigned by the laboratory to each test plate (1 test plate = 1 experiment)

4016 <sup>2</sup>The substance code is used by the laboratory to track the main substance being tested during the experiment (controls are not reflected in the  
 4017 substance code)

4018 <sup>3</sup>Plate induction values are calculated as the averaged highest non-adjusted E2 values divided by the averaged non-adjusted DMSO values for  
 4019 each experiment.

4020 <sup>4</sup>EC<sub>50</sub> values for the E2 reference standard were not calculated for plates that did not meet acceptance criteria

4021 <sup>5</sup>Plate reduction was calculated as the averaged highest non-adjusted ral/E2 values divided by the averaged non-adjusted ral/E2 values for each  
 4022 experiment.

4023 <sup>6</sup>IC<sub>50</sub> values for the ral/E2 reference standard were not calculated for plates that did not meet acceptance criteria

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4024 **Table A-12 (Continued) Experiments Conducted to Evaluate Abnormal Responses and/or**  
 4025 **Decreased Viability**

Quality Testing of Materials							
Experiment I.D. <sup>1</sup>	Substance Code <sup>2</sup>	Date	Plate Induction <sup>3</sup>	EC <sub>50</sub> (µg/mL) <sup>4</sup>	Plate Reduction <sup>5</sup>	IC <sub>50</sub> (µg/mL) <sup>6</sup>	Rationale for Unacceptability
bg1, 11-12-03, Media QC, G418 treated cells	E2	03/12/06	1.9	NC	N/A	N/A	Low Induction; Abnormal Morphology
bg1, 11-12-03, Media QC, Non-G418 treated cells	E2	03/12/06	1.7	NC	N/A	N/A	Low Induction; Abnormal Morphology
bg1,111203 agonist,antagonist coming flasks	E2	03/12/06	1.8	NC	N/A	N/A	Low Induction; Abnormal Morphology
bg1,111203 agonist,antagonist falcon flasks	E2	03/12/06	1.8	NC	N/A	N/A	Low Induction; Abnormal Morphology
bg1-DB agonist,antagonist, via QC	E2	03/12/06	1.2	NC	N/A	N/A	Low Induction; Abnormal Morphology
N0001 Trip	E2	03/18/06	1.6	NC	N/A	N/A	Abnormal Morphology
N0002 Trip	E2	03/18/06	1.5	NC	N/A	N/A	Abnormal Morphology
bg13801,Denisons background experiment 24hrs	E2	03/21/06	2.5	NC	N/A	N/A	Low Induction; Abnormal Morphology
bg13801,Denisons background experiment 48hrs	E2	03/21/06	2.0	NC	N/A	N/A	Low Induction; Abnormal Morphology

4026 Abbreviations: N/A = Not Applicable; NC = Not Calculated; Ral = Raloxifene

4027 <sup>1</sup>Experiment I.D. is the name assigned by the laboratory to each test plate (1 test plate = 1 experiment)

4028 <sup>2</sup>The substance code is used by the laboratory to track the main substance being tested during the experiment (controls are not reflected in the  
 4029 substance code)

4030 <sup>3</sup>Plate induction values are calculated as the averaged highest non-adjusted E2 values divided by the averaged non-adjusted DMSO values for  
 4031 each experiment.

4032 <sup>4</sup>EC<sub>50</sub> values for the E2 reference standard were not calculated for plates that did not meet acceptance criteria

4033 <sup>5</sup>Plate reduction was calculated as the averaged highest non-adjusted ral/E2 values divided by the averaged non-adjusted ral/E2 values for each  
 4034 experiment.

4035 <sup>6</sup>IC<sub>50</sub> values for the ral/E2 reference standard were not calculated for plates that did not meet acceptance criteria

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DO NOT CITE, QUOTE, OR DISTRIBUTE

4036 **Table A-12 (Continued) Experiments Conducted to Evaluate Abnormal Responses and/or**  
 4037 **Decreased Viability**

Quality Testing of Materials							
Experiment I.D. <sup>1</sup>	Substance Code <sup>2</sup>	Date	Plate Induction <sup>3</sup>	EC <sub>50</sub> (µg/mL) <sup>4</sup>	Plate Reduction <sup>5</sup>	IC <sub>50</sub> (µg/mL) <sup>6</sup>	Rationale for Unacceptability
bg1-3801 f-cf-c, cell supply QC	E2	03/24/06	6.2	NC	N/A	N/A	Acceptable
BG-1 3-8-01	E2	03/24/06	6.2	3.02 x 10 <sup>-5</sup>	N/A	N/A	Acceptable
DB 2-7-06	E2	03/26/06	4.5	1.38 x 10 <sup>-5</sup>	N/A	N/A	Acceptable
bg1DB3706 F25 2 days,luc and via QC	E2	03/26/06	4.5	NC	N/A	N/A	Acceptable
Test No Falcon	E2	03/27/06	7.5	1.57 x 10 <sup>-5</sup>	N/A	N/A	Acceptable
bgMERE	E2	03/29/06	4.4	1.40 x 10 <sup>-5</sup>	N/A	N/A	Acceptable
bg1DB3706 RPMI no phenol red luc and via QC	E2	03/29/06	2.9	NC	N/A	N/A	Low Induction; Abnormal Morphology
bgMERE 11203 luc and via QC	E2	03/29/06	7.0	NC	N/A	N/A	Acceptable
bg1 N0008 RF R&D	E2	04/08/06	1.7	NC	N/A	N/A	Low Induction; Abnormal Morphology
N0005 ag-2 fails induction	E2	04/08/06	1.8	NC	N/A	N/A	Low Induction; Abnormal Morphology
DMSO Test, 3 point BE	E2	04/14/06	3.7	NC	N/A	N/A	Abnormal Morphology

4038 Abbreviations: N/A = Not Applicable; NC = Not Calculated; Ral = Raloxifene

4039 <sup>1</sup>Experiment I.D. is the name assigned by the laboratory to each test plate (1 test plate = 1 experiment)

4040 <sup>2</sup>The substance code is used by the laboratory to track the main substance being tested during the experiment (controls are not reflected in the substance code)

4042 <sup>3</sup>Plate induction values are calculated as the averaged highest non-adjusted E2 values divided by the averaged non-adjusted DMSO values for each experiment.

4044 <sup>4</sup>EC<sub>50</sub> values for the E2 reference standard were not calculated for plates that did not meet acceptance criteria

4045 <sup>5</sup>Plate reduction was calculated as the averaged highest non-adjusted ral/E2 values divided by the averaged non-adjusted ral/E2 values for each experiment.

4047 <sup>6</sup>IC<sub>50</sub> values for the ral/E2 reference standard were not calculated for plates that did not meet acceptance criteria