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)

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List of Acronyms and Abbreviations

AR	Androgen receptor
ATP	Adenosine triphosphate
BBP	Butylbenzyl phthalate
BRD	Background review document
CASRN	Chemical Abstracts Service Registry Number
CV	Coefficient of variation
DBA	Dibenzo[*a*,*h*]anthracene
DMEM	Dulbecco's modification of Eagle's medium
DMSO	Dimethyl Sulfoxide
DMSO Control	1% v/v DMSO in tissue culture medium
E2	17β-Estradiol
E2 control	2.5×10^{-5} μg/mL 17β-estradiol control used in the BG1Luc ER TA antagonist assay
E2 reference Standard	10-point serial dilution of 17β-Estradiol Reference Standard for the BG1Luc ER TA agonist assay
EC_{50}	Half–maximal effective concentration
EE	17α-ethinyl estradiol
EDSP	Endocrine Disruptor Screening Program
EDWG	Endocrine Disruptor Working Group
EFM	Dulbecco's modification of Eagle's medium (DMEM) containing 4.5 g/L glucose, with sodium pyruvate, without phenol, containing 1% penicillin/streptomycin, 2% L-glutamine, and 5% charcoal/dextran treated FBS
EPA	U.S. Environmental Protection Agency
ER	Estrogen receptor
EtOH	Ethanol
Flavone control	25 μg/mL flavone with 2.5×10^{-5} μg/mL 17β-estradiol; used as the positive control in the BG1Luc ER TA antagonist assay
FBS	Fetal bovine serum (charcoal/dextran treated)
FR	Federal Register
G418	Gentamycin
GLP	Good Laboratory Practices (OECD 1998)
IC_{50}	Concentration of the test substance that inhibits the reference estrogen response by 50%
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
MEM	Minimum essential medium
Methoxychlor	*p,p'*-Methoxychlor

Methoxychlor control	3.13 μg/mL methoxychlor positive control for the BG1Luc ER TA agonist assay
MMTV	Mouse mammary tumor virus
NICEATM	National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods
Nonylphenol	*p*-n-Nonylphenol
NTP	National Toxicology Program
NTPSI	National Toxicology Program Substances Inventory
OECD	Organisation for Economic Co-operation and Development
o,p'-DDT	1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane
QC	Quality control
Raloxifene	Raloxifene HCl
Ral/E2 reference standard	Nine-point serial dilution of raloxifene with a fixed concentration of 2.5×10^{-5} μg/mL 17β-estradiol reference standard for the BG1Luc ER TA antagonist assay
RLU	Relative light units
Std Dev	Standard deviation
SOP	Standard operating procedure
TA	Transcriptional activation
XDS	Xenobiotic Detection Systems

Preface

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

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

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

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

ICCVAM considered the Panel's conclusions, recommendations, and public comments received in response to the *FR* notice. ICCVAM then developed test method recommendations that included minimum procedural standards and a list of 78 reference substances that should be used to standardize and validate *in vitro* ER and AR binding and TA test methods. In June 2003, ICCVAM issued an *FR* notice (NIEHS 2003) announcing the availability of a report defining these recommendations and minimum procedural standards entitled, "ICCVAM Evaluation of *In Vitro* Test Methods for Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays," (ICCVAM 2003), as well as the final BRDs (ICCVAM 2002d, 2002b, 2002a, 2002c). The *FR* notice also requested the nomination of in *vitro test* methods for use in the EDSP as part of the Tier I screening battery of *in vitro* and *in vivo* test assays that will be used to reach weight-of-evidence decisions on whether to conduct large multi-generational Tier 2 *in vivo* studies.

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

disruptor test methods (ICCVAM 2003). Based on the NICEATM pre-screen evaluation, ICCVAM recommended that:

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

NICEATM exercised a pre-validation coordination option in its support contract to conduct and manage a study to standardize BG1Luc ER TA protocols and to conduct additional antagonist testing. The study was initiated in October 2005 and was conducted at XDS.

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

Executive Summary

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

Selection and Standardization of Reference Standards and Controls
Reference standards and controls selected and standardized for the agonist assay were:

- A 10-point serial dilution of 17β-estradiol (E2) as the reference standard
- A 1% volume/volume (v/v) solution of dimethyl sulfoxide (DMSO) as the solvent control
- 3.13 μg/mL methoxychlor as a weak acting positive control.

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

- A nine-point serial dilution of raloxifene with a fixed concentration of 2.5×10^{-5} μg/mL E2 as the reference standard
- A 1% volume/volume (v/v) solution of DMSO as the solvent control
- 2.5×10^{-5} μg/mL E2 as the E2 control
- 25 μg/mL flavone with 2.5×10^{-5} μg/mL E2 as a weak acting positive control.

Historical data for the reference standards and controls were generated from 10 independent experiments. These data were used to establish quality control measures for subsequent experiments.

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

Testing of Coded Substances in Agonist and Antagonist Protocols

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

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

Problems Encountered During Testing of Coded Substances

Technical errors were made when making serial dilutions in individual experiments for atrazine, corticosterone, diethylstilbestrol, and 17α-ethinyl estradiol, resulting in the exclusion of certain data points from single replicates of these individual experiments. Early in the study, cells that were being cultured for use in the assay did not perform to previously established historical norms, or exhibited decreased viability. A series of qualifying experiments indicated that the likely cause of these cell culture problems was a combination of factors including contaminated lots of gentamicin, L-glutamine, fetal bovine serum, and tissue culture flasks. Based on this information, protocols were specifically modified to test the performance of these components before use in cell culture.

Concordance of Testing Results with ICCVAM Published Data

For each reference substance, there was agreement among the replicate experiments in terms of the classification of the substance as being positive or negative in the agonist or antagonist assays. Estrogenic activity for substances tested using the standardized agonist protocol exhibited 100% concordance with ICCVAM published data (ICCVAM 2003, 2006), classifying six substances (bisphenol A, bisphenol B, *o,p'*-DDT, diethylstilbestrol, 17α-ethinyl estradiol, and flavone) as ER agonists and two (atrazine and corticosterone) as negative. The relative activities of the ER agonists, based on their calculated EC_{50} concentrations, were in agreement with ICCVAM reported median activities. In terms of estrogenic antagonist activity, there was 75% concordance with ICCVAM published data. The classification of four substances (dibenzo[*a,h*]anthracene, flavone, genistein, and tamoxifen) as ER antagonists and two (butylbenzyl phthalate and progesterone) as negative for ER antagonism agreed with the ICCVAM published data. Two substances (*p,*n-nonylphenol and *o,p'*-DDT) classified as ER antagonists in the

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[1] EC_{50} = half–maximal effective concentration

[2] IC_{50} = Concentration of the test substance inhibiting the reference estrogen response by 50%

ICCVAM published data were classified as negative in the BG1Luc ER TA protocol standardization study. Although these substances caused a significant decrease in ER TA activity, they also caused a significant decrease in cell viability over the same concentration range. Thus, these two substances were classified as cytotoxic rather than as estrogenic antagonists. There was also a high degree of correlation between the visual observation and CellTiter-Glo® methods of assessing cell viability for all substances tested.

1.0 Introduction

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

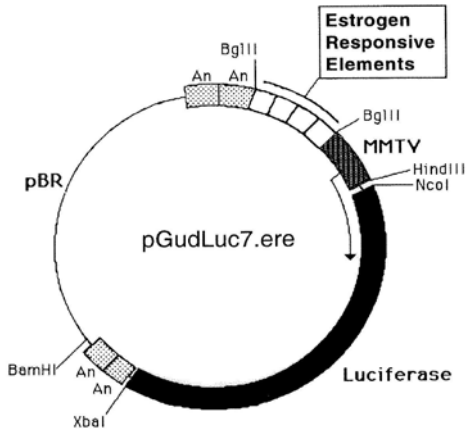
- Standardize procedures for using the BG1Luc ER TA to identify ER agonists and antagonists
- Standardize procedures for a quantitative test of cell viability for use with the BG1Luc ER TA agonist and antagonist assays
- Develop two Good Laboratory Practice (GLP)-compliant (OECD 1998, 2004) protocols: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

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

2.0 Overview of the BG1Luc ER TA

The BG1Luc ER TA measures whether and to what extent a substance induces or inhibits TA activity via ER mediated pathways in recombinant BG-1Luc4E2 cells (Rogers and Denison 2000; Rogers and Denison 2002). The BG-1Luc4E2 cell line was derived from BG-1 immortalized adenocarcinoma cells that endogenously express ER and have been have been stably transfected with the plasmid pGudLuc7.ERE. This plasmid contains four copies of a synthetic oligonucleotide containing the estrogen response element upstream of the mouse mammary tumor viral (MMTV) promoter and the firefly luciferase gene **(Figure 2-1)**. BG1 cells that were transfected with the reporter gene construct and stable transfectants were selected by growth in minimum essential medium (MEM) containing gentamycin (G418) (Rogers and Denison 2000; Rogers and Denison 2002). Luciferase expression is driven by ligand binding of the estrogen receptor.

Figure 2-1 pGudLuc7.ERE Plasmid



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

The BG1Luc ER TA assay has been proposed by XDS for use in the U.S. Environmental Protection Agency (EPA) Endocrine Disruptor Screening Program (EDSP) as part of the Tier I screening battery of *in vitro* and *in vivo* test assays that will be used to reach weight-of-evidence decisions on whether to conduct large multi-generational Tier 2 *in vivo* studies.

3.0 Overview of the Protocol Standardization Study Design

The purpose of the study was to test eight coded substances for agonism and eight coded substances for antagonism to determine whether the results were reproducible. Also integral to the study design was the standardization of reference standards and controls, and the development of a quantitative method to assess cell viability.

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

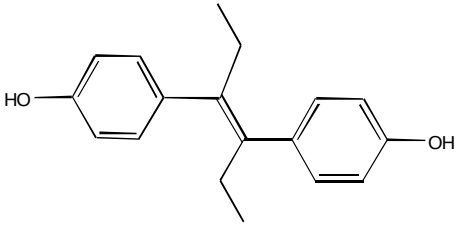
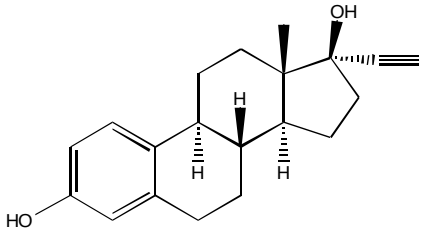
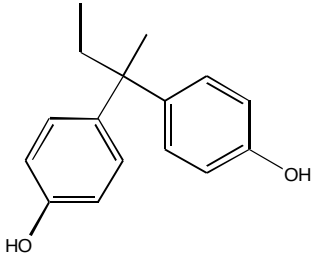
- Their inclusion on the subset of minimum substances recommended by ICCVAM for validating *in vitro* ER assays
- Their ER agonist activity classification, including those that are negative for agonism:
  - Strongly active = half maximal effective concentration [EC_{50}] value was <0.001 µM
  - Moderately active = EC_{50} value was between 0.001 and 0.1µM
  - Weakly active = EC_{50} value was >0.1 µM
- Their ER antagonist classification:
  - Uniformly active in multiple assays
  - Active in the majority of assays in which it was tested
  - Active in the single assay in which it was tested
  - Uniformly negative in all assays
- Substances were also included that would likely be cytotoxic in the assay or that might pose solubility problems

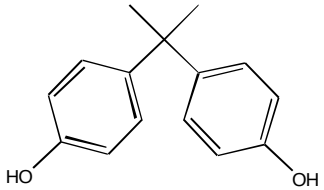
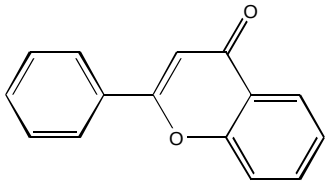
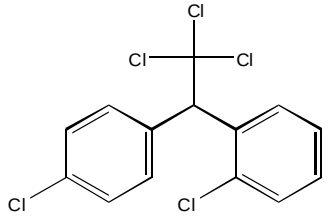
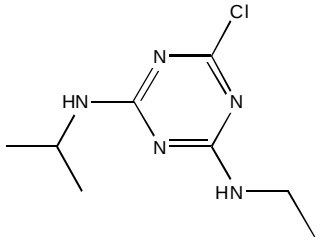
The selected substances for protocol standardization, eight for agonism and eight for antagonism, are detailed in **Table 3-1**.

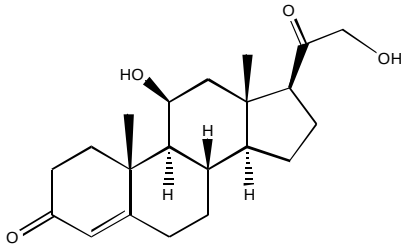
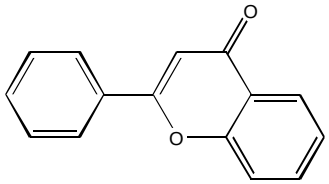
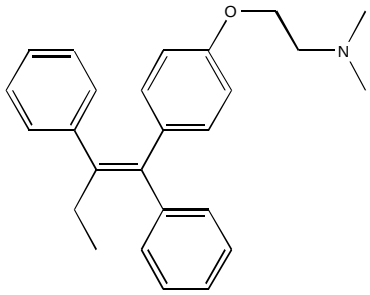
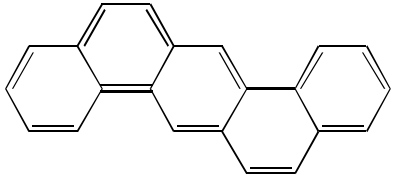
The study was conducted in the following sequence:

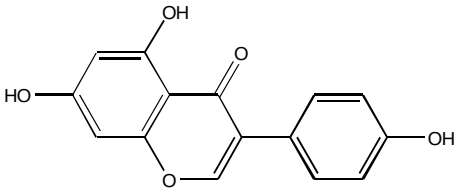
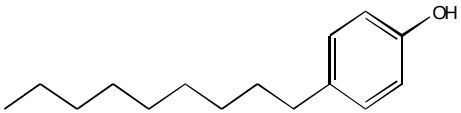
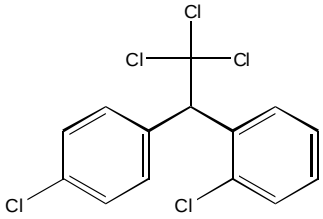
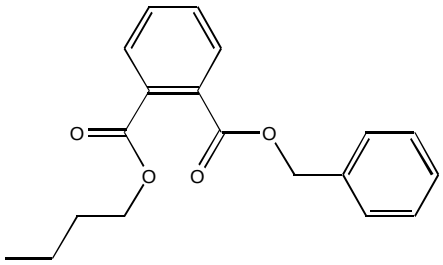
- Selection of a positive control for agonist assays
- Selection of an antagonist reference standard and controls
- Development of historical databases
- Standardization of procedures for evaluation of cell viability
- Range finder testing of eight coded substances for agonism
- Range finder testing of eight coded substances for antagonism
- Comprehensive testing of eight coded substances for agonism
- Comprehensive testing of eight coded substances for antagonism

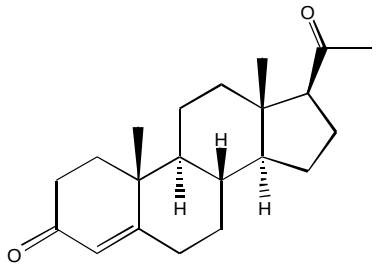
Table 3-1 Substances Selected for BG1Luc ER TA Protocol Standardization

Selected for Agonism (A) or Antagonism (Z)	Substance	CASRN	Structure	ER Agonist Activity[1,2]	ER Antagonist Activity[1,3]	Comments
A	Diethylstilbestrol	56-53-1		+++	-	
A	17α-Ethinyl estradiol	57-63-6		+++	-	
A	Bisphenol B	77-40-7		++		

Selected for Agonism (A) or Antagonism (Z)	Substance	CASRN	Structure	ER Agonist Activity[1,2]	ER Antagonist Activity[1,3]	Comments
A	Bisphenol A	80-5-7		+	-	
A	Flavone	525-82-6		+	###	
A	*o,p'* –DDT[4,5]	789-2-6		+	#	Cytotoxic
A	Atrazine	1912-24-9		-	-	Cytotoxic

Selected for Agonism (A) or Antagonism (Z)	Substance	CASRN	Structure	ER Agonist Activity[1,2]	ER Antagonist Activity[1,3]	Comments
A	Corticosterone	50-22-6		-	-	Negative for Agonism
Z	Flavone[4]	525-82-6		+-	###	
Z	Tamoxifen	10540-29-1		-	###	Cytotoxic
Z	Dibenzo[*a*,*h*] anthracene	53-70-3		-	##	

Selected for Agonism (A) or Antagonism (Z)	Substance	CASRN	Structure	ER Agonist Activity[1,2]	ER Antagonist Activity[1,3]	Comments
Z	Genistein	446-72-0		+	#	Insoluble
Z	*p* -n -Nonylphenol	104-40-5		++	#	
Z	*o,p'* –DDT[4,5]	789-2-6		+	#	Cytotoxic
Z	Butylbenzyl phthalate	85-68-7		++	-	Negative for Antagonism

Selected for Agonism (A) or Antagonism (Z)	Substance	CASRN	Structure	ER Agonist Activity[1,2]	ER Antagonist Activity[1,3]	Comments
Z	Progesterone	57-83-0		+	-	Negative for Antagonism

[1] Data on agonist and antagonist activities were derived from (ICCVAM 2003, 2006)

[2] +++ Indicates that the substance was relatively active (half maximal effective concentration [EC50] value was <0.001 µM); ++ indicates that the substance was moderately active (EC50 value was between 0.001 and 0.1µM): + indicates that the substance was weakly active (EC50 value was >0.1 µM); +- indicates that 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.

[3] ### 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 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.

[4] 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 acting as a positive in both the agonist and antagonist assay.

[5] *o,p'*-DDT = 1,1,1-Trichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl)ethane

4.0 Initial Protocol Development

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

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

5.0 Selection and Standardization of Reference Standards and Controls

5.1 Standardization of Agonist Reference Standard

ICCVAM Guidelines (ICCVAM 2003, 2006) recommend the use of E2 as the reference standard for ER TA agonist assays; therefore, this substance was retained as the reference standard for the BG1Luc ER TA agonist protocol. In order to maximize the number of concentrations and replicates of coded substance that could be tested on a single plate, experiments were conducted to determine the optimal number of E2 reference standard concentrations and replicates per plate.

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

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

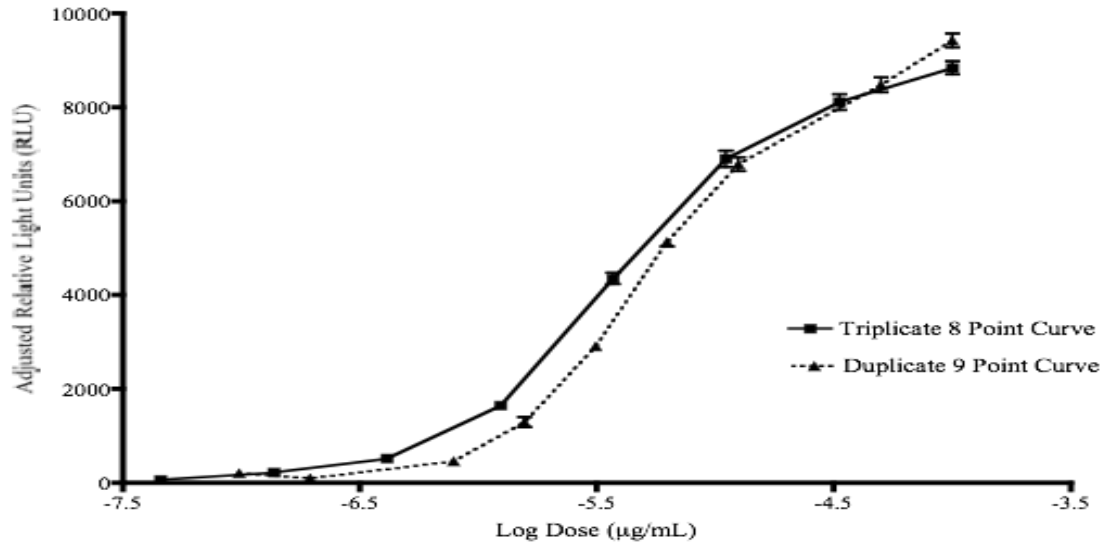
Eight-Point Half-Log Dilution[1]	Nine-Point Serial Dilution[1]
1.00×10^{-4}	1.00×10^{-4}
3.33×10^{-5}	5.00×10^{-5}
1.11×10^{-5}	1.25×10^{-5}
3.70×10^{-6}	6.25×10^{-6}
1.23×10^{-6}	3.13×10^{-6}
4.12×10^{-7}	1.56×10^{-6}
1.37×10^{-7}	7.83×10^{-7}
4.57×10^{-8}	1.95×10^{-7}
-	9.77×10^{-8}

Abbreviations: E2 = 17β–estradiol

[1] Concentrations are presented as μg/mL.

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

Figure 5-1 Comparison of Eight-Point Triplicate and Nine-Point Duplicate E2 Configurations



Abbreviations: E2 = 17β–estradiol
Each line represents the mean and standard deviation of 10 separate experiments.

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

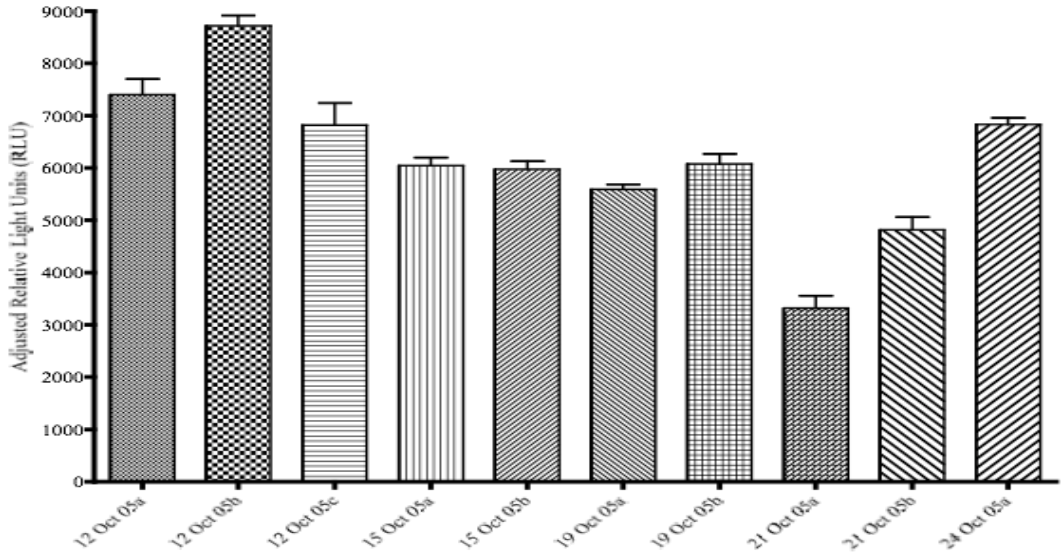
5.2 Selection and Standardization of Agonist Controls

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

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

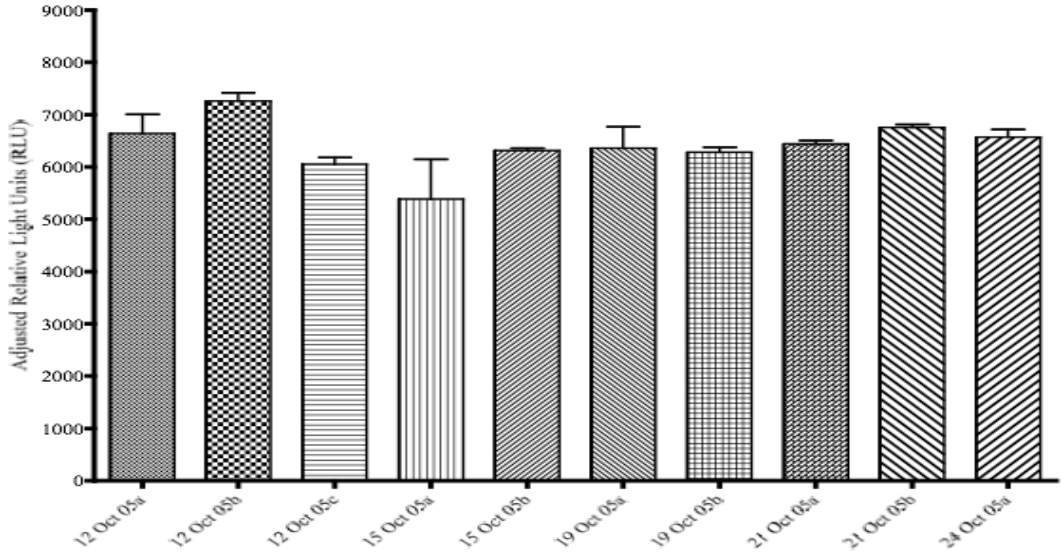
The three substances were evaluated in 10 independent experiments over a two-week period at concentrations that had previously been determined to have similar ER TA activities in terms of magnitude of response as E2, but at a significantly higher concentration than E2. The resulting data was evaluated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

Figure 5-2 Evaluation of Kaempferol as an Agonist Positive Control



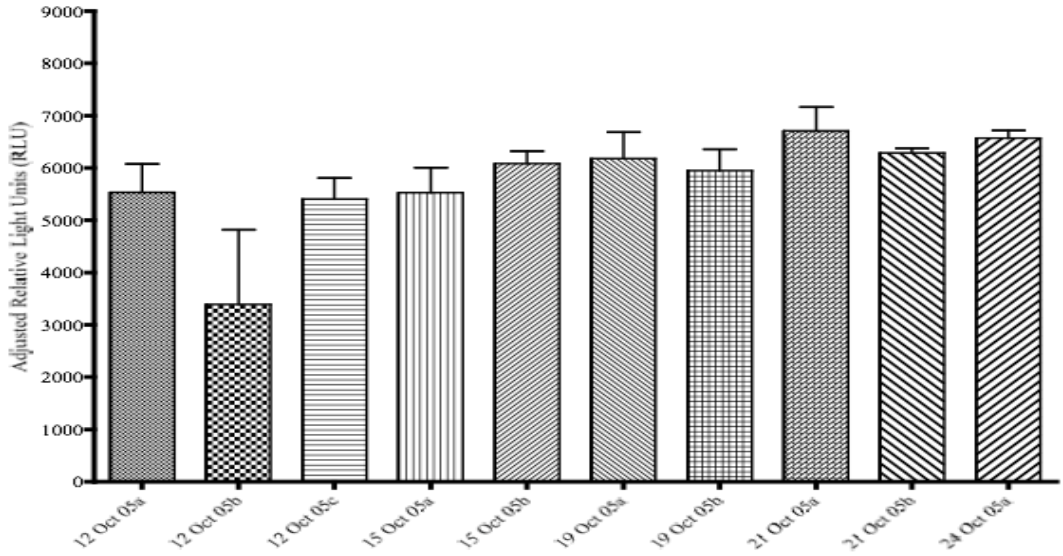
Each bar represents the mean and standard deviation of triplicate wells.
Letters after the date on bar labels indicate that the experiment was performed multiple times on the same day.

Figure 5-3 Evaluation of Methoxychlor as an Agonist Positive Control



Each bar represents the mean and standard deviation of triplicate wells.
Letters after the date on bar labels indicate that the experiment was performed multiple times on the same day.

Figure 5-4 Evaluation of Zearalenone as an Agonist Positive Control



Each bar represents the mean and standard deviation of triplicate wells.
Letters after the date on bar labels indicate that the experiment was performed multiple times on the same day.

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

5.3 Selection and Standardization of Antagonist Reference Standard

During the initial development of the BG1Luc ER TA antagonist protocol, XDS used tamoxifen as a reference standard. However, tamoxifen requires metabolic activation to 4-hydroxytamoxifen and was cytotoxic at the higher concentrations of the reference standard needed to establish saturation of response. Therefore, an objective of the study was to select and standardize the use of an alternative reference standard. Although ICCVAM Guidelines (ICCVAM 2003, 2006) recommend the use of ICI 182,780 as a reference standard in ER TA antagonist assays, this substance has limited commercial availability (ICCVAM 2006). As an alternative, raloxifene HCl (raloxifene), a strong estrogen antagonist that is listed in the ICCVAM Guidelines (ICCVAM 2003, 2006) for validation testing, was evaluated for use as the reference standard in the BG1Luc ER TA antagonist assay. In order to maximize the number of concentrations and replicates of coded substance that could be examined on a single plate, experiments were conducted to determine the optimal number of raloxifene reference standard concentrations and replicates per plate. Two raloxifene reference standard configurations were compared, an eight-point, half-log dilution with samples run in triplicate, and a nine-point, serial dilution with samples run in duplicate (**Table 5-2**). These concentrations of raloxifene were combined with a fixed concentration of 2.5×10^{-5} µg/mL E2 (Ral/E2) to establish the concentration-response curve for antagonism.

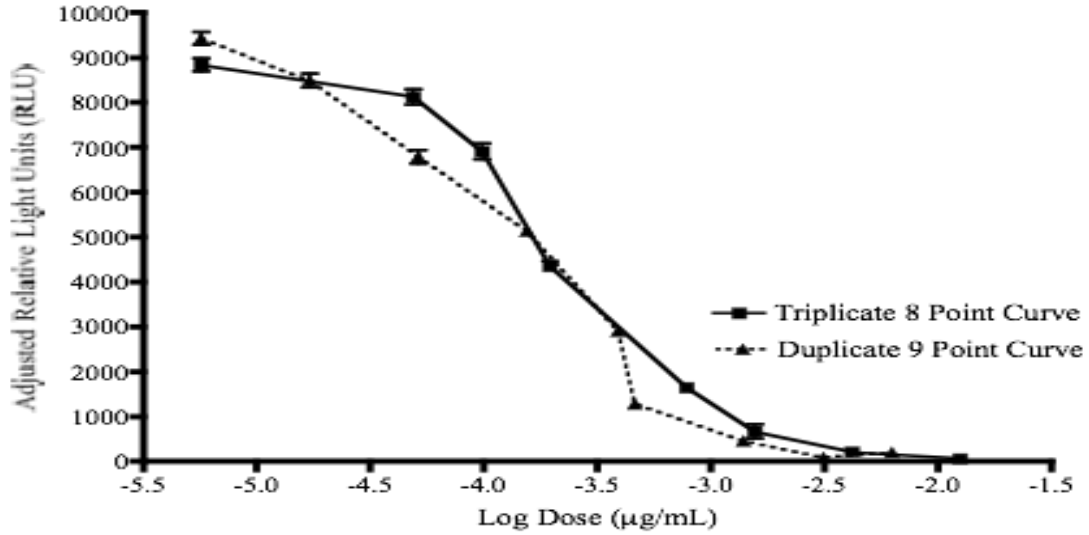
Table 5-2 Concentrations of Raloxifene Tested in Eight-Point Half-Log vs. Nine-Point Serial Dilution Ral/E2 Design

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

Abbreviations: Ral/E2 = concentrations of raloxifene with a fixed concentration of 2.5×10^{-5} μg/mL 17β–estradiol

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

Figure 5-5 Comparison of Eight-Point Triplicate and Nine-Point Duplicate Ral/E2 Configurations



Abbreviations: Ral/E2 = concentrations of raloxifene with a fixed concentration of 2.5×10^{-5} μg/mL 17β–estradiol
Each line represents the mean and standard deviation of 10 separate experiments.

Results indicated that the duplicate nine-point curve had more data points that fell within the linear portion of the concentration-response curve. In order to maximize the testing of coded substances as

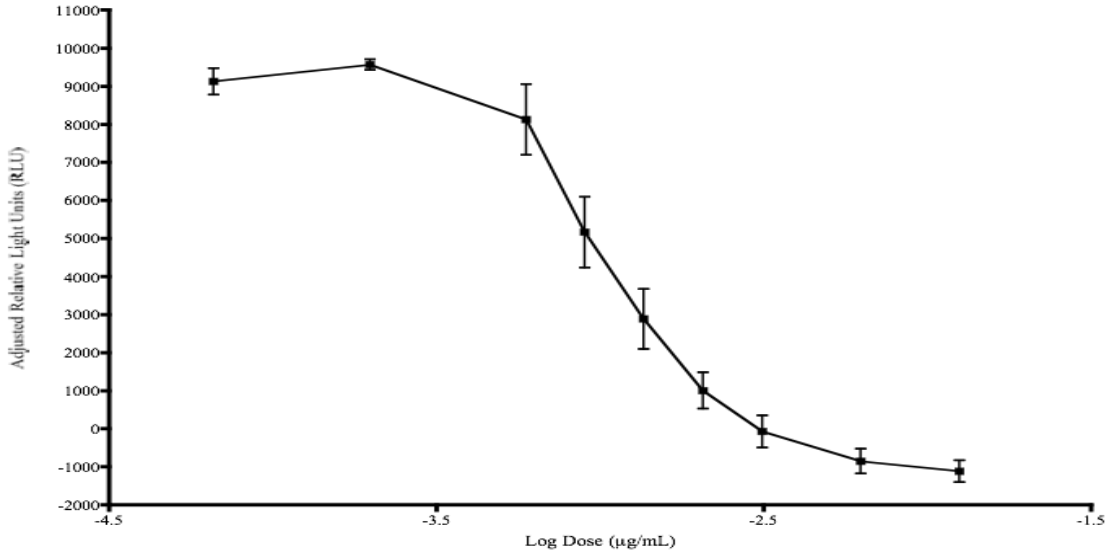
11 point, double serial dilutions in triplicate on a 96-well plate, the duplicate Ral/E2 reference standard configuration was selected for use in the antagonist assay.

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

Table 5-3 Concentrations of Raloxifene in Reference Standard

Raloxifene Concentration (μg/mL)		
1.25×10^{-2}	1.56×10^{-3}	1.95×10^{-4}
6.25×10^{-3}	7.81×10^{-4}	9.77×10^{-5}
3.13×10^{-3}	3.91×10^{-4}	4.88×10^{-5}

Figure 5-6 Evaluation of Ral/E2 as an Antagonist Reference Standard



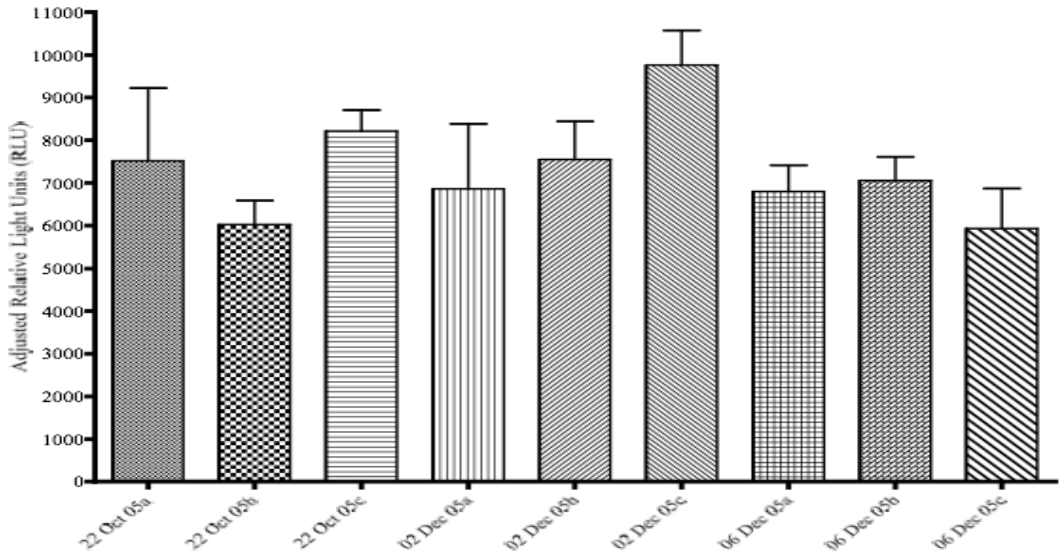
Abbreviations: Ral/E2 = concentrations of raloxifene with a fixed concentration of 2.5×10^{-5} μg/mL 17β–estradiol
The line represents the mean and standard deviation of 10 separate experiments.

5.4 Selection and Standardization of Antagonist Controls

ICCVAM Guidelines (ICCVAM 2003, 2006) also recommend the inclusion of a weak positive control that would reduce the ability of the reference estrogen to induce maximum ER TA in the test system by 70 to 90% in an antagonist assay. The purpose of a weak positive control is to facilitate the demonstration of the sensitivity and reproducibility of the assay. Three substances, dibenzo[*a*,*h*]anthracene (DBA), flavone, and tamoxifen, were selected from recommended substances for ER TA test methods listed in the ICCVAM Guidelines (ICCVAM 2003, 2006) and evaluated as potential antagonist positive controls.

These three candidates were evaluated for their potential to reduce the induction of ER TA caused by 2.5×10^{-5} µg/mL of E2 in multiple independent experiments over a two week period at concentrations that had been determined by XDS in previous experiments to cause a decrease in ER TA by approximately 70%. Results (**Figures 5-7, 5-8,** and **5-9**) were evaluated for consistency of response.

Figure 5-7 Evaluation of DBA as an Antagonist Positive Control

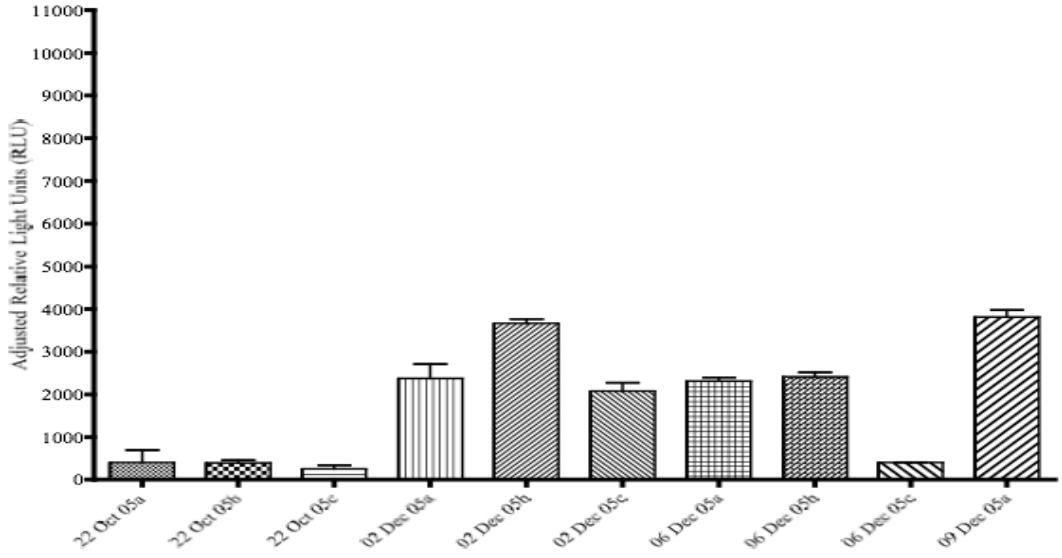


Abbreviations: DBA = dibenzo[*a*,*h*]anthracene

Each bar represents the mean and standard deviation of triplicate wells.

Letters after the date on bar labels indicate that the experiment was performed multiple times on the same day.

Figure 5-8 Evaluation of Flavone as an Antagonist Positive Control

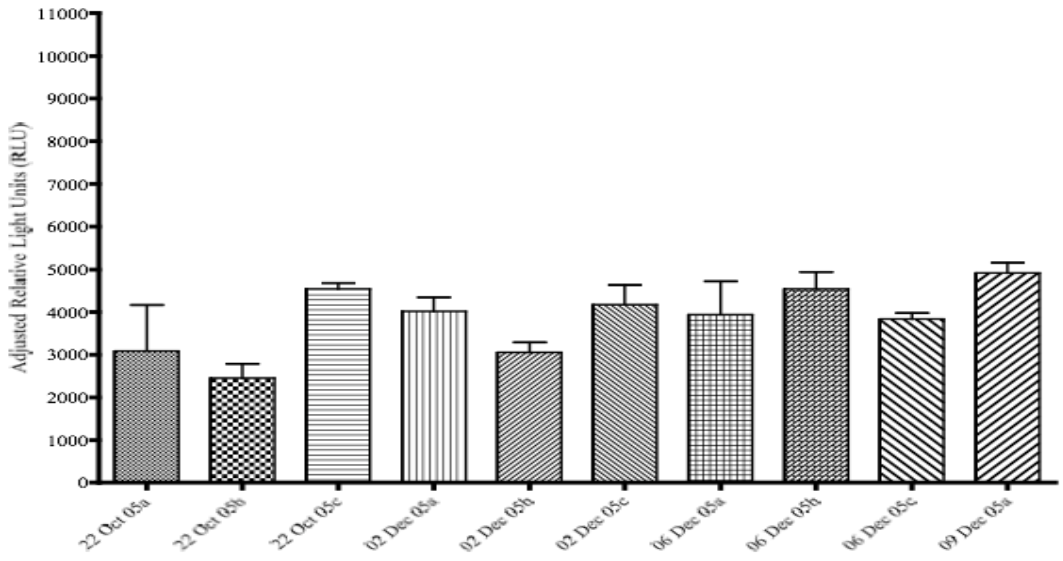


Each bar represents the mean and standard deviation of triplicate wells.

Letters after the date on bar labels indicate that the experiment was performed multiple times on the same day.

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

Figure 5-9 Evaluation of Tamoxifen as an Antagonist Positive Control



Each bar represents the mean and standard deviation of triplicate wells.
Letters after the date on bar labels indicate that the experiment was performed multiple times on the same day.

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

5.5 Summary of Selected Reference Standards and Controls

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

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

Use	Substance Name	CASRN	Supplier	Catalog Number	Purity	ER TA Agonist Activity[1,2]	ER TA Antagonist Activity[1,3]
Solvent	Dimethyl sulfoxide	67-68-5	Sigma-Aldrich Corp	D8418	99.9%	-	-
Agonist Reference Standard	17β-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	17β-estradiol	50-28-2	Sigma-Aldrich Corp	E8875	98%	+++	-

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

[1] Data on agonist and antagonist activities were derived from (ICCVAM 2006)

[2] +++ Indicates that the substance was strongly active (EC_{50} value was <0.001 μM); + indicates that the substance was weakly active (EC_{50} value was >0.1 μM), or a positive response was reported without an EC_{50} value;
- indicates that the substance was uniformly negative in multiple assays.

[3] ### Indicates that the substance was uniformly positive in multiple assays; - indicates that the substance was uniformly negative in multiple assays.

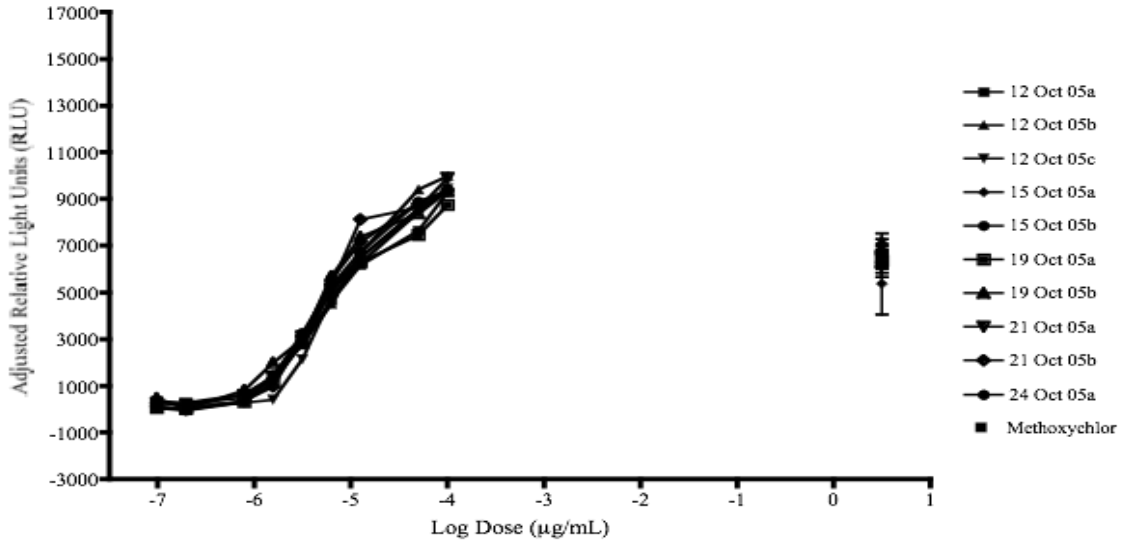
6.0 Historical Databases

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

6.1 Agonist Historical Database

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

Figure 6-1 Agonist Historical Database



Each line represents the 17β–estradiol reference standard for a single experiment. Each point on the line represents the mean and standard deviation of duplicate wells.

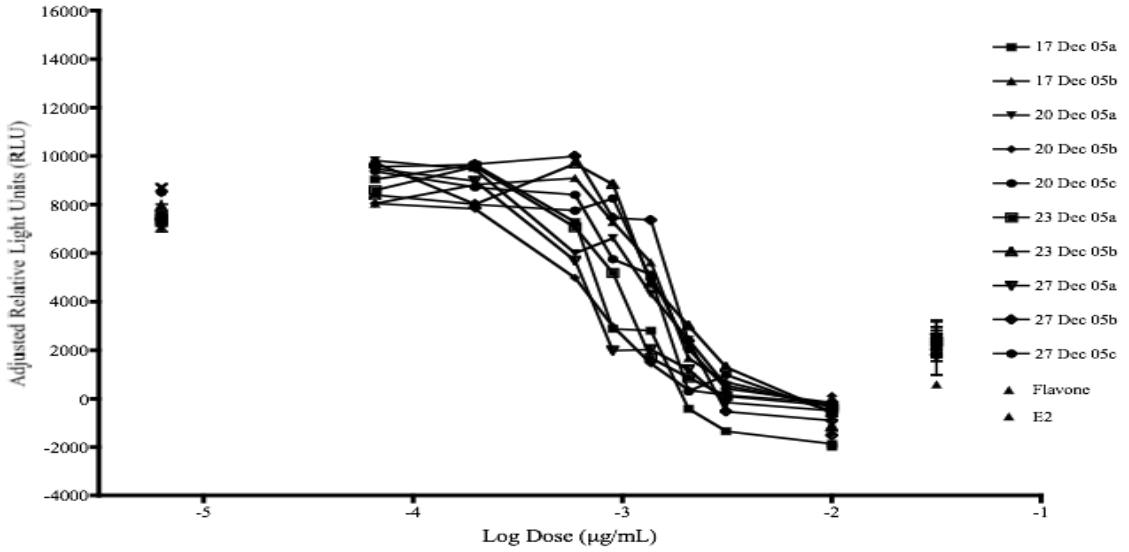
Each point at "0" on the abscissa represents the methoxychlor control for a single experiment (mean and standard deviation of triplicate wells).

Letters after the date on line labels indicate that the experiment was performed multiple times on the same day.

6.2 Antagonist Historical Database

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

Figure 6-2 Antagonist Historical Database



Each line represents the Ral/E2 (concentrations of raloxifene with a fixed concentration of 2.5 x 10^{-5} µg/mL 17β-estradiol) reference standard for a single experiment. Each point on the line represents the mean and standard deviation of duplicate wells.

Each point at "0" on the abscissa represents the flavone control for a single experiment (mean and standard deviation of triplicate wells).

Each point at "-7" on the abscissa represents the 17β–estradiol control for a single experiment (mean and standard deviation of triplicate wells).

Letters after the date on line labels indicate that the experiment was performed multiple times on the same day.

7.0 Assessment of Cell Viability

7.1 Qualitative Evaluation of Cell Viability

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

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

7.2 Quantitative Evaluation of Cell Viability

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

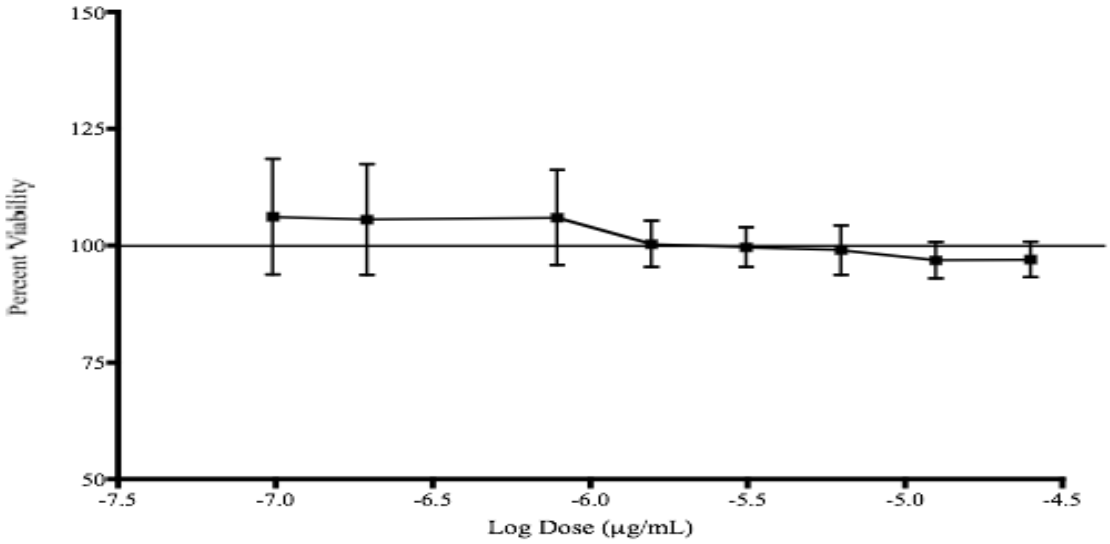
7.3 CellTiter-Blue®

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

7.4 CellTiter-Glo®

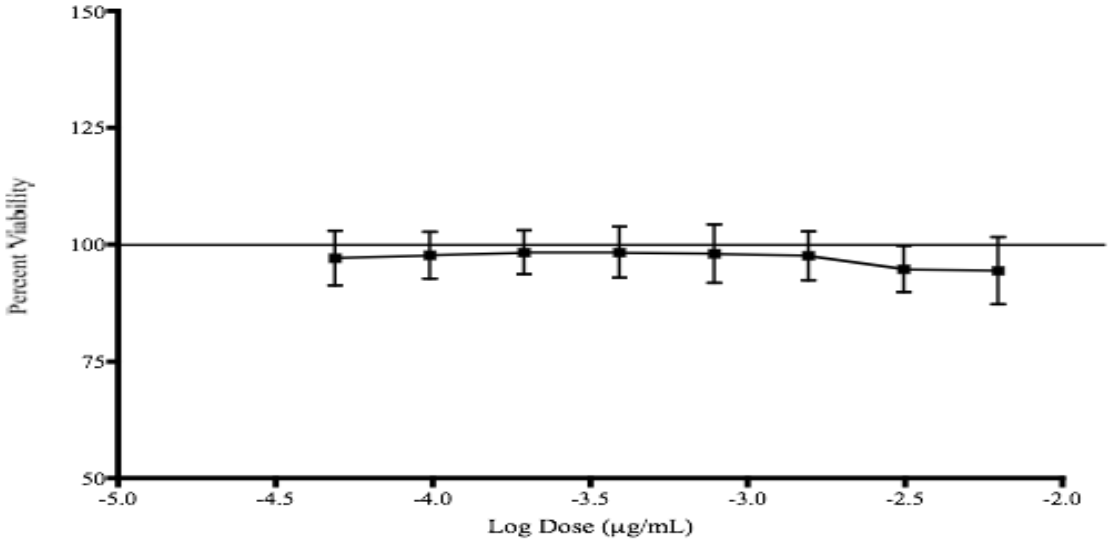
CellTiter-Glo® is a method of determining the number of viable cells in culture based on quantitation of adenosine triphosphate (ATP) in viable cells. This method requires the use of concurrent parallel experimental plates because the assay format results in cell lysis and generation of a luminescent signal proportional to the amount of ATP present. Results for CellTiter-Glo® testing using the E2 reference standard and the Ral/E2 reference standard are presented in **Figures 7-1** and **7-2**. Based on these results, the CellTiter-Glo® method was selected to quantitatively measure cytotoxicity for the protocol standardization study.

Figure 7-1 CellTiter-Glo® Agonist Viability Testing Trials



Graph represents the mean and standard deviation of 10 replicate experiments.
Horizontal line represents 100% viability as measured in dimethyl sulfoxide control.

Figure 7-2 CellTiter-Glo® Antagonist Viability Testing Trials



Graph represents the mean and standard deviation of 10 replicate experiments.
Horizontal line represents 100% viability as measured in dimethyl sulfoxide control.

7.3 Cell Viability Limit

Examination of CellTiter-Glo® cell viability data for the E2 and Ral/E2 reference standards demonstrated that viability for these reference standards did not fall below 80% (**Figures 7-1** and **7-2**). No decrease in response in the BG1Luc ER TA resulted from this level of reduction in cell viability (**Figures 6-1** and **6-2**), and therefore, the limit for cell viability was set at 80%. Test substance concentrations that reduced

the percentage of viable cells below 80% were classified as cytotoxic and were not used to assess ER TA activity.

8.0 Procedures for Testing of Coded Substances

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

8.1 Coded Test Substances

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

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	*o,p'*-DDT	789-02-6	ChemService, Inc	PS-698	98%
N0006	R00108	Diethylstilbestrol	56-53-1	Sigma-Aldrich Corp	D4628	99%
N0007	R00109	17α-ethinyl estradiol	57-63-6	Sigma-Aldrich Corp	E4876	99%
N0008	R00110	Flavone[1]	525-82-6	Sigma-Aldrich Corp	F2003	99%
N0009	R00111A	Butylbenzyl phthalate	85-68-7	Sigma-Aldrich Corp	308501	98%
N0010	R00119A	Dibenzo[*a,h*] anthracene	53-70-3	Sigma-Aldrich Corp	48574	99%
N0011	R00122A	Genistein	446-72-0	Sigma-Aldrich Corp	G6649	99%
N0012	R00112A	Flavone[1]	525-82-6	Sigma-Aldrich Corp	F2003	99%
N0013	R00120A	*p*-n-nonylphenol	104-40-5	Alfa Aesar, Corp	A15609	100%
N0014	R0013A	Progesterone	57-83-0	Sigma-Aldrich Corp	P8783	100%
N0015	R00121A	*o,p'*-DDT[1]	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

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

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

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

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

8.2 Lot-to-Lot Consistency of Test Substances

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

9.0 General Procedure for Agonist Testing

Agonist range finder experiments were conducted by testing substances at serial log concentrations. Results from range finder testing were then used to select starting concentrations for comprehensive testing of test substances. Agonist range finder and comprehensive testing were conducted on 96-well plates using 10 concentrations of E2 in duplicate as the reference standard (**Table 9-1**). Four replicate wells of the DMSO control and three replicate wells of the methoxychlor control were included on each plate. In order to avoid edging effects[3], wells on the perimeter of the plate were not used for experiments. These wells did not contain cells but did contain cell culture media to prevent drying out of experimental wells.

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

E2 Concentrations (μg/mL)		
1.00×10^{-4}	6.25×10^{-6}	1.95×10^{-7}
5.00×10^{-5}	3.13×10^{-6}	9.78×10^{-8}
2.50×10^{-5}	1.56×10^{-6}	
1.25×10^{-5}	7.83×10^{-7}	

Abbreviations: E2 = 17β-estradiol

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

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log EC50 - X)\text{HillSlope}}}$$

where Y=response (i.e., relative light units), X is the logarithm of the test substance concentration, Bottom is the minimum response, Top is the maximum response, log EC_{50} is the logarithm of X as the response midway between Top and Bottom, and HillSlope describes the steepness of the curve. The model calculates the best fit for the Top, Bottom, HillSlope, and EC_{50} parameters.

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

- Induction – Plate induction (i.e., the highest E2 reference standard RLU value divided by the averaged DMSO solvent RLU value) must be greater than three fold.

[3] 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.

- Reference standard results – Calculated E2 reference standard EC_{50} values must be within 2.5 times the standard deviation of the historical database EC_{50} mean values.
- DMSO control results - DMSO control RLU values must be within 2.5 times the standard deviation of the historical database solvent control mean RLU values.
- Positive control results – Methoxychlor control RLU values must be within 2.5 times the standard deviation of the historical database methoxychlor control mean RLU values.

10.0 Agonist Testing

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

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

Table 10-1 Test Substances for Agonist Testing

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

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

[1] Data on agonist activities were derived from ICCVAM (ICCVAM 2006)

[2] +++ Indicates that the substance was strongly active (EC_{50} value was <0.001 μM); ++ indicates that the substance was moderately active (EC_{50} value was between 0.001 and 0.1 μM); + indicates that the substance was weakly active (EC_{50} value was >0.1 μM), or a positive response was reported without an EC_{50} value; - indicates that the substance was uniformly negative in multiple assays.

[3] Information on solubility and cytotoxicity were derived from the scientific literature.

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

10.1 Agonist Range Finding

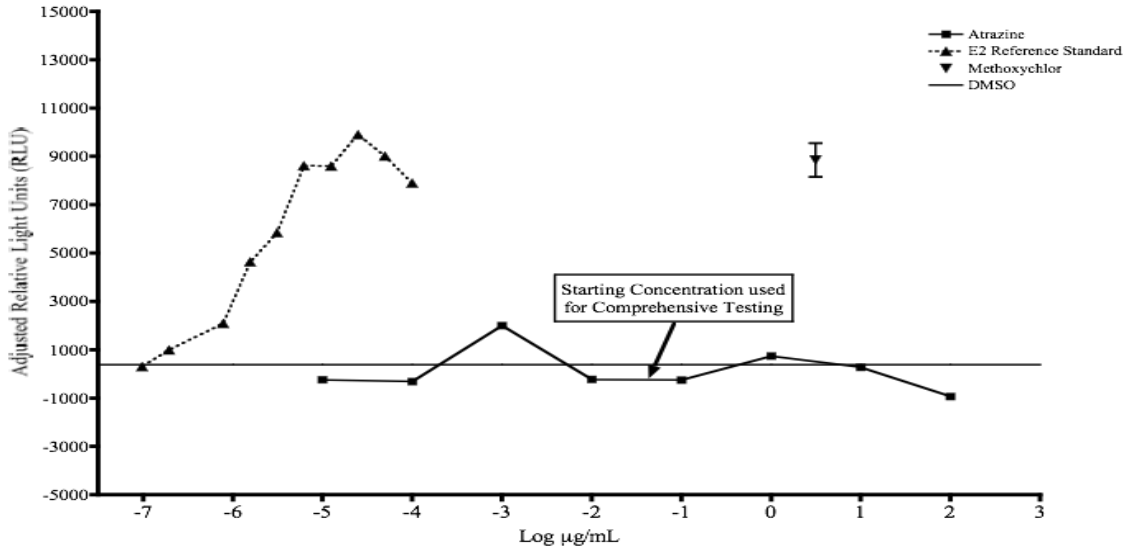
Agonist range finding for coded substances consisted of eight-point, logarithmic serial dilutions, with each concentration tested in a single well of the 96-well plate. All agonist range finder experiments used the same concentrations of test substance (**Table 10-2**). Concentrations for comprehensive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

Table 10-2 Agonist Range Finder Concentrations for Coded Substances

Range Finder Concentrations (µg/mL)		
100	0.1	1.00×10^{-4}
10	1.00×10^{-2}	1.00×10^{-5}
1	1.00×10^{-3}	

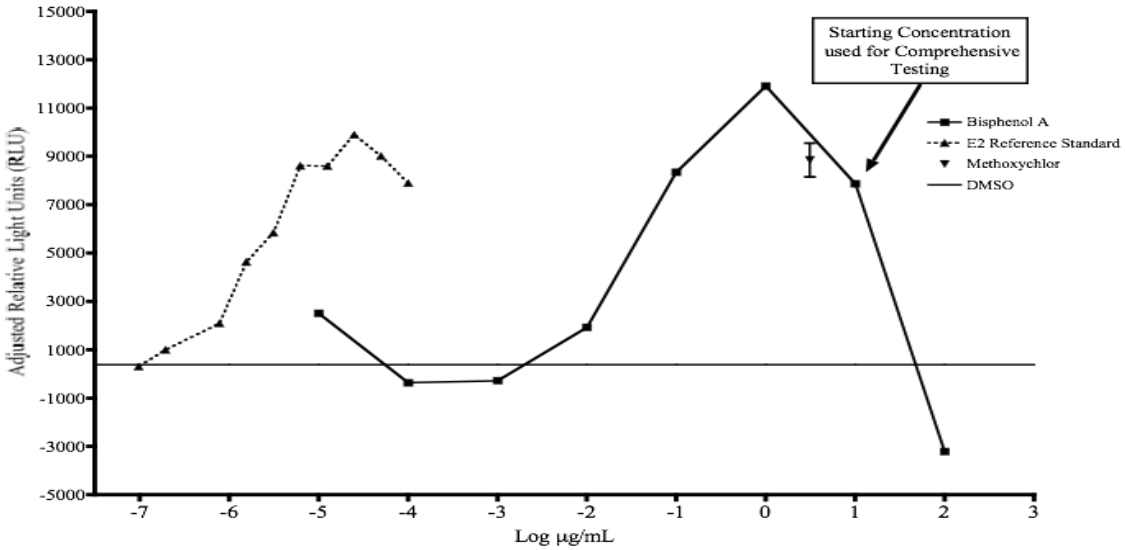
Results for agonist range finder experiments are presented in **Figures 10-1** through **10-8**.

Figure 10-1 Agonist Range Finder for N0001 – Atrazine



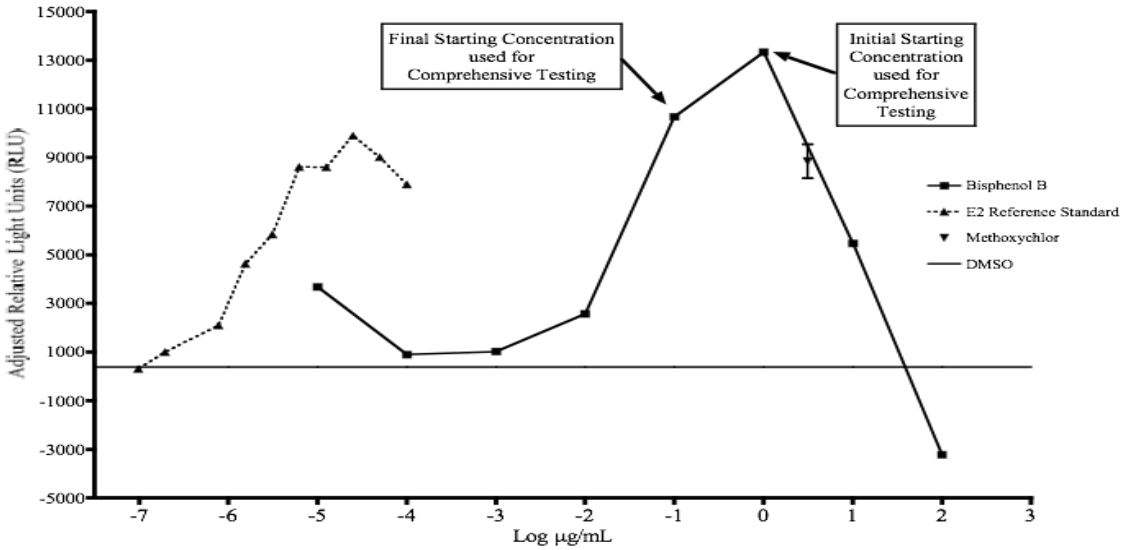
Abbreviations: E2 = 17ß-estradiol; Methoxychlor = 3.13 µg/mL methoxychlor control; DMSO = dimethyl sulfoxide;
Horizontal line represents the mean of four DMSO control replicates plus three times the standard deviation of the DMSO control mean.

Figure 10-2 Agonist Range Finder for N0002 - Bisphenol A



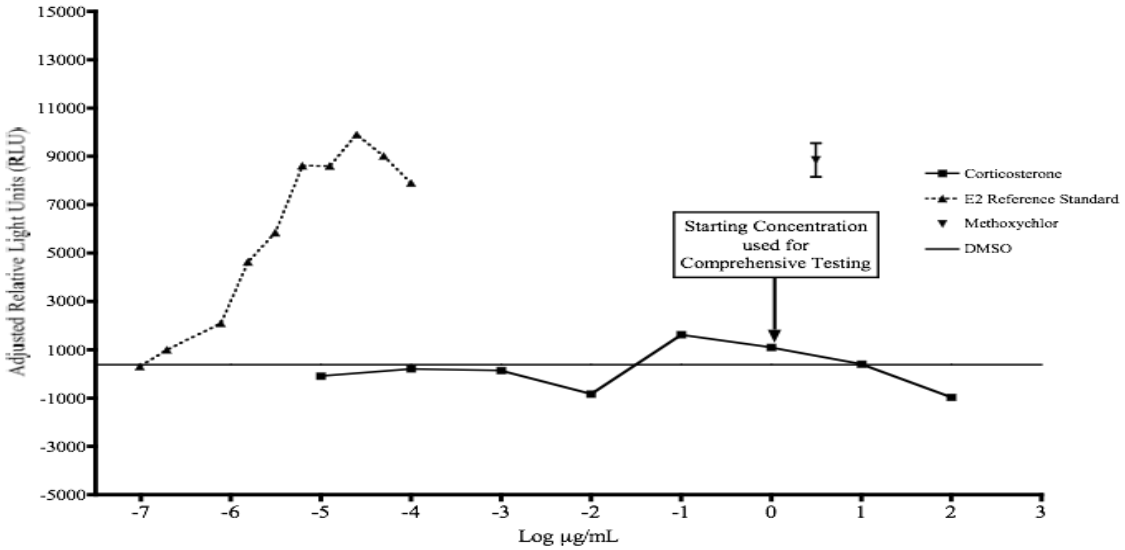
Abbreviations: E2 = 17ß-estradiol; Methoxychlor = 3.13 µg/mL methoxychlor control; DMSO = dimethyl sulfoxide;
Horizontal line represents the mean of four DMSO control replicates plus three times the standard deviation of the DMSO control mean.

Figure 10-3 Agonist Range Finder for N0003 - Bisphenol B



Abbreviations: E2 = 17ß-estradiol; Methoxychlor = 3.13 µg/mL methoxychlor control; DMSO = dimethyl sulfoxide;
Horizontal line represents the mean of four DMSO control replicates plus three times the standard deviation of the DMSO control mean.

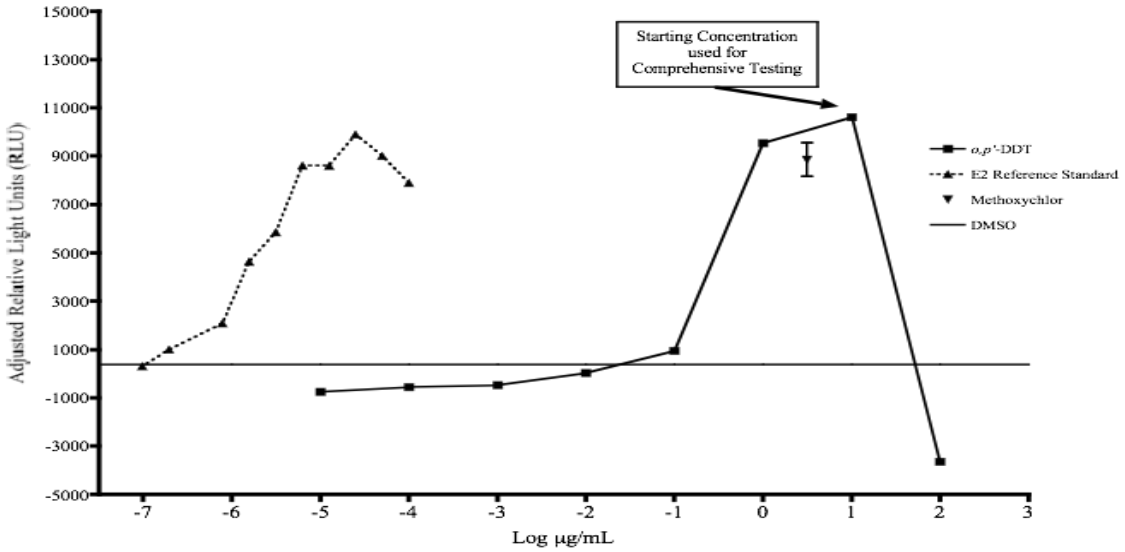
Figure 10-4 Agonist Range Finder for N0004 – Corticosterone



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

Horizontal line represents the mean of four DMSO control replicates plus three times the standard deviation of the DMSO control mean.

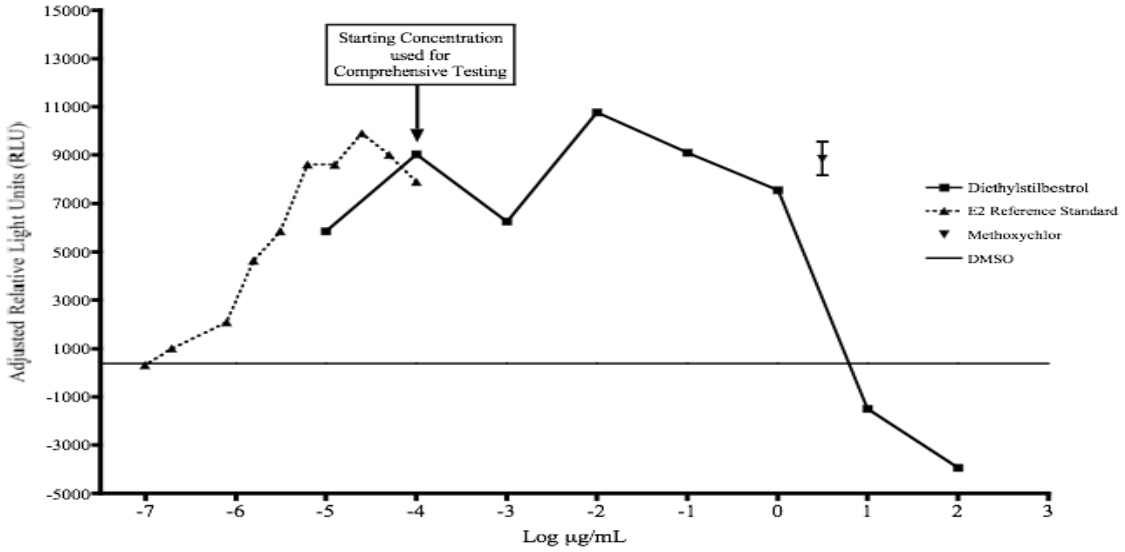
Figure 10-5 Agonist Range Finder for N0005 - *o,p'*-DDT



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

Horizontal line represents the mean of four DMSO control replicates plus three times the standard deviation of the DMSO control mean.

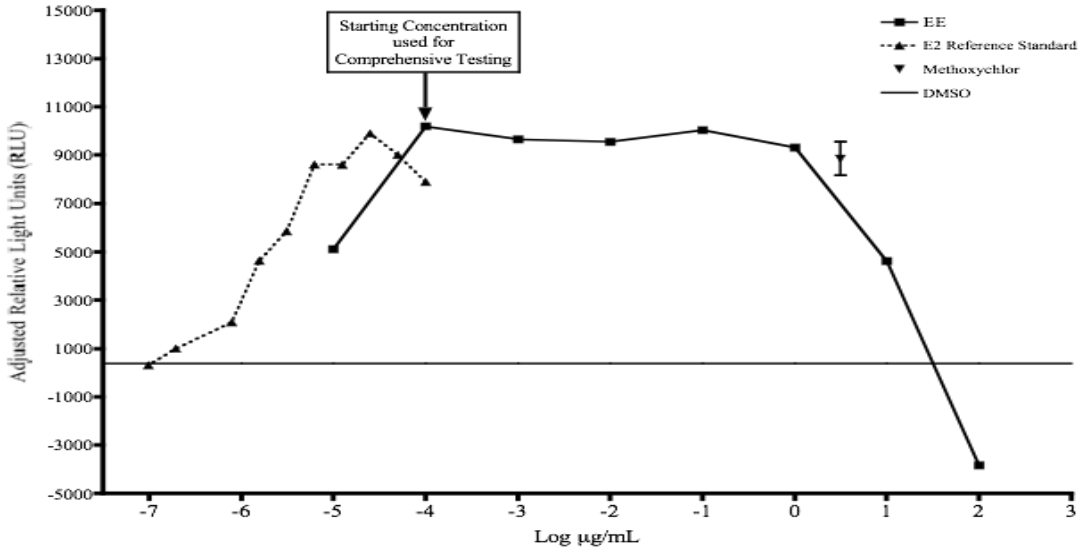
Figure 10-6 Agonist Range Finder for N0006 – Diethylstilbestrol



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

Horizontal line represents the mean of four DMSO control replicates plus three times the standard deviation of the DMSO control mean.

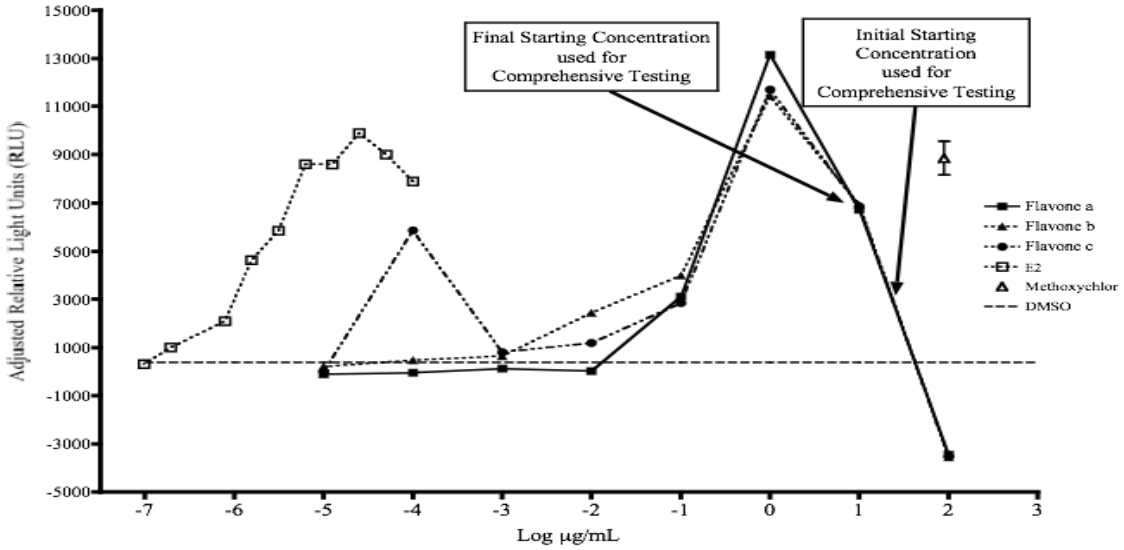
Figure 10-7 Agonist Range Finder for N0007 – EE



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

Horizontal line represents the mean of four DMSO control replicates plus three times the standard deviation of the DMSO control mean.

Figure 10-8 Agonist Range Finder for N0008 – Flavone



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

Horizontal line represents the mean of four DMSO control replicates plus three times the standard deviation of the DMSO control mean.

Each line represents a single flavone experiment replicate. Flavone range finding was repeated in triplicate after an abnormal initial range finder experiment.

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.

Due to concerns about possible experimental error, flavone range finding was repeated in triplicate (see also **Section 15.0**).

Visual observations for cell viability were conducted for all experimental plates just prior to BG1Luc ER TA evaluation. Cell viability testing (i.e., CellTiter-Glo®) was conducted in parallel plates on the same day. Comparisons of cell viability data from CellTiter-Glo® assays and visual observations are shown in **Table 10-3**.

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

Substance	Concentration (µg/mL)	CellTiter-Glo®	Visual Observation Score[1]
N0001 - Atrazine	100	93%	1
	10	104%	1
	1	99%	1
	0.1	99%	1
	1.00×10^{-2}	107%	1
	1.00×10^{-3}	90%	1
	1.00×10^{-4}	98%	1
	1.00×10^{-5}	107%	1
N0002 - Bisphenol A	**100[2]**	**6%**	**4**
	10	105%	1
	1	99%	1
	0.1	108%	1
	1.00×10^{-2}	105%	1
	1.00×10^{-3}	95%	1
	1.00×10^{-4}	109%	1
	1.00×10^{-5}	96%	1
N0003 - Bisphenol B	**100**	**6%**	**4**
	10	102%	1
	1	100%	1
	0.1	105%	1
	1.00×10^{-2}	108%	1
	1.00×10^{-3}	106%	1
	1.00×10^{-4}	102%	1
	1.00×10^{-5}	102%	1
N0004 - Corticosterone	100	80%	2
	10	94%	1
	1	97%	1
	0.1	102%	1
	1.00×10^{-2}	104%	1
	1.00×10^{-3}	103%	1
	1.00×10^{-4}	107%	1
	1.00×10^{-5}	103%	1

Table 10-3 CellTiter-Glo® and Visual Observation Data for Agonist Range Finder Experiments (cont'd)

Substance	Concentration (µg/mL)	CellTiter-Glo®	Visual Observation Score[1]
N0005 - *o,p'*-DDT	**100**	**12%**	**4**
	10	94%	1
	1	103%	1
	0.1	93%	1
	1.00×10^{-2}	97%	1
	1.00×10^{-3}	101%	1
	1.00×10^{-4}	101%	1
	1.00×10^{-5}	108%	1
N0006 - Diethylstilbestrol	**100**	**6%**	**4**
	10	111%	1
	1	111%	1
	0.1	107%	1
	1.00×10^{-2}	99%	1
	1.00×10^{-3}	92%	1
	1.00×10^{-4}	96%	1
	1.00×10^{-5}	101%	1
N0007 - EE	**100**	**30%**	**3**
	10	96%	1
	1	104%	1
	0.1	107%	1
	1.00×10^{-2}	112%	1
	1.00×10^{-3}	104%	1
	1.00×10^{-4}	102%	1
	1.00×10^{-5}	93%	1
N0008 - Flavone	**100**	**12%**	**4**
	10	92%	1
	1	102%	1
	0.1	103%	1
	1.00×10^{-2}	101%	1
	1.00×10^{-3}	92%	1
	1.00×10^{-4}	102%	1
	1.00×10^{-5}	100%	1

Abbreviations: *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane; EE = 17α-ethinyl estradiol

[1] Visual observations are scored using the scale provide in **Table 7.1**

[2] Bolded text indicates substances and concentrations that caused a decrease in cell viability below 80%

Six of the eight substances caused a decrease in cell viability at the highest concentration used for range finder testing. The decrease in cell viability was observed with both visual observations and CellTiter-Glo®.

10.2 Agonist Comprehensive Testing

10.2.1 N0001 – Atrazine

Atrazine was selected for agonist testing because it was listed as negative for ER agonist activity in the ICCVAM Guidelines (ICCVAM 2003, 2006) and was indicated as potentially cytotoxic (Freyberg 2005).

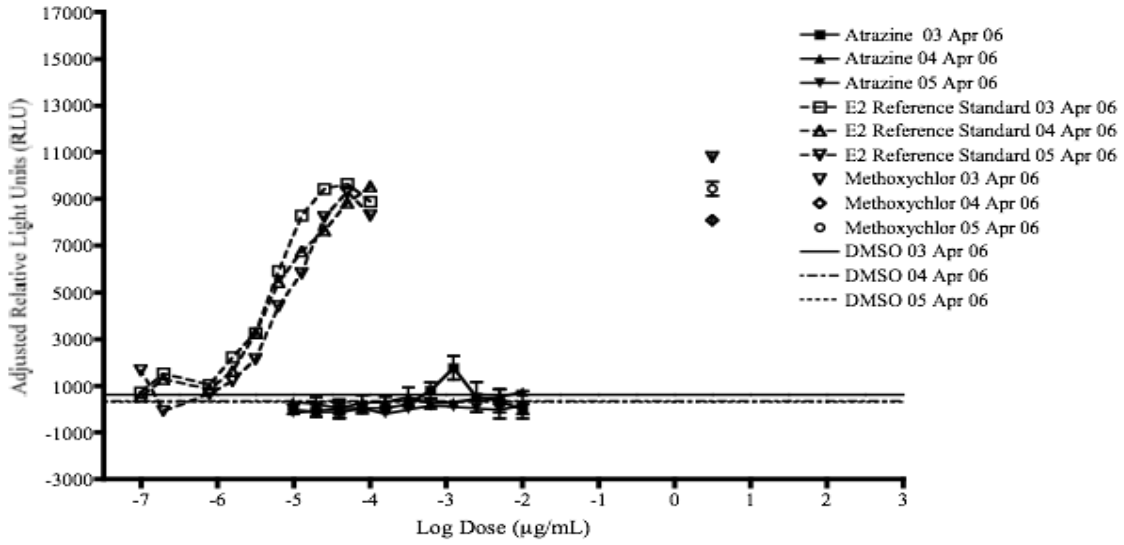
The highest concentration of atrazine used for comprehensive testing was 1.00×10^{-2} µg/mL. This concentration was selected as the starting point for a double serial dilution because it was a single log dilution higher than the concentration giving the highest adjusted RLU value during range finder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

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

N0001 – Atrazine (µg/mL)		
1.00×10^{-2}	6.25×10^{-4}	3.91×10^{-5}
5.00×10^{-3}	3.13×10^{-4}	1.95×10^{-5}
2.50×10^{-3}	1.56×10^{-4}	9.77×10^{-5}
1.25×10^{-3}	7.81×10^{-5}	

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

Figure 10-9 Agonist Comprehensive Testing for N0001 – Atrazine: Individual Experiments



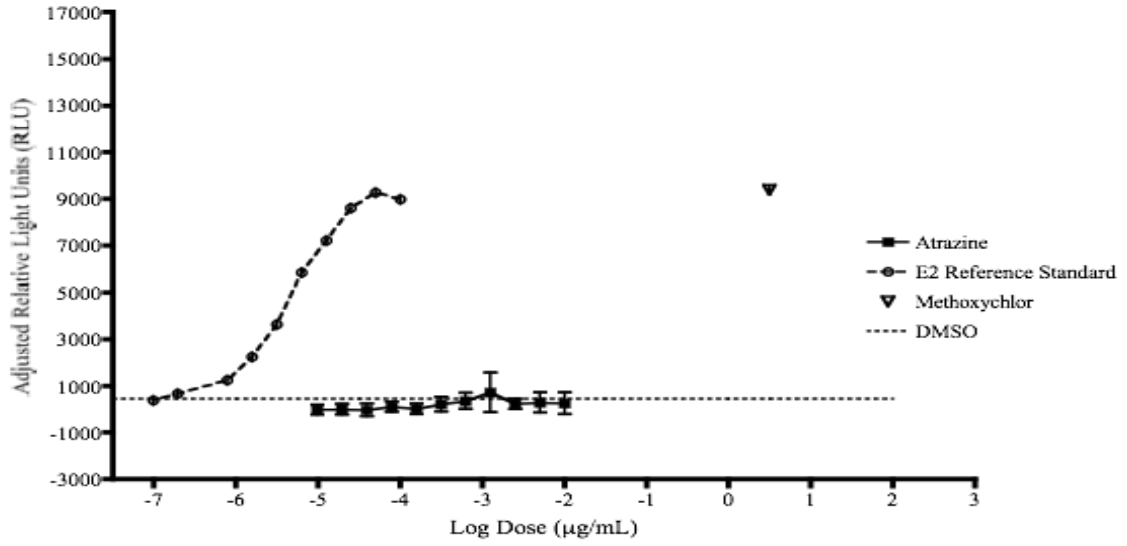
Abbreviations: E2 = 17ß-estradiol; Methoxychlor = 3.13 µg/mL methoxychlor control; DMSO = dimethyl sulfoxide;
Horizontal lines represent the mean of four DMSO control replicates plus three times the standard deviation of the DMSO control mean. Values must be above this line in order to be considered positive for agonism.

Atrazine was negative for agonism at all concentrations tested on 4 April 06 and 5 April 06. On 3 April 06, one concentration of atrazine (1.25×10^{-3} µg/mL) yielded a positive response. However, because this

response was only observed for a single concentration in a single experiment, atrazine was classified as a negative for agonism.

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

Figure 10-10 Agonist Comprehensive Testing for N0001 – Atrazine: Averaged Experiments



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

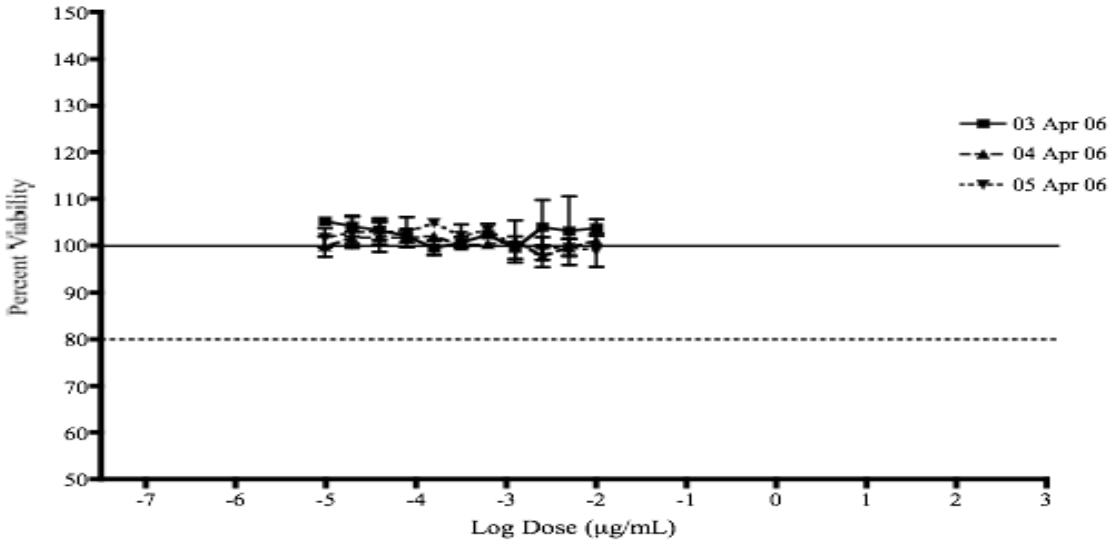
Historical mean and standard deviation of the E2 reference standard.

Historical mean and standard deviation of the methoxychlor control.

Horizontal line represents the historical mean of DMSO vehicle control 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.

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

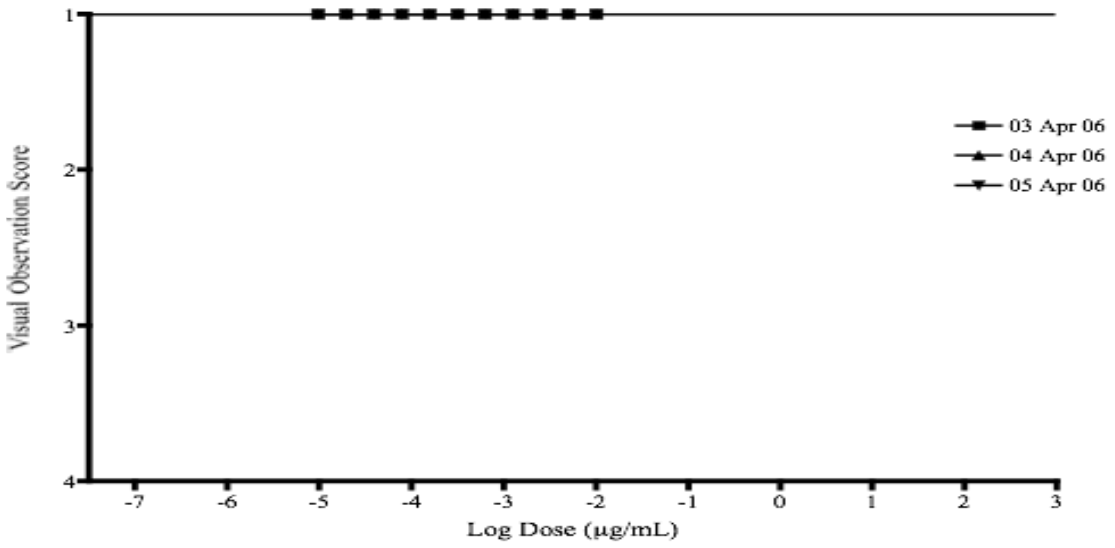
Figure 10-11 CellTiter-Glo® Viability Assessment for N0001 – Atrazine



Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

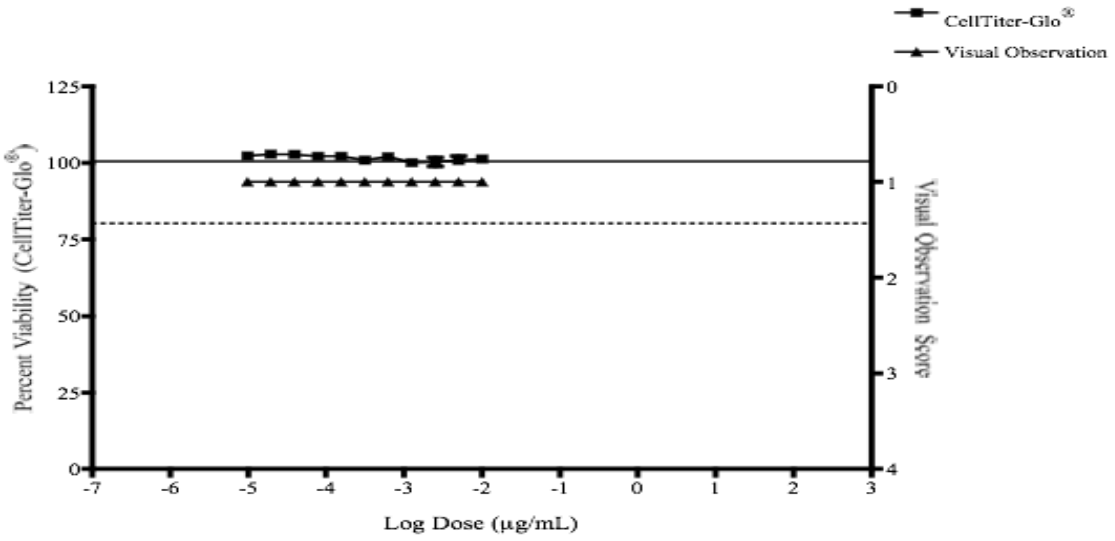
Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability below this line are considered cytotoxic and are not included in the evaluation of agonist activity.

Figure 10-12 Visual Observation Viability Assessment for N0001 – Atrazine



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 ≥ 2 are considered cytotoxic and are not included in the evaluation of antagonist activity.

Figure 10-13 Combined Qualitative and Quantitative Viability Assessments for N0001 – Atrazine



Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

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.

10.2.2 N0002 – Bisphenol A

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

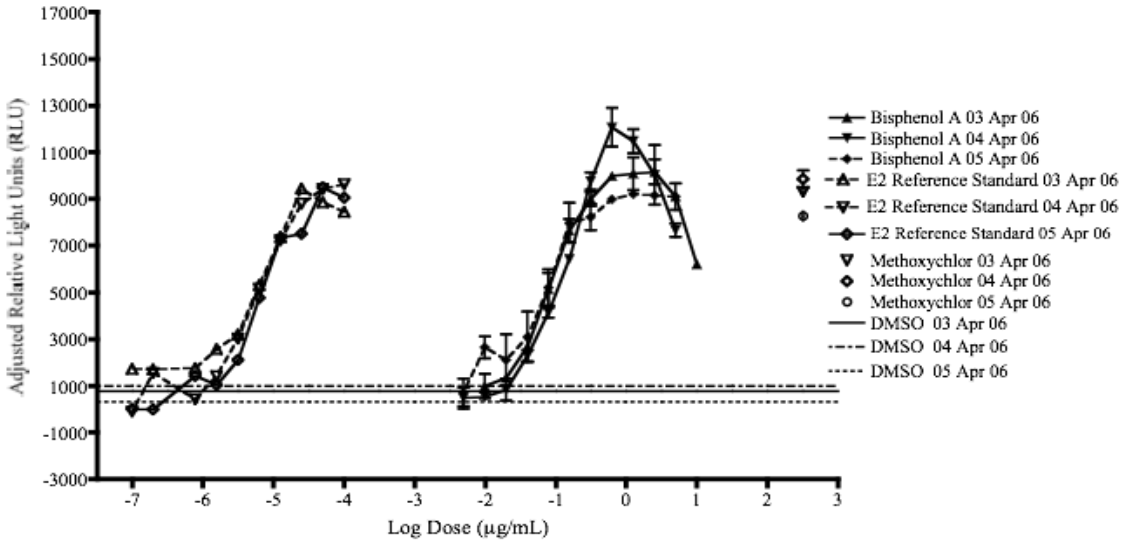
The highest concentration of bisphenol A used in comprehensive testing was 10 µg/mL. This concentration was selected as the starting point because it was a single log dilution higher than the concentration giving the highest adjusted RLU value during range finder testing. The concentrations of bisphenol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

Table 10-5 Concentrations of N0002 - Bisphenol A Used in Comprehensive Testing

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

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

Figure 10-14 Agonist Comprehensive Testing for N0002 – Bisphenol A: Individual Experiments



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

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.

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.

Bisphenol A showed agonist activity in the three experiments that were conducted. EC_{50} values for individual experiments are shown in **Table 10-6**.

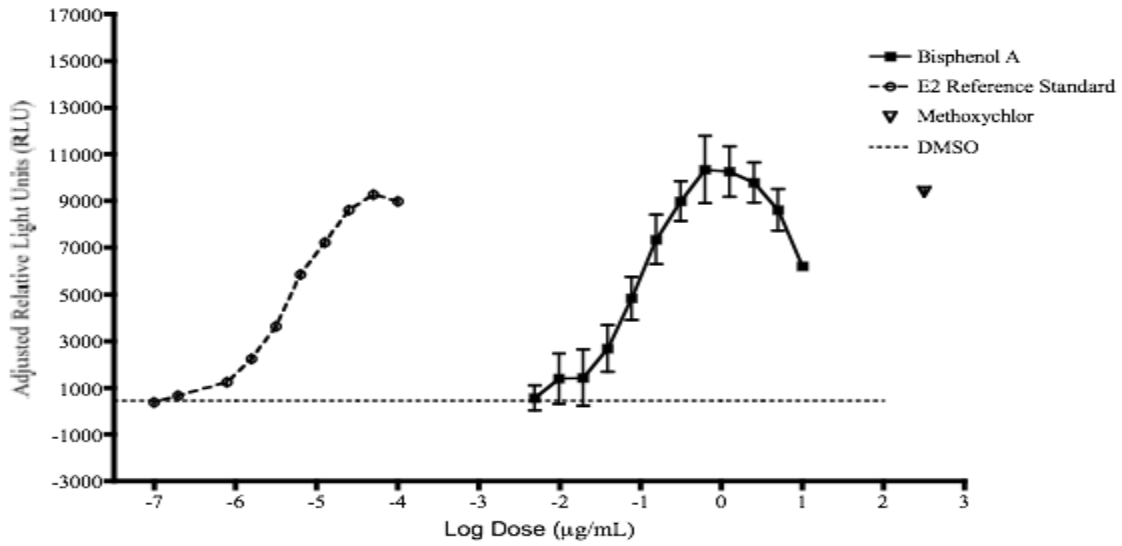
Table 10-6 Individual EC_{50} Values for N0002 – Bisphenol A

Experiment Date	EC_{50} (µg/mL)
3 April 06	7.55×10^{-2}
4 April 06	0.11
5 April 06	8.00×10^{-2}

Abbreviations: EC_{50} = half-maximal effect concentration

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

Figure 10-15 Agonist Comprehensive Testing for N0002 – Bisphenol A: Averaged Experiments



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

Historical mean and standard deviation of the E2 reference standard.

Historical mean and standard deviation of the methoxychlor control).

Horizontal line represents the historical mean of DMSO vehicle control 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.

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.

Bisphenol A was positive for agonism at all but the lowest concentration tested (7.96×10^{-2} µg/mL). The averaged EC_{50} value (**Table 10-7**) was calculated as the mean of three experiments.

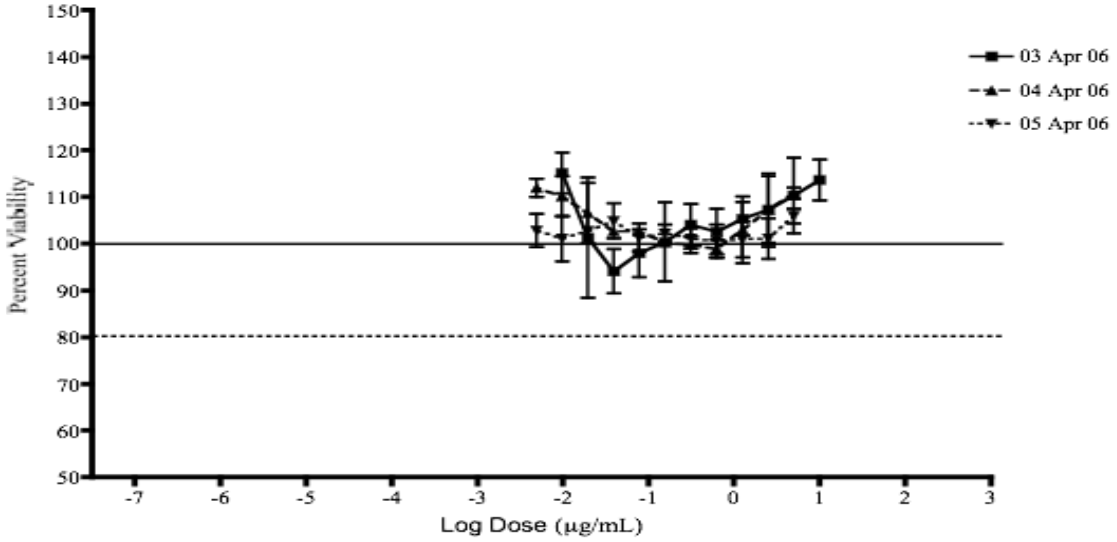
Table 10-7 Averaged EC_{50} Value for N0002 – Bisphenol A

EC_{50} (µg/mL)	STD DEV	CV
8.76×10^{-2}	1.75×10^{-2}	20%

Abbreviations: EC_{50} = half-maximal effect concentration; STD DEV = Standard Deviation of the Mean; CV = Coefficient of Variation

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

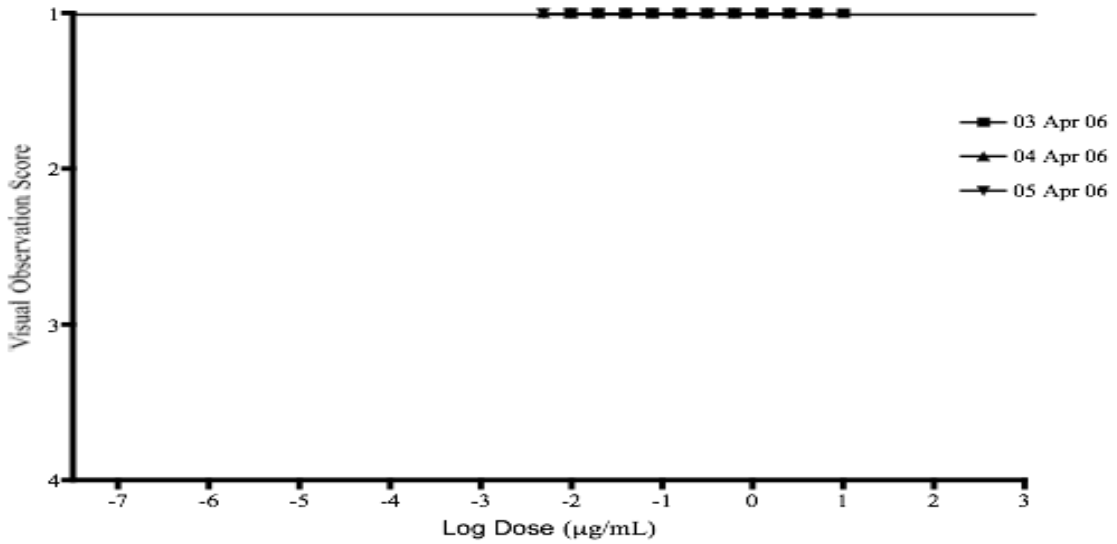
Figure 10-16 CellTiter-Glo® Viability Assessment for N0002 – Bisphenol A



Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

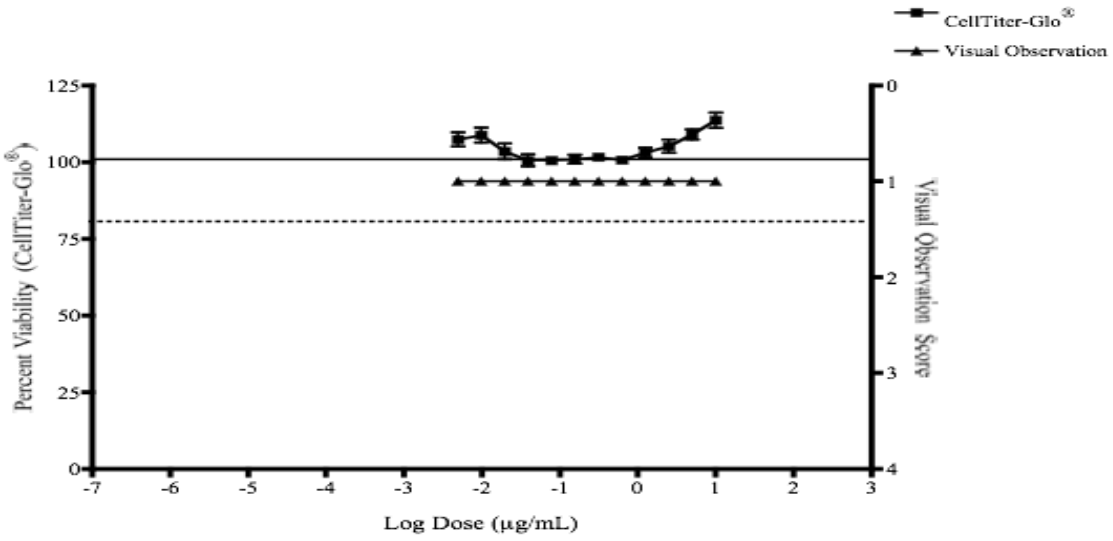
Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability below this line are considered cytotoxic and are not included in the evaluation of agonist activity.

Figure 10-17 Visual Observation Viability Assessment for N0002 – Bisphenol A



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 ≥ 2 are considered cytotoxic and are not included in the evaluation of antagonist activity.

Figure 10-18 Combined Qualitative and Quantitative Viability Assessment for N0002 – Bisphenol A



Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

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.

10.2.3 N0003 – Bisphenol B

Bisphenol B was selected for agonist testing because it was listed as moderately positive for ER agonist activity in the ICCVAM Guidelines (ICCVAM 2003, 2006).

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

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

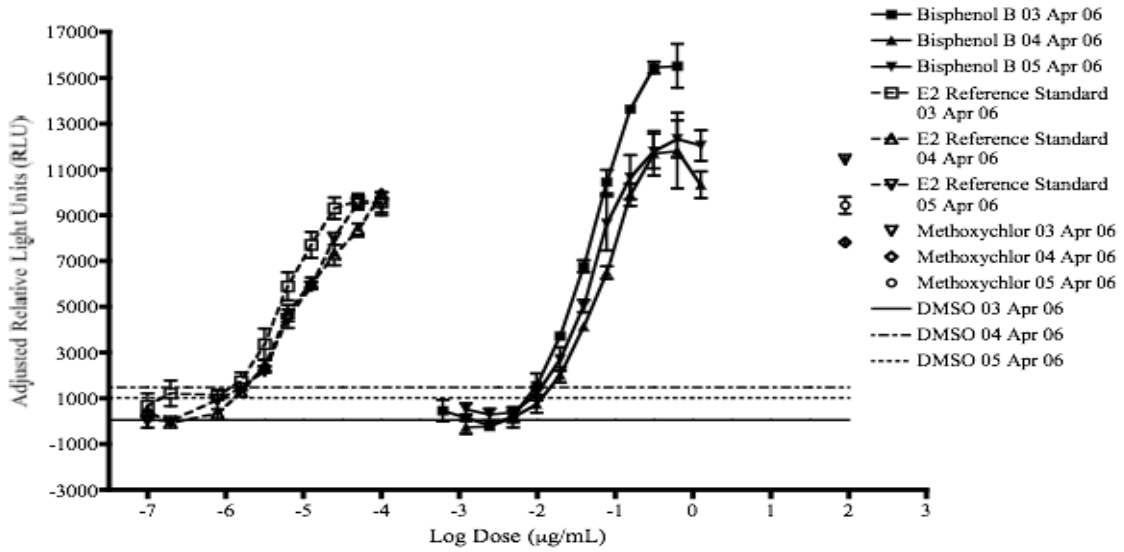
N0003 – Bisphenol B (µg/mL)		
1.25*	7.81×10^{-2}	4.88×10^{-3}
0.63#	3.91×10^{-2}	2.44×10^{-3}
0.31	1.95×10^{-2}	1.22×10^{-3}
0.16	9.77×10^{-3}	6.10×10^{-4}

* Final starting concentration for bisphenol B testing

\# Initial starting concentration for bisphenol B testing

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

Figure 10-19 Agonist Comprehensive Testing for N0003 – Bisphenol B: Individual Experiments



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

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.

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.

Bisphenol B showed agonist activity at the majority of concentrations tested. EC_{50} values for individual experiments are shown in **Table 10-9**.

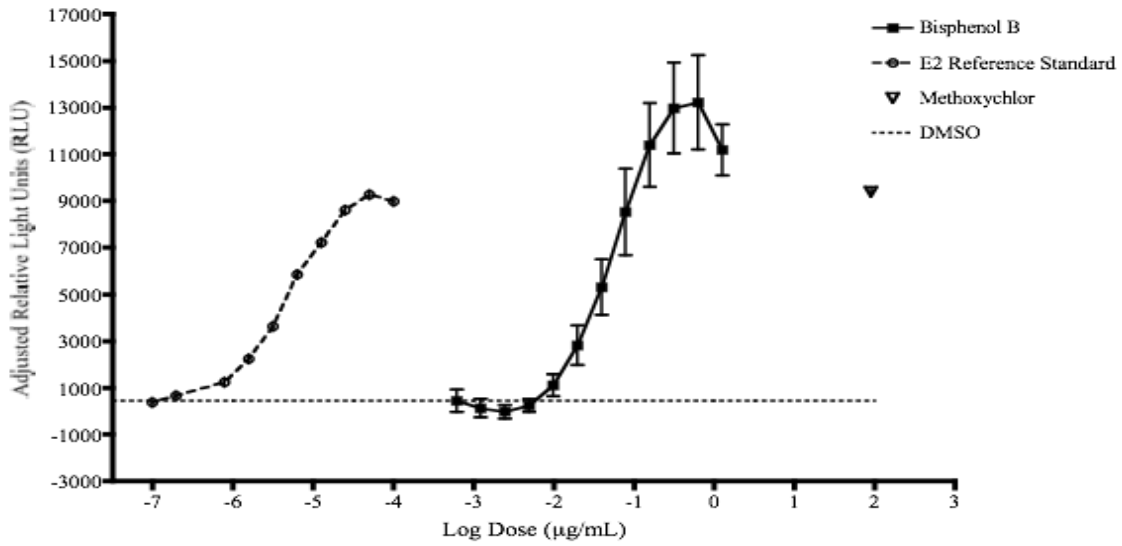
Table 10-9 Individual EC_{50} Values for N0003 – Bisphenol B

Experiment Date	EC_{50} (µg/mL)
3 April 06	4.90×10^{-2}
4 April 06	5.70×10^{-2}
5 April 06	4.90×10^{-2}

EC_{50} = half-maximal effect concentration

Results of averaged agonist experiments for bisphenol B are shown in **Figure 10-20**.

Figure 10-20 Agonist Comprehensive Testing for N0003 – Bisphenol B: Averaged Experiments



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

Historical mean and standard deviation of the E2 reference standard.

Historical mean and standard deviation of the positive methoxychlor control.

Horizontal line represents the historical mean of DMSO vehicle control 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.

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.

Bisphenol B was positive for agonism at the majority of concentrations tested. The averaged EC_{50} value (**Table 10-10**) was calculated as the mean of three experiments.

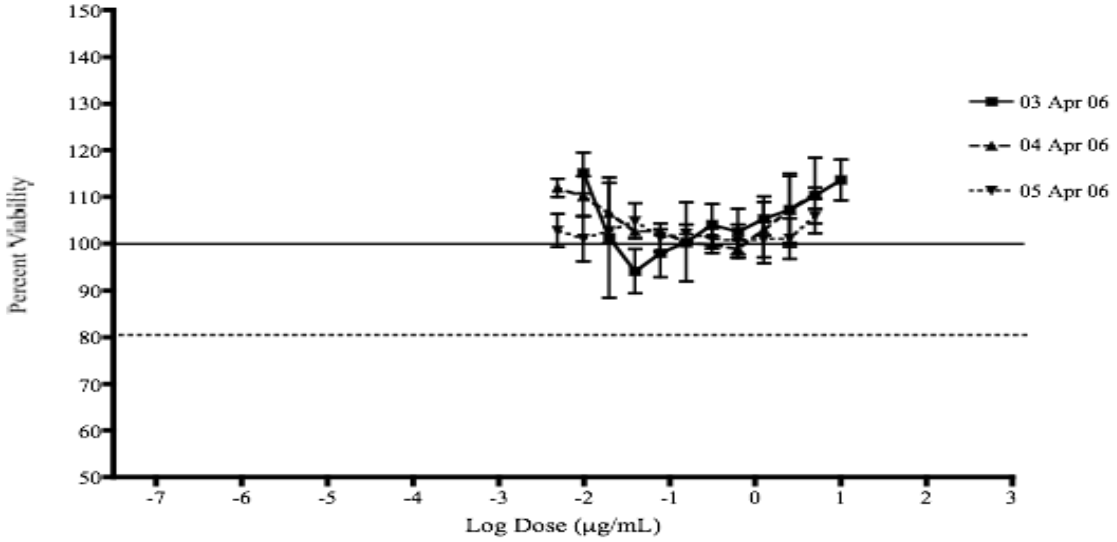
Table 10-10 Averaged EC_{50} Value for N0003 – Bisphenol B

EC_{50} (µg/mL)	STD DEV	CV
5.16×10^{-2}	4.63×10^{-3}	9%

Abbreviations: EC_{50} = half-maximal effect concentration; STD DEV = Standard Deviation of the Mean; CV = Coefficient of Variation

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

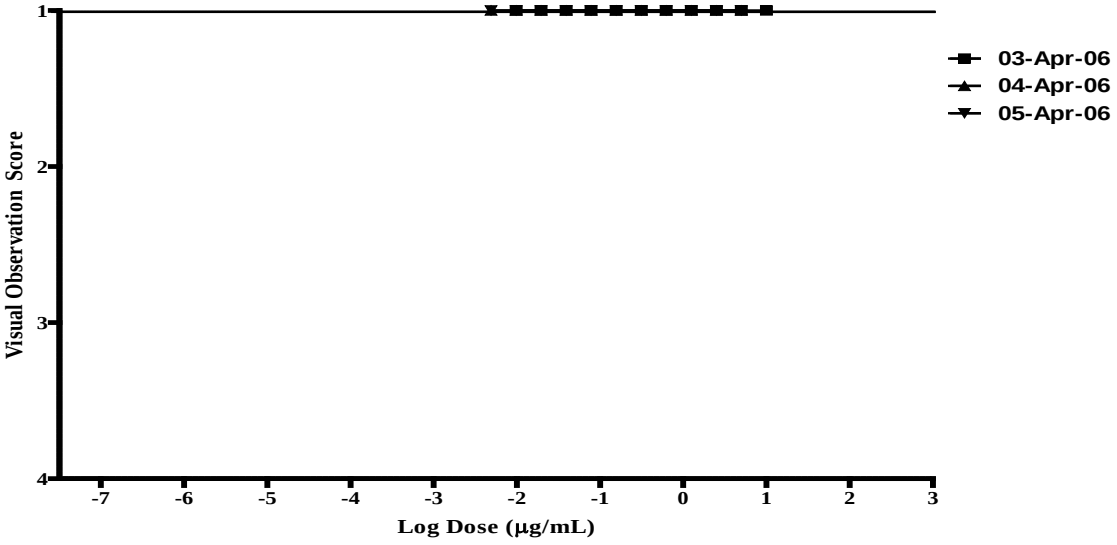
Figure 10-21 CellTiter-Glo® Viability Assessment for N0003 – Bisphenol B



Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

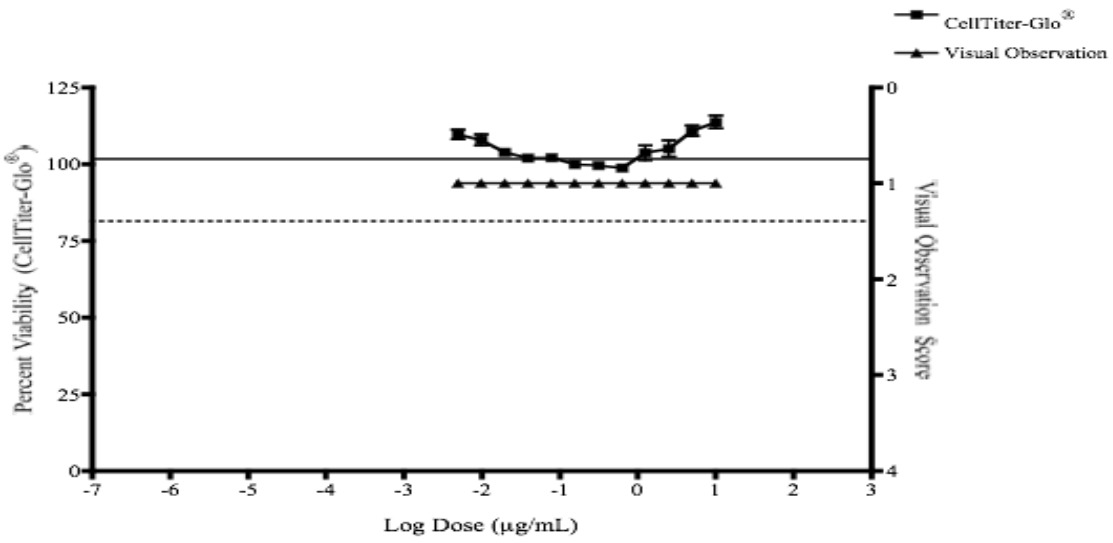
Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability below this line are considered cytotoxic and are not included in the evaluation of agonist activity.

Figure 10-22 Visual Observation Viability Assessment for N0003 – Bisphenol B



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 ≥ 2 are considered cytotoxic and are not included in the evaluation of antagonist activity.

Figure 10-23 Combined Qualitative and Quantitative Viability Assessment for N0003 – Bisphenol B



Solid horizontal line indicates 100% cell viability as measured in DMSO control.

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.

10.2.4 N0004 – Corticosterone

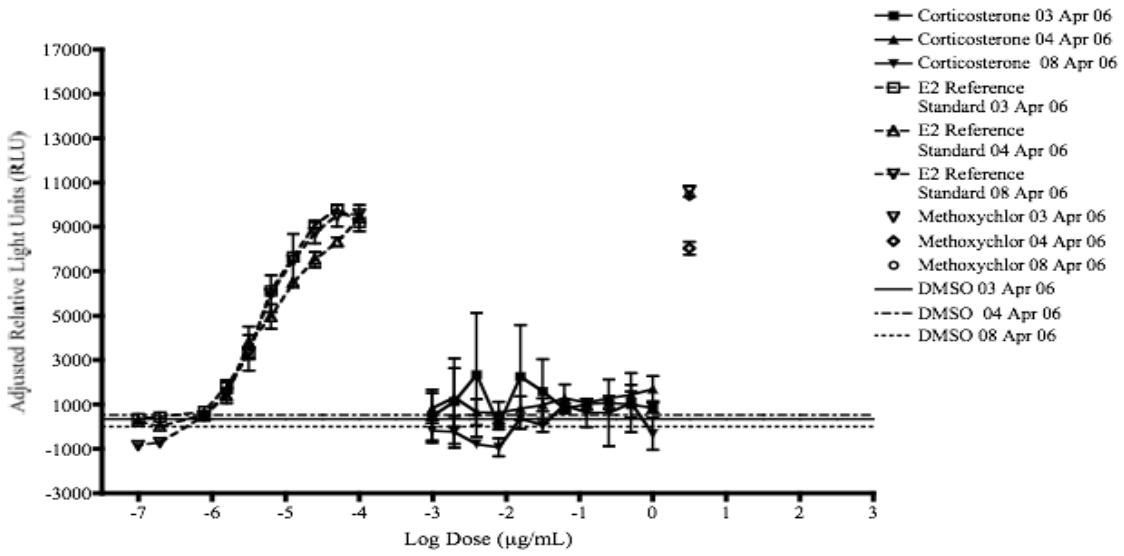
Corticosterone was selected for agonist testing because it was listed as negative for ER agonist activity in the ICCVAM Guidelines (ICCVAM 2003, 2006). The highest concentration of corticosterone used in comprehensive testing was 1 µg/mL. This concentration was selected as the starting point for a double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

Table 10-11 Concentrations of N0004 – Corticosterone Used in Comprehensive Testing

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

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

Figure 10-24 Agonist Comprehensive Testing for N0004 – Corticosterone: Individual Experiments



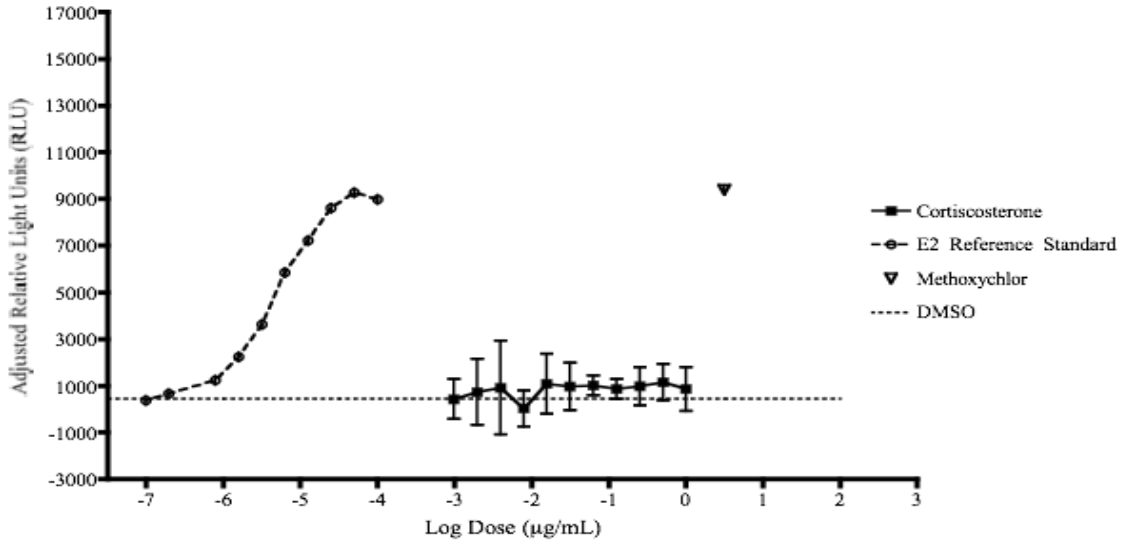
Abbreviations: E2 = 17ß-estradiol; Methoxychlor = 3.13 µg/mL methoxychlor control; DMSO = dimethyl sulfoxide.

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

On 3 April 2006, there were three concentrations of corticosterone (3.91×10^{-3}, 1.56×10^{-2}, 3.13×10^{-2} µg/mL) that yielded a positive response. However, this response was only observed in a single experiment, so corticosterone was classified as negative for agonism.

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

Figure 10-25 Agonist Comprehensive Testing for N0004 – Corticosterone: Averaged Experiments



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

Historical mean and standard deviation of the E2 reference standard.

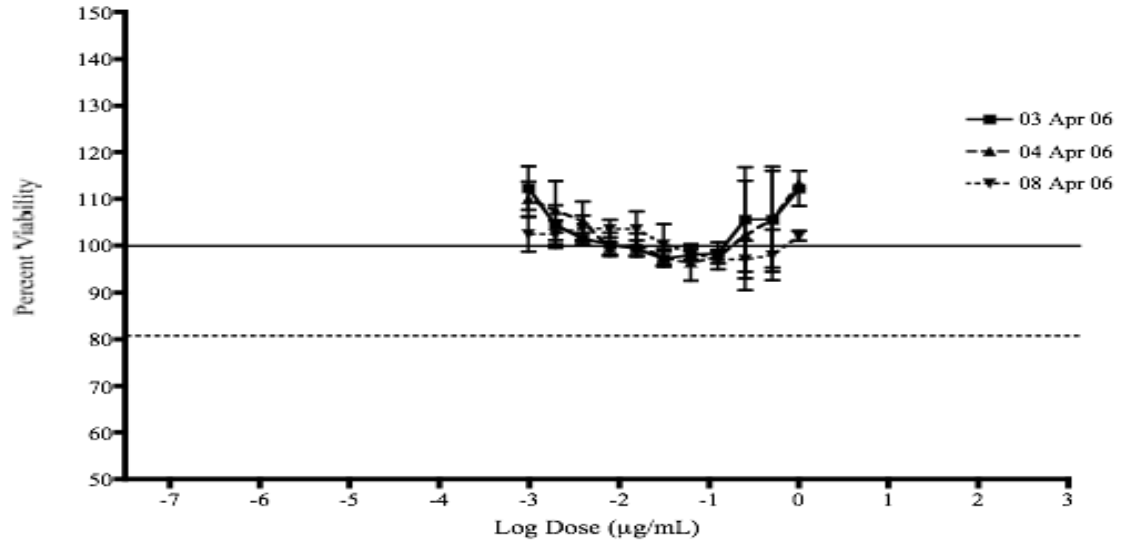
Historical mean and standard deviation of the methoxychlor control.

Horizontal line represents the historical mean of DMSO vehicle control 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.

Corticosterone was negative for agonism at all concentrations tested.

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

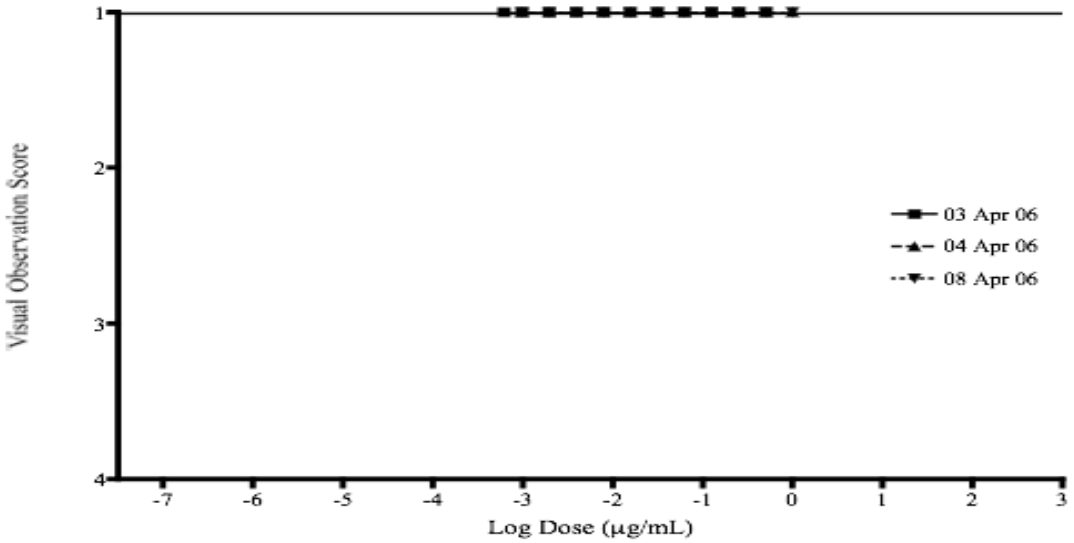
Figure 10-26 CellTiter-Glo® Viability Assessment for N0004 – Corticosterone



Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

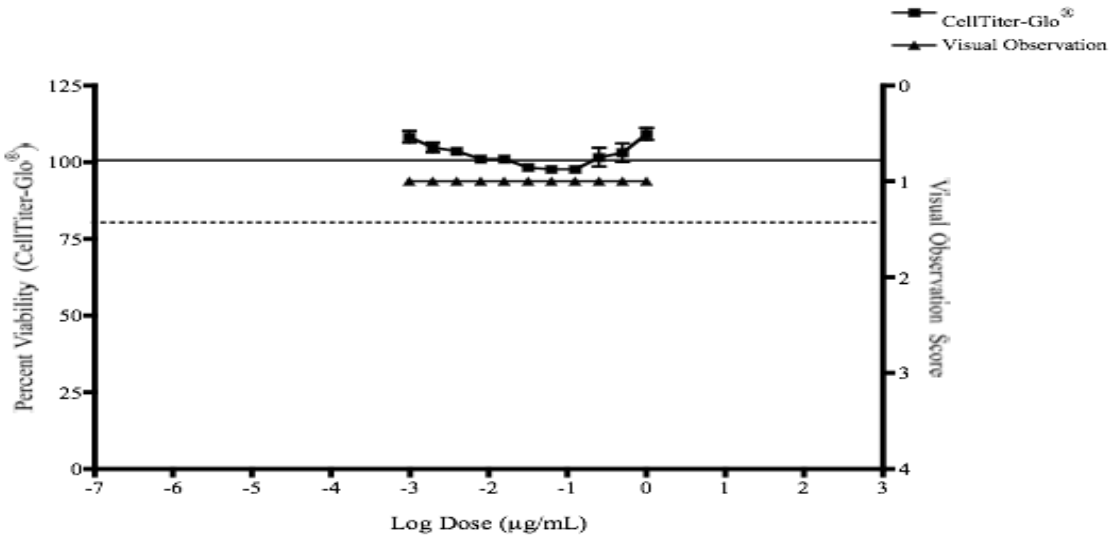
Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability below this line are considered cytotoxic and are not included in the evaluation of agonist activity.

Figure 10-27 Visual Observation Viability Assessment for N0004 – Corticosterone



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 ≥ 2 are considered cytotoxic and are not included in the evaluation of antagonist activity.

Figure 10-28 Combined Qualitative and Quantitative Viability Assessment for N0004 – Corticosterone



Solid horizontal line indicates 100% cell viability as measured in DMSO control.

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.

10.2.5 N0005 – *o,p'*-DDT

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

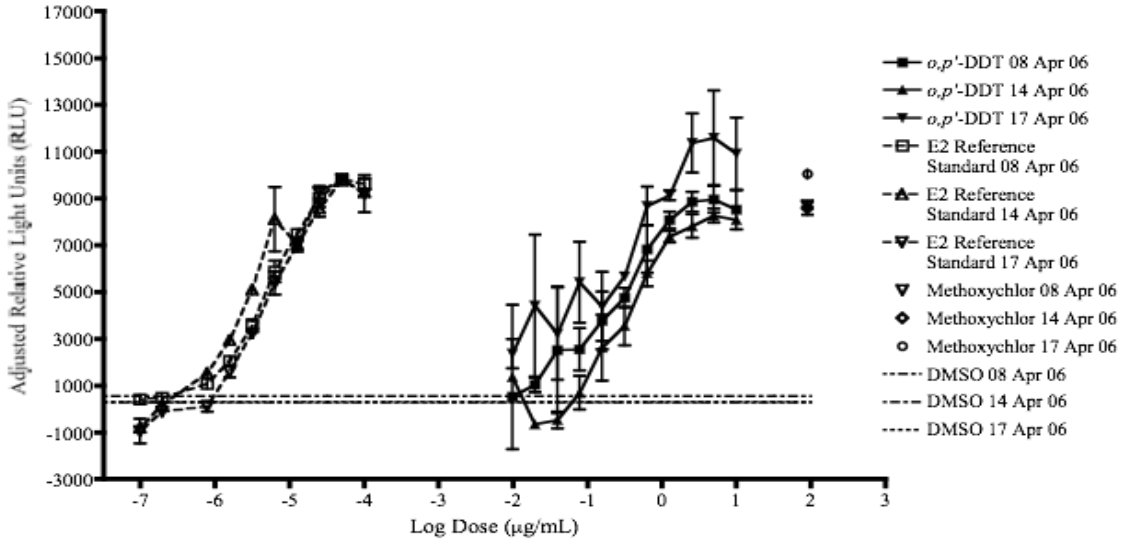
Table 10-12 Concentrations of *o,p'*-DDT Used in Comprehensive Testing

N0005 – *o,p'*-DDT (µg/mL)		
10	0.63	3.91×10^{-2}
5	0.31	1.95×10^{-2}
2.5	0.16	9.77×10^{-3}
1.25	7.81×10^{-2}	

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

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

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



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

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.

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.

o,p'-DDT showed agonist activity in all experiments conducted. EC_{50} values for individual experiments are shown in **Table 10-13**.

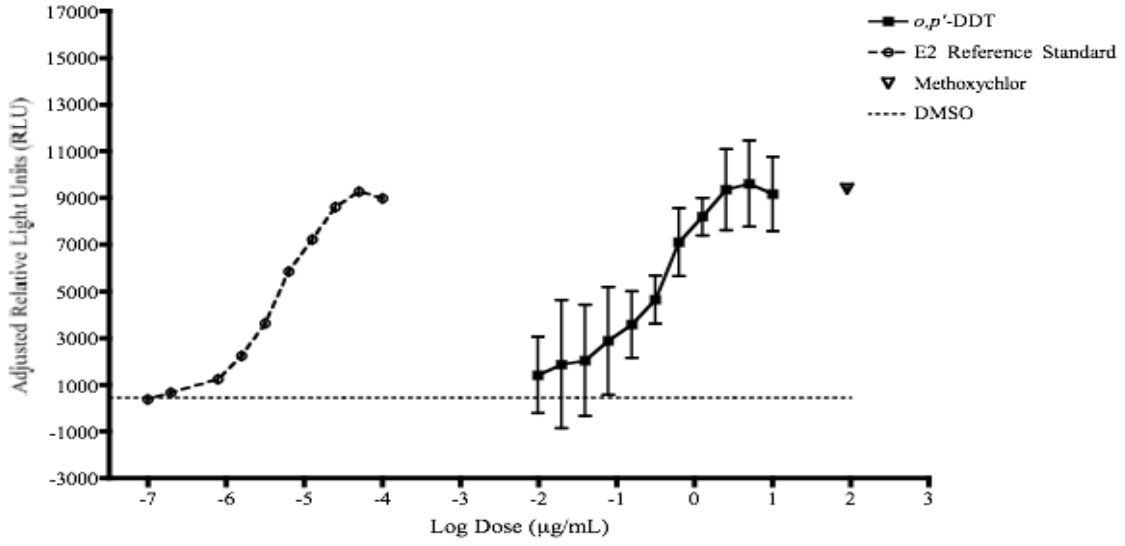
Table 10-13 Individual EC_{50} Values for N0005 – *o,p'*-DDT

Experiment Date	EC_{50} (µg/mL)
8 April 06	0.28
14 April 06	0.34
17 April 06	0.53

Abbreviations: EC_{50} = half-maximal effect concentration; *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane

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

Figure 10-30 Agonist Comprehensive Testing for N0005 – *o,p'*-DDT: Averaged Experiments



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

Historical mean and standard deviation of the E2 reference standard.

Historical mean and standard deviation of the methoxychlor control).

Horizontal line represents the historical mean of DMSO vehicle control 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.

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.

o,p'-DDT was positive for agonism at the majority of concentrations tested. The averaged EC_{50} (**Table 10-14**) value was calculated as the mean of three experiments.

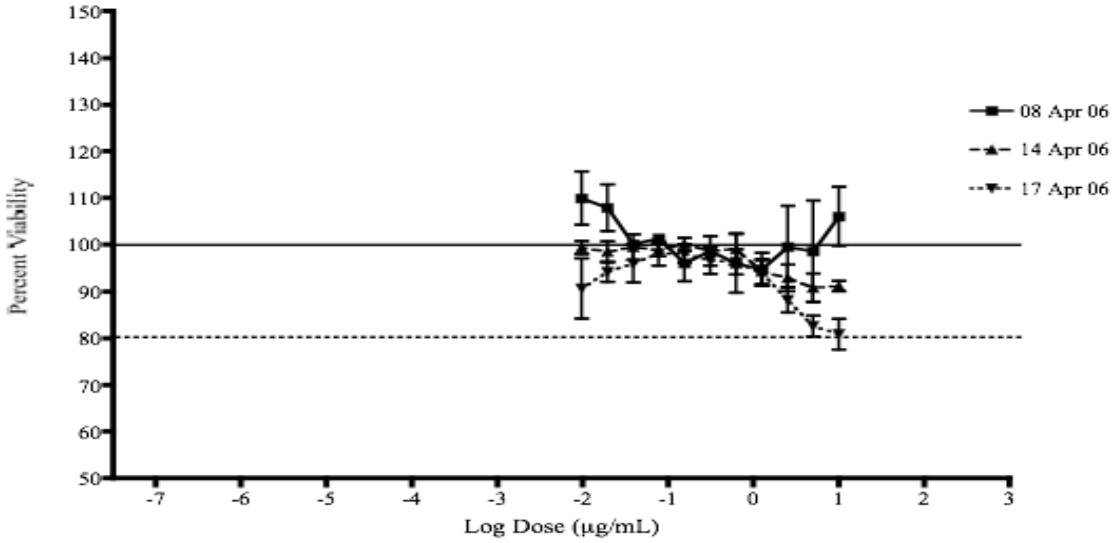
Table 10-14 Averaged EC_{50} Value for N0005 – *o,p'*-DDT

EC_{50} (µg/mL)	STD DEV	CV
0.38	0.13	34%

Abbreviations: EC_{50} = half-maximal effect concentration; *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane; STD DEV = Standard Deviation; CV = Coefficient of Variation

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

Figure 10-31 CellTiter-Glo® Viability Assessment for N0005 – *o.p'*-DDT

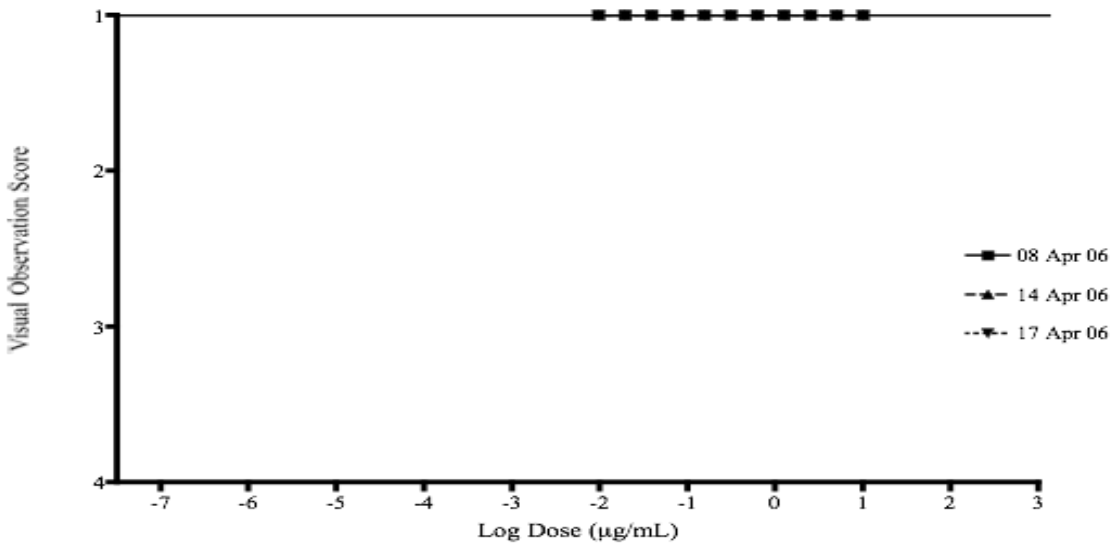


Abbreviations: *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane.

Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

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.

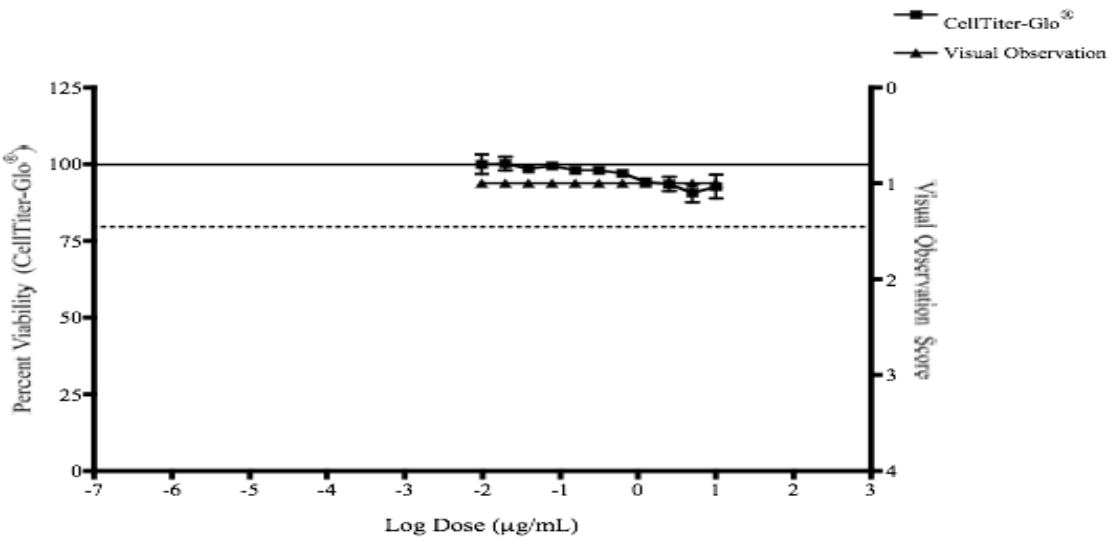
Figure 10-32 Visual Observation Viability Assessment for N0005 – *o,p'*-DDT



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

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 ≥ 2 are considered cytotoxic and are not included in the evaluation of antagonist activity.

Figure 10-33 Combined Qualitative and Quantitative Viability Assessment for N0005 – *o,p'*-DDT



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

Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

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.

10.2.6 N0006 – Diethylstilbestrol

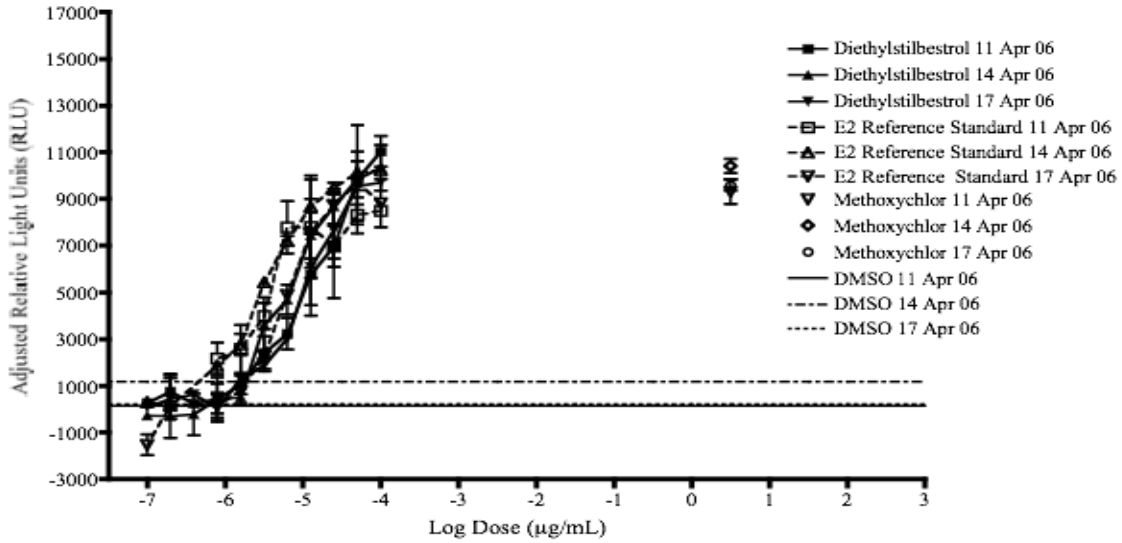
Diethylstilbestrol was selected for agonist testing because it was listed as strongly positive for ER agonist activity in the ICCVAM Guidelines (ICCVAM 2003, 2006). The highest concentration of diethylstilbestrol used in comprehensive testing was 1.00×10^{-4} µg/mL. This concentration was selected as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

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

N0006 – Diethylstilbestrol (µg/mL)		
1.00×10^{-4}	6.25×10^{-6}	3.91×10^{-7}
5.00×10^{-5}	3.13×10^{-6}	1.95×10^{-7}
2.50×10^{-5}	1.56×10^{-6}	9.77×10^{-8}
1.25×10^{-5}	7.81×10^{-7}	

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

Figure 10-34 Agonist Comprehensive Testing for N0006 – Diethylstilbestrol: Individual Experiments



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

Diethylstilbestrol showed agonist activity in all experiments conducted. EC_{50} values for individual experiments are shown in **Table 10-16**.

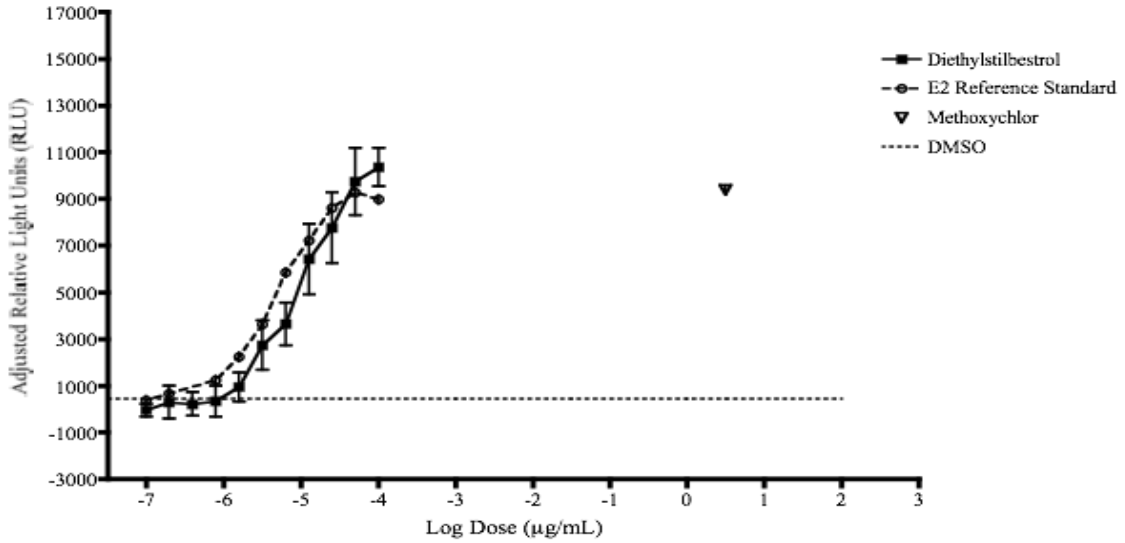
Table 10-16 Individual EC_{50} Values for N0006 – Diethylstilbestrol

Experiment Date	EC_{50} (µg/mL)
8 April 06	2.02×10^{-5}
14 April 06	6.60×10^{-6}
17 April 06	1.09×10^{-5}

Abbreviations: EC_{50} = half-maximal effect concentration

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

Figure 10-35 Agonist Comprehensive Testing for N0006 – Diethylstilbestrol: Averaged Experiments



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

Historical mean and standard deviation of the E2 reference standard.

Historical mean and standard deviation of the methoxychlor control.

Horizontal line represents the historical mean of DMSO vehicle control 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.

Diethylstilbestrol was positive for agonism at the majority of concentrations tested. The averaged EC_{50} (**Table 10-17**) value was calculated as the mean of three experiments.

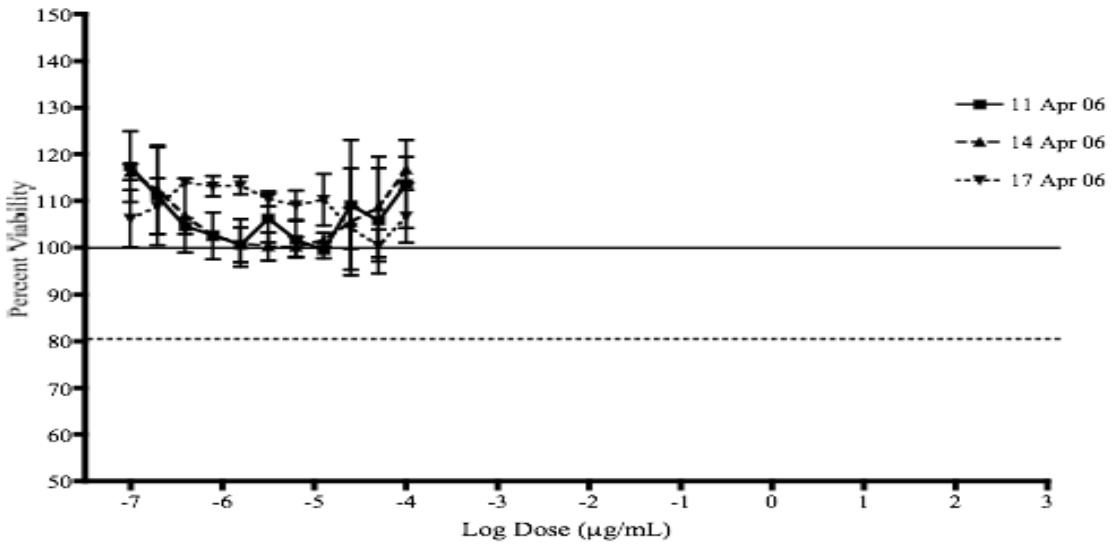
Table 10-17 Averaged EC_{50} Value for N0006 – Diethylstilbestrol

EC_{50} (µg/mL)	STD DEV	CV
1.26×10^{-5}	7.00×10^{-6}	55%

Abbreviations: EC_{50} = half-maximal effect concentration; STD DEV = Standard Deviation of the Mean; CV = Coefficient of Variation

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

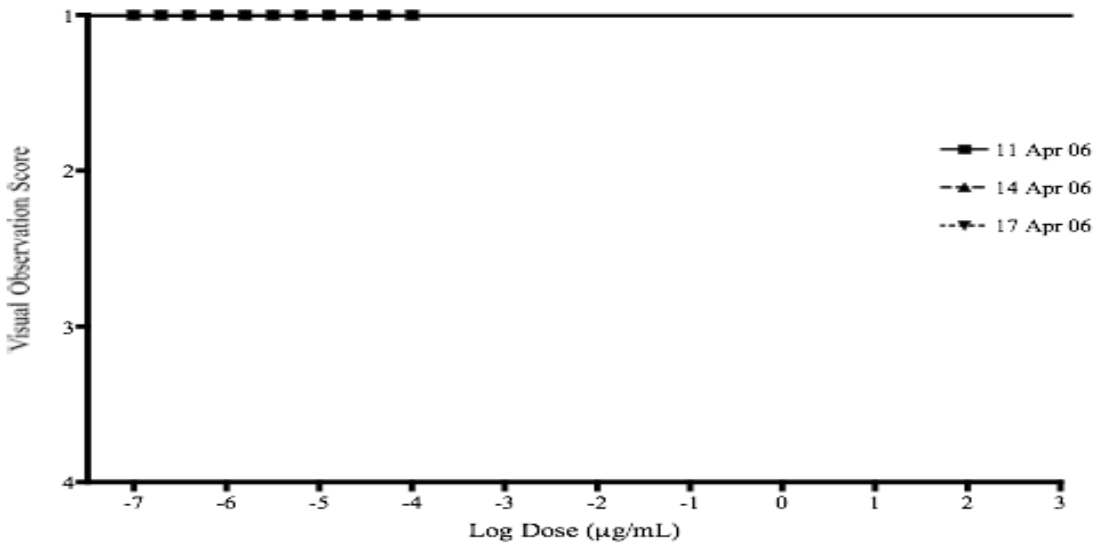
Figure 10-36 CellTiter-Glo® Viability Assessment for N0006 – Diethylstilbestrol



Solid horizontal line indicates 100% cell viability as measured in DMSO control.

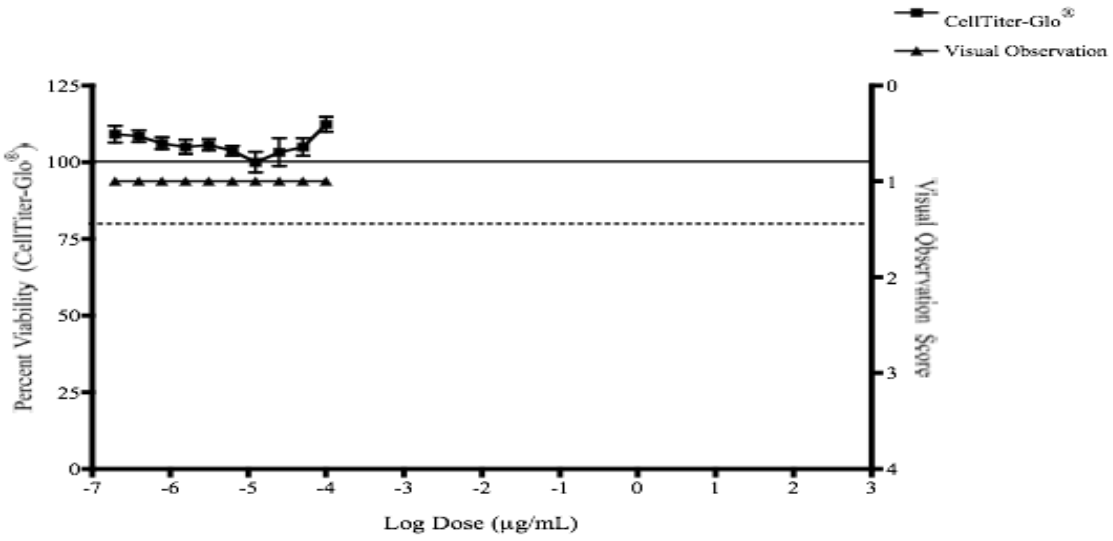
Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability below this line are considered cytotoxic and are not included in the evaluation of agonist activity.

Figure 10-37 Visual Observation Viability Assessment for N0006 – Diethylstilbestrol



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 ≥ 2 are considered cytotoxic and are not included in the evaluation of antagonist activity.

Figure 10-38 Combined Qualitative and Quantitative Viability Assessment for N0006 – Diethylstilbestrol



Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

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.

10.2.7 N0007 – EE

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

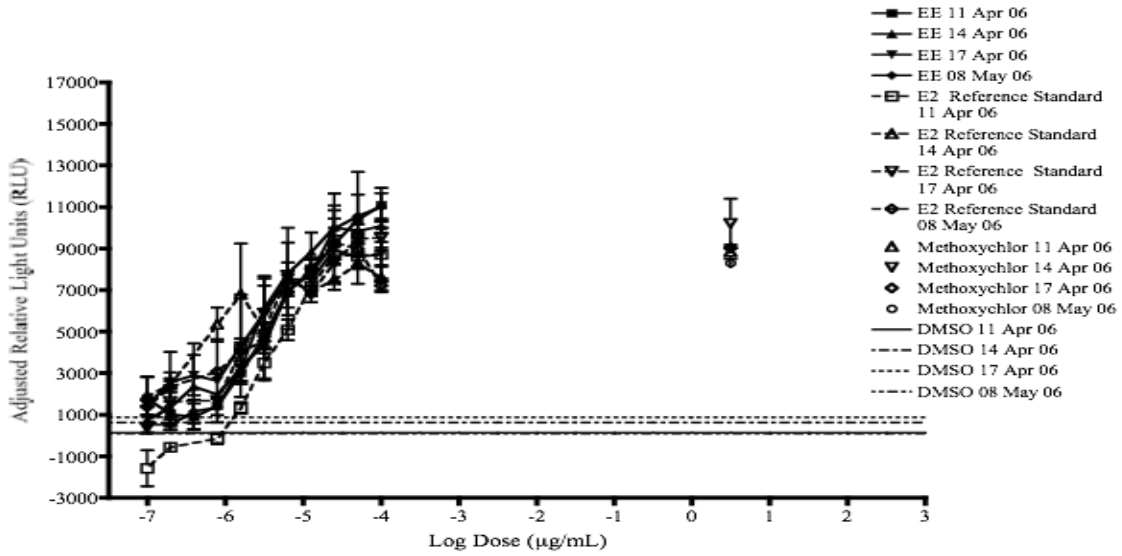
Table 10-18 Concentrations of N0007 - EE Used in Comprehensive Testing

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

Abbreviations: EE = 17α-ethinyl estradiol

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

Figure 10-39 Agonist Comprehensive Testing for N0007 – EE: Individual Experiments



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

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.

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

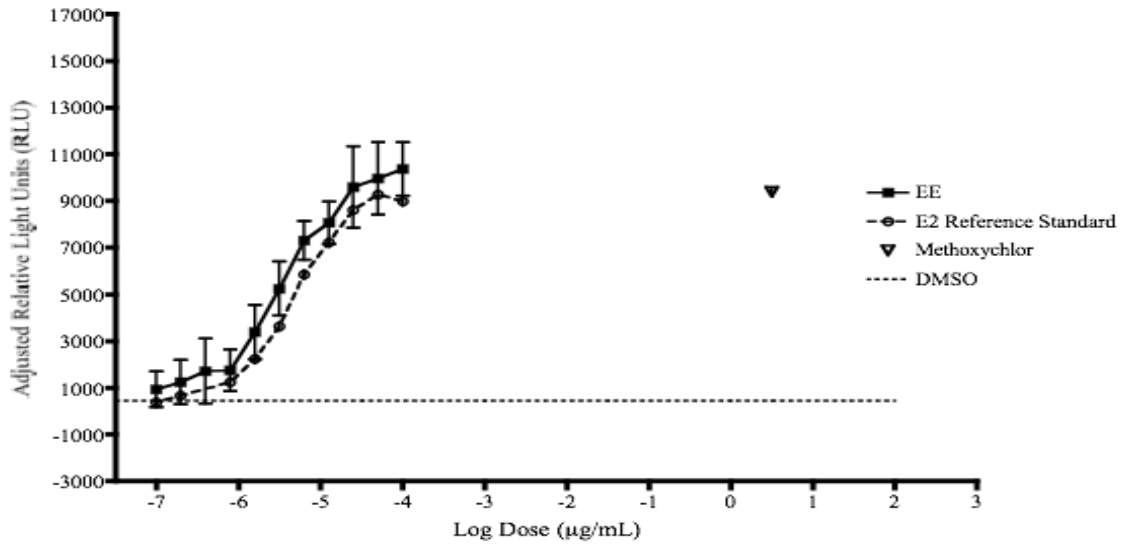
Table 10-19 Individual EC_{50} Values for N0007 – EE

Experiment Date	EC_{50} (µg/mL)
11 April 06	5.07×10^{-6}
14 April 06	3.00×10^{-6}
17 April 06	Not Calculated
8 May 06	4.90×10^{-6}

Abbreviations: EC_{50} = half-maximal effect concentration; EE = 17α-ethinyl estradiol

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

Figure 10-40 Agonist Comprehensive Testing for N0007 – EE: Averaged Experiments



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

Historical mean and standard deviation of the E2 reference standard.

Historical mean and standard deviation of the methoxychlor control.

Horizontal line represents the historical mean of DMSO vehicle control 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.

EE was positive for agonism at the majority of concentrations tested. The averaged EC_{50} (**Table 10-20**) value was calculated as the mean of three experiments.

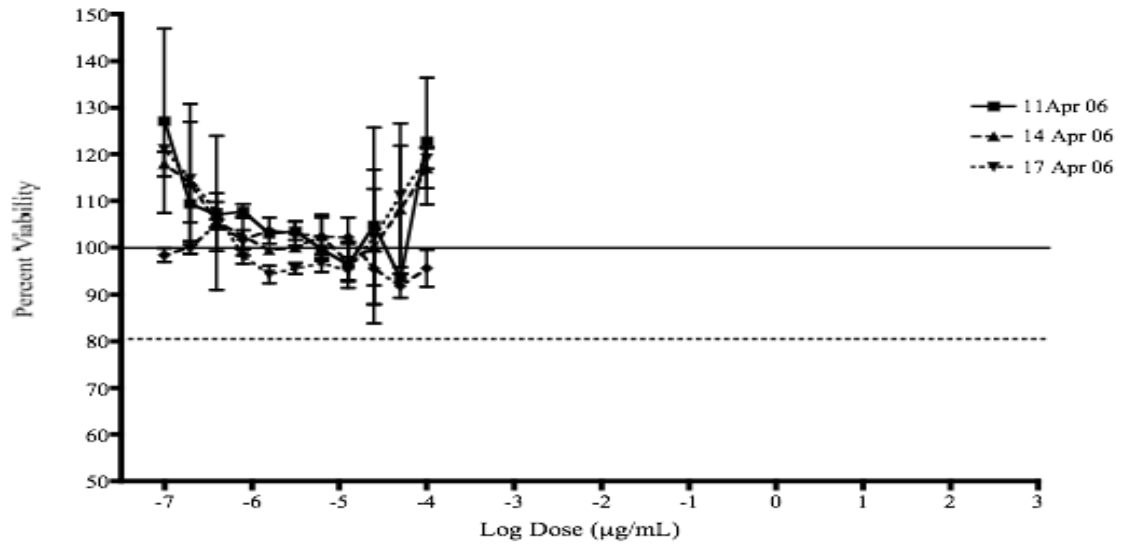
Table 10-20 Averaged EC_{50} Value for N0007 – EE

EC_{50} (µg/mL)	STD DEV	CV
3.87×10^{-6}	1.31×10^{-6}	34%

Abbreviations: CV = Coefficient of Variation ;EC_{50} = half-maximal effect concentration; EE = 17α-ethinyl estradiol; STD DEV = Standard Deviation of the Mean

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

Figure 10-41 CellTiter-Glo® Viability Assessment for N0007 – EE

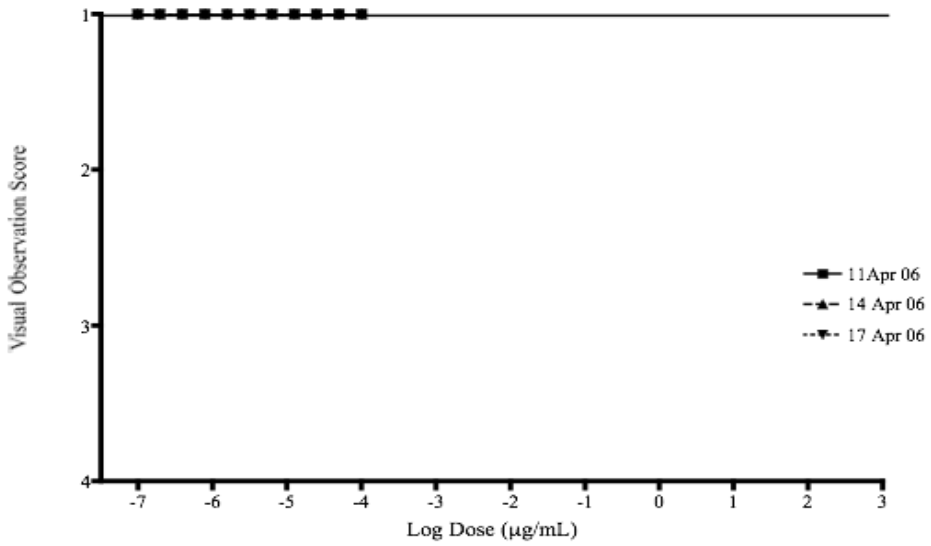


Abbreviations: EE = 17α-ethinyl estradiol

Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

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.

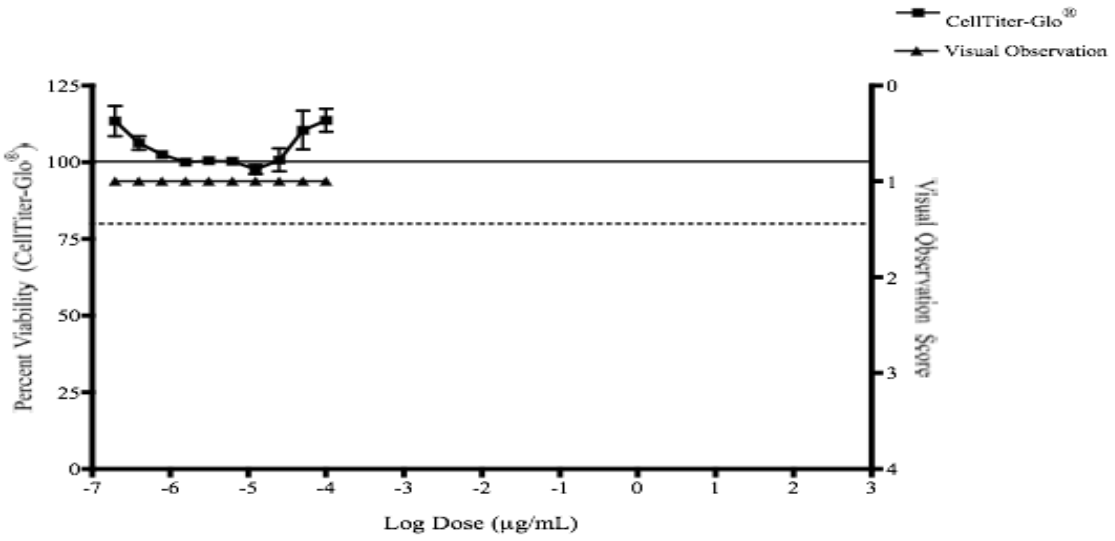
Figure 10-42 Visual Observation Viability Assessment for N0007 – EE



Abbreviations: EE = 17α-ethinyl estradiol

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 ≥ 2 are considered cytotoxic and are not included in the evaluation of antagonist activity.

Figure 10-43 Combined Qualitative and Quantitative Viability Assessments for N0007 – EE



Abbreviations: EE = 17α-ethinyl estradiol

Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

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.

10.2.8 N0008 – Flavone

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

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

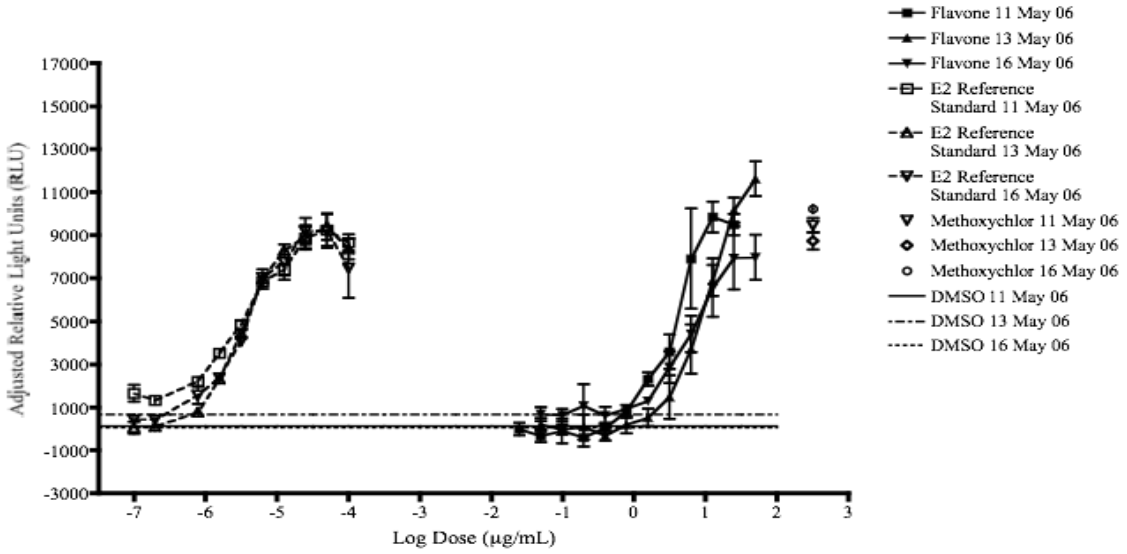
N0008 – Flavone (µg/mL)		
50[#]	3.13	0.2
25[*]	1.56	9.77×10^{-2}
12.5	0.78	4.88×10^{-2}
6.25	0.39	2.44×10^{-2}

[#] Final starting concentration for flavone testing.

[*] Initial starting concentration for flavone testing.

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

Figure 10-44 Agonist Comprehensive Testing for N0008 – Flavone: Individual Experiments



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

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.

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.

Flavone showed agonist activity at the majority of concentrations tested. EC_{50} values for individual experiments are shown in **Table 10-22**.

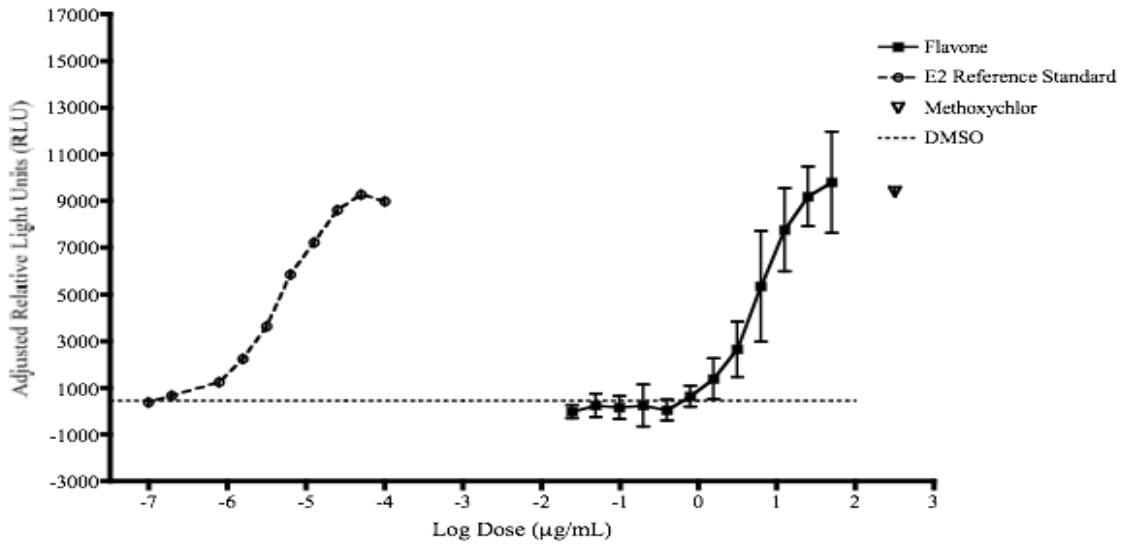
Table 10-22 Individual EC_{50} Values for N0008 – Flavone

Experiment Date	EC_{50} (µg/mL)
11 May 06	3.64
13 May 06	11
16 May 06	6.13

Abbreviations: EC_{50} = half-maximal effect concentration;

Results of averaged agonist experiments for flavone are shown in **Figure 10-45**.

Figure 10-45 Agonist Comprehensive Testing for N0008 – Flavone: Averaged Experiments



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

Historical mean and standard deviation of the E2 reference standard.

Historical mean and standard deviation of the methoxychlor control.

Horizontal line represents the historical mean of DMSO vehicle control 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.

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.

Flavone was positive for agonism at the majority of concentrations tested. The averaged EC_{50} (**Table 10-23**) value was calculated as the mean of three experiments.

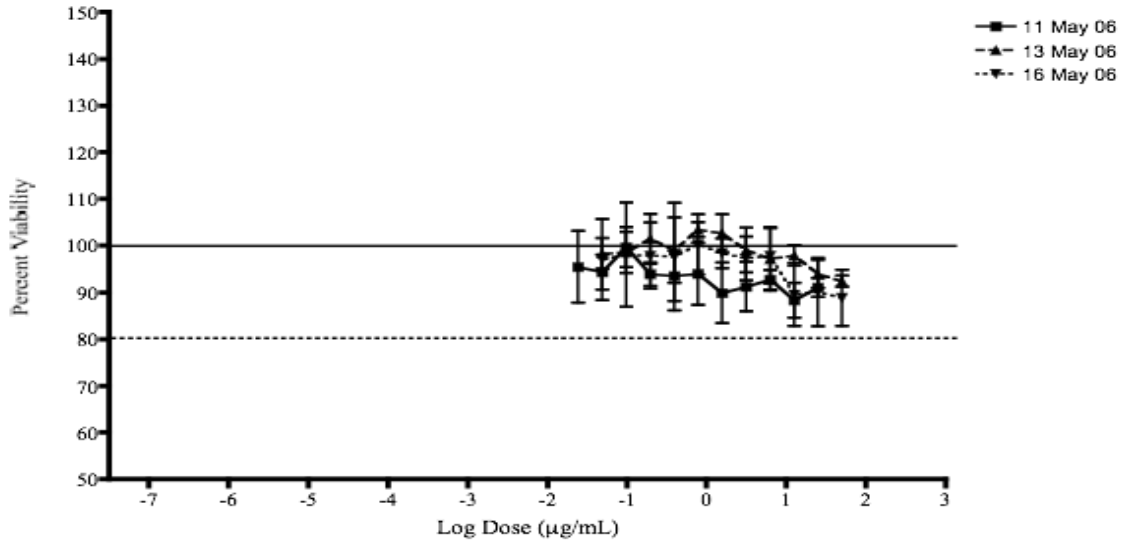
Table 10-23 Averaged EC_{50} Value for N0008 – Flavone

EC_{50} (µg/mL)	STD DEV	CV
6.88	3.67	53%

Abbreviations: EC_{50} = half-maximal effect concentration; STD DEV = Standard Deviation of the Mean; CV = Coefficient of Variation

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

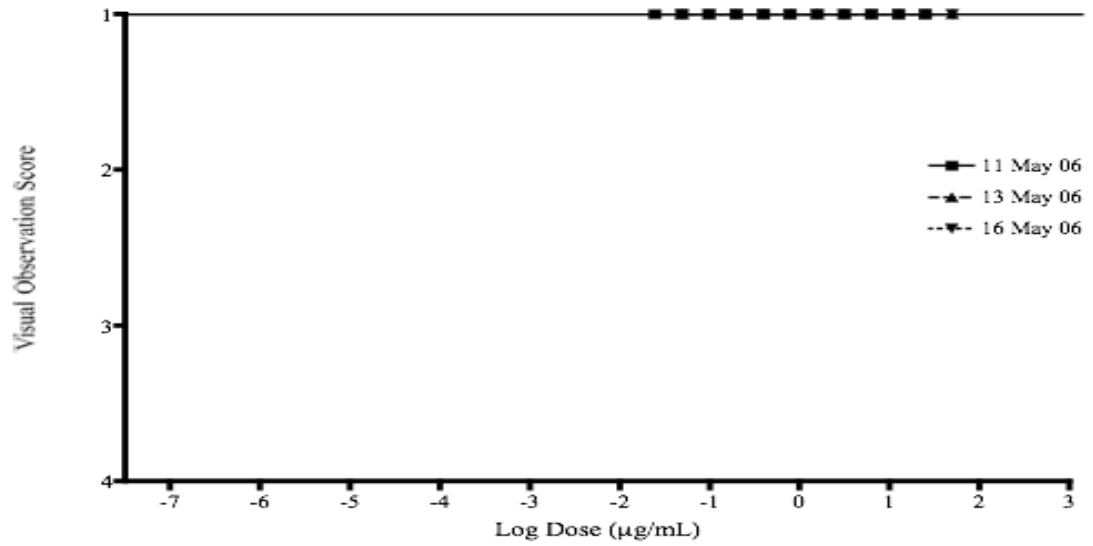
Figure 10-46 CellTiter-Glo® Viability Assessment for N0008 – Flavone



Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

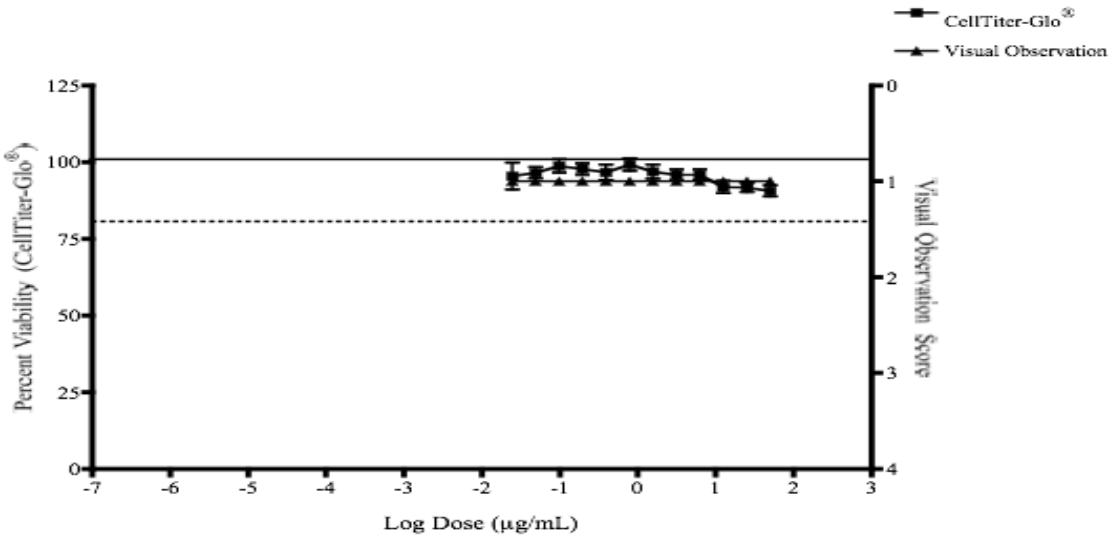
Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability below this line are considered cytotoxic and are not included in the evaluation of agonist activity.

Figure 10-47 Visual Observation Viability Assessment for N0008 – Flavone



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 ≥ 2 are considered cytotoxic and are not included in the evaluation of antagonist activity.

Figure 10-48 Combined Qualitative and Quantitative Viability Assessment for N0008 – Flavone



Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

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.

11.0 General Procedure for Antagonist Testing

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

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

Raloxifene Concentrations (µg/mL)		
1.25 x 10^{-2}	1.56 x 10^{-3}	1.95 x 10^{-4}
6.25 x 10^{-3}	7.81 x 10^{-4}	9.77 x 10^{-5}
3.13 x 10^{-3}	3.91 x 10^{-4}	4.88 x 10^{-5}

Abbreviations: Ral/E2 = concentrations of raloxifene with a fixed concentration of 2.5 x 10^{-5} µg/mL 17β–estradiol

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

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\text{logIC50} - X)\text{HillSlope}}}$$

where Y= response (i.e., relative light units), X is the logarithm of concentration, Bottom is the minimum response, Top is the maximum response, log IC_{50} is the logarithm of X as the response midway between Top and Bottom, and HillSlope describes the steepness of the curve. The model calculates the best fit for the Top, Bottom, HillSlope, and IC_{50} parameters.

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

- Reduction – Plate reduction (i.e., the highest Ral/E2 reference standard RLU value divided by the lowest Ral/E2 reference standard RLU value) must be greater than three-fold.
- Reference standard results – Calculated Ral/E2 reference standard IC_{50} values must be within 2.5 times the standard deviation of the historical database IC_{50} mean values.

[4] 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.

- DMSO control results - DMSO control RLU values must be within 2.5 times the standard deviation of the historical database DMSO control mean RLU values.
- Flavone and E2 control results – Flavone and E2 control RLU values must be within 2.5 times the standard deviation of the historical database flavone and E2 control mean RLU values.

12.0 Antagonist Testing

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

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

Table 12-1 Test Substances for Antagonist Testing

Code	Substance Name	CASRN	ER TA Antagonist Activity[1,2,3]	Additional Basis for Selection[4]
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	*p*-n-nonylphenol	104-40-5	#	
N0014	Progesterone	57-83-0	-	
N0015	*o*,*p'*-DDT	789-02-6	#	Cytotoxic
N0016	Tamoxifen	10540-29-1	###	Cytotoxic

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

[1] Data on antagonist activities were derived from ICCVAM (2006)

[2] ### 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 tested; # indicates that the substance was positive in the single assay in which it was tested; #- indicates the substance was positive in one assay but was also negative in one or more assays; - indicates that the substance was uniformly negative in multiple assays

[3] Antag = Antagonist

[4] Information on solubility and cytotoxicity were derived from the scientific literature.

12.1 Antagonist Range Finding

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

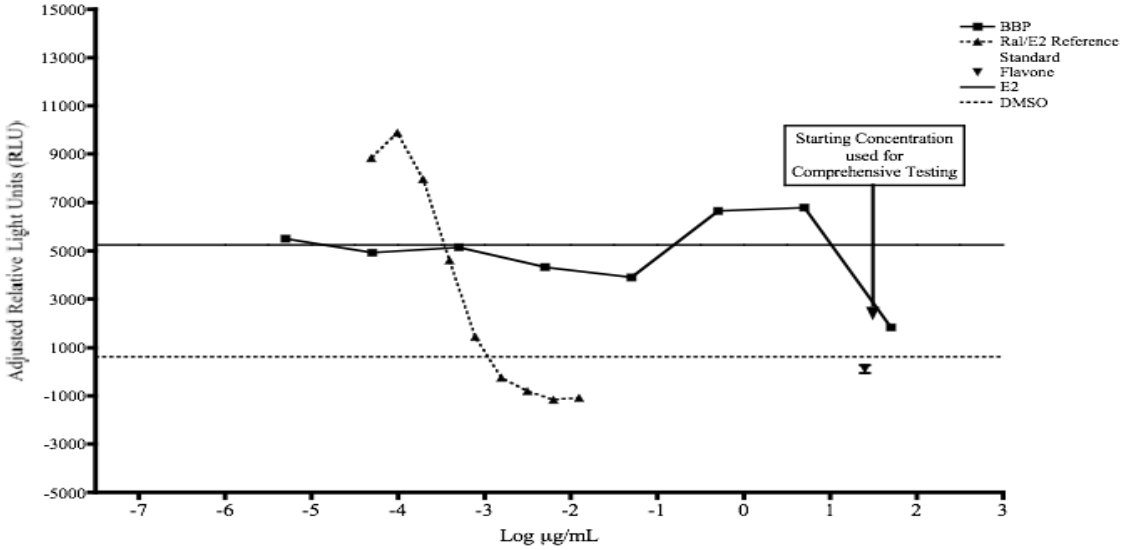
Table 12-2 Antagonist Range Finder Concentrations for Coded Substances

Range Finder Concentrations (µg/mL)		
50	5.00×10^{-2}	5.00×10^{-5}
5	5.00×10^{-3}	5.00×10^{-6}
0.5	5.00×10^{-4}	

All concentrations of test substance were run in conjunction with 2.50×10^{-3}µg/mL E2.

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

Figure 12-1 Antagonist Range Finder for N0009 – BBP

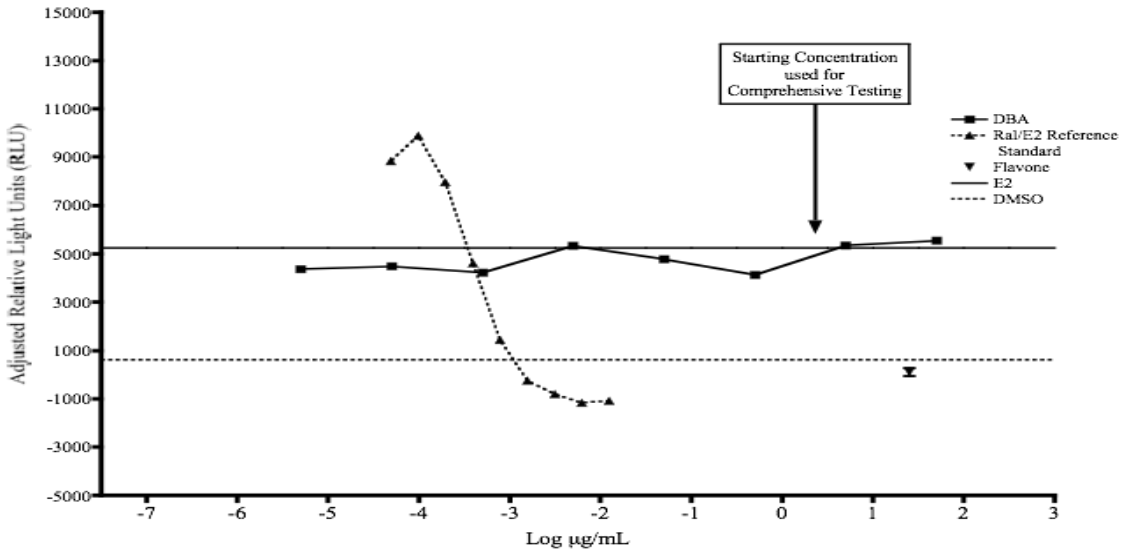


Abbreviations: BBP = Butylbenzyl phthalate; Ral/E2 Reference Standard = varying concentrations of raloxifene with a fixed concentration of 2.5×10^{-5} µg/mL 17β–estradiol; Flavone = 25 µg/mL Flavone + 2.5×10^{-5} µM 17β–estradiol; E2 = 2.5×10^{-5} µg/mL 17β–estradiol; DMSO = dimethyl sulfoxide.

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.

Dashed horizontal line represents the mean of three DMSO control replicates plus three times the standard deviation of the DMSO control mean.

Figure 12-2 Antagonist Range Finder for N0010 – DBA

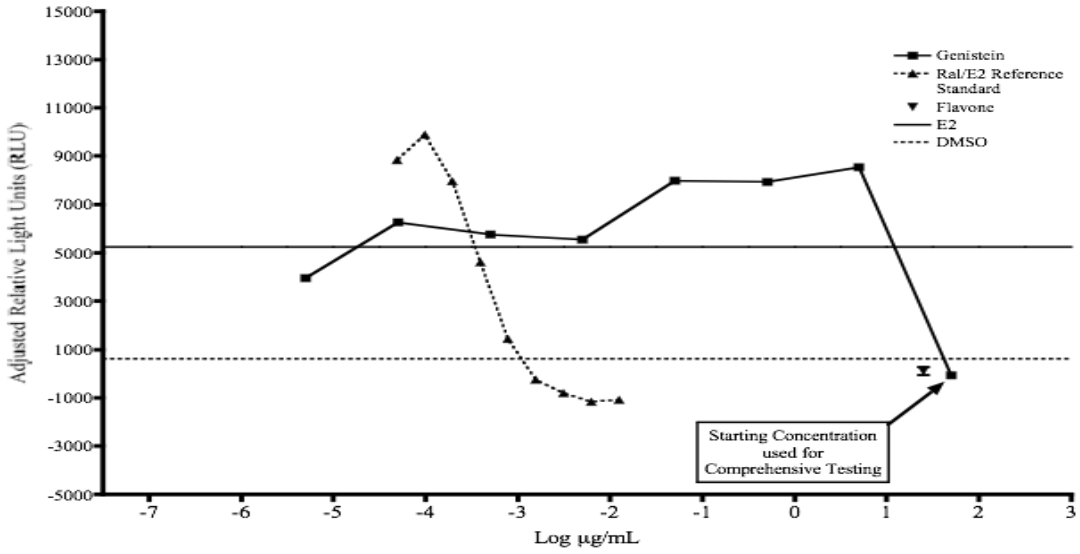


Abbreviations: DBA = Dibenzo [a,h] anthracene; Ral/E2 Reference Standard = varying concentrations of raloxifene with a fixed concentration of 2.5 x 10^{-5} µg/mL 17β–estradiol; Flavone = 25 µg/mL Flavone + 2.5 x 10^{-5} µM 17β–estradiol; E2 = 2.5 x 10^{-5} µg/mL 17β–estradiol; DMSO = dimethyl sulfoxide.

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.

Dashed horizontal line represents the mean of three DMSO control replicates plus three times the standard deviation of the DMSO control mean.

Figure 12-3 Antagonist Range Finder for N0011 – Genistein

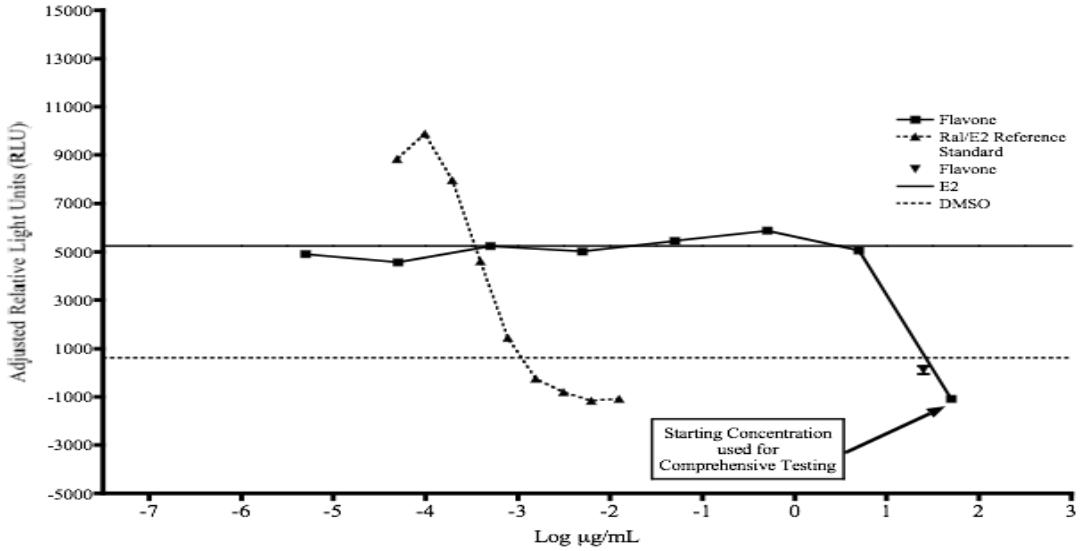


Abbreviations: Ral/E2 Reference Standard = varying concentrations of raloxifene with a fixed concentration of 2.5 x 10^{-5} µg/mL 17β–estradiol; Flavone = 25 µg/mL Flavone + 2.5 x 10^{-5} µM 17β–estradiol; E2 = 2.5 x 10^{-5} µg/mL 17β–estradiol; DMSO = dimethyl sulfoxide.

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.

Dashed horizontal line represents the mean of three DMSO control replicates plus three times the standard deviation of the DMSO control mean.

Figure 12-4 Antagonist Range Finder for N0012 – Flavone

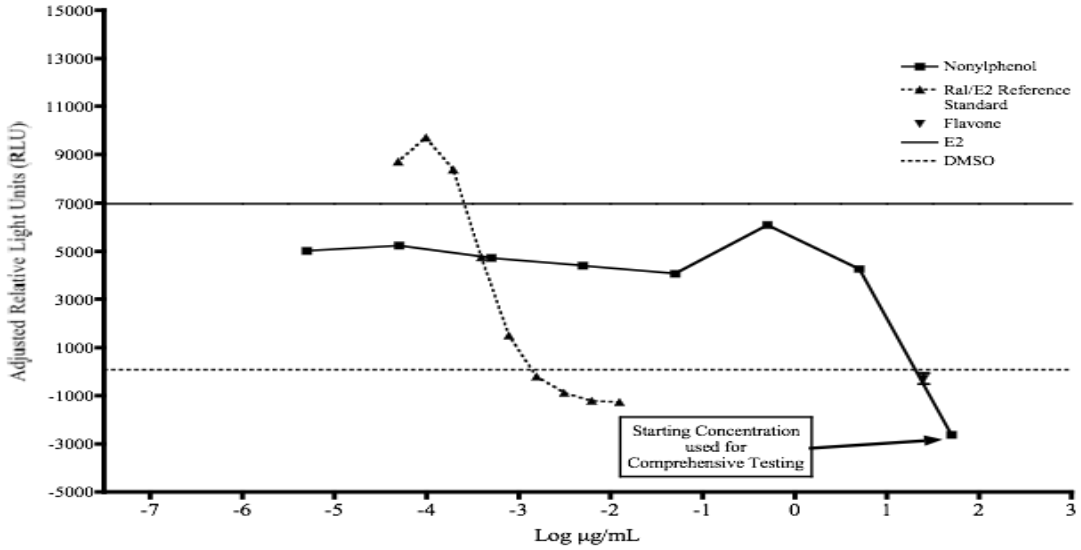


Abbreviations: Ral/E2 Reference Standard = varying concentrations of raloxifene with a fixed concentration of 2.5 x 10^{-5} µg/mL 17β–estradiol; Flavone = 25 µg/mL Flavone + 2.5 x 10^{-5} µM 17β–estradiol; E2 = 2.5 x 10^{-5} µg/mL 17β–estradiol; DMSO = dimethyl sulfoxide.

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.

Dashed horizontal line represents the mean of three DMSO control replicates plus three times the standard deviation of the DMSO control mean.

Figure 12-5 Antagonist Range Finder for N0013 – Nonylphenol

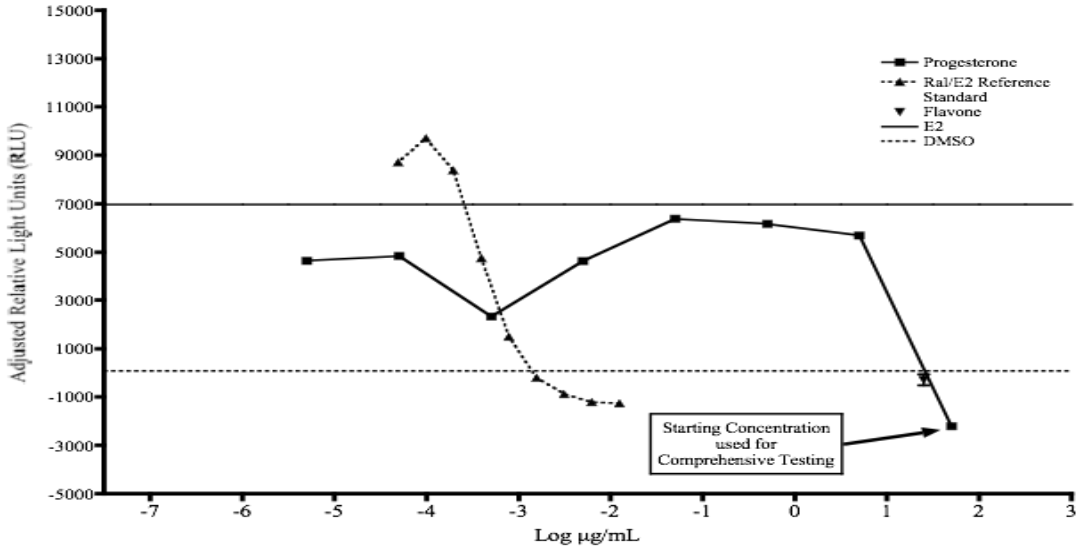


Abbreviations: Ral/E2 Reference Standard = varying concentrations of raloxifene with a fixed concentration of 2.5 x 10^{-5} µg/mL 17β–estradiol; Flavone = 25 µg/mL Flavone + 2.5 x 10^{-5} µM 17β–estradiol; E2 = 2.5 x 10^{-5} µg/mL 17β–estradiol; DMSO = dimethyl sulfoxide.

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.

Dashed horizontal line represents the mean of three DMSO control replicates plus three times the standard deviation of the DMSO control mean.

Figure 12-6 Antagonist Range Finder for N0014 – Progesterone

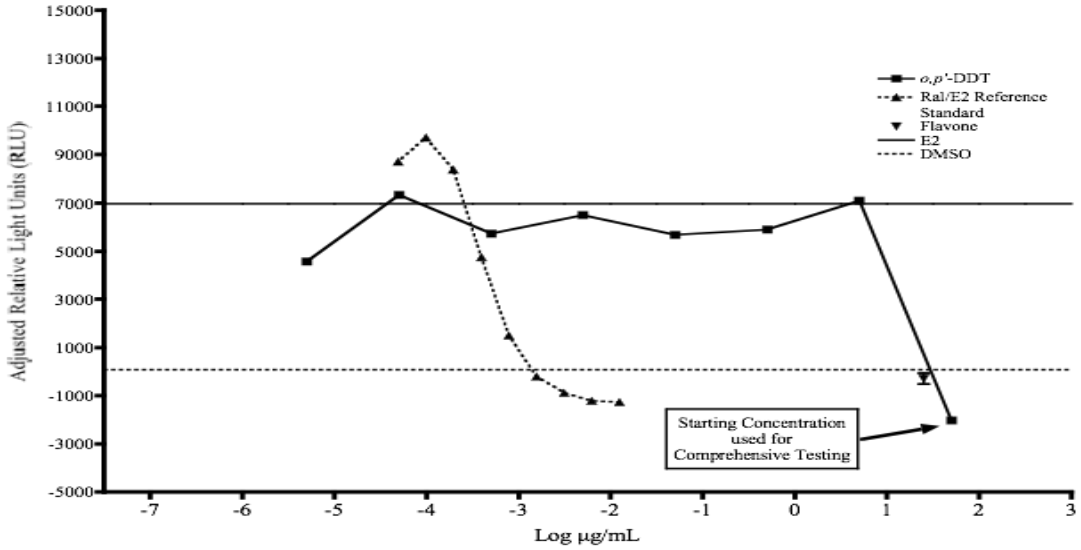


Abbreviations: Ral/E2 Reference Standard = varying concentrations of raloxifene with a fixed concentration of 2.5 x 10^{-5} µg/mL 17β–estradiol; Flavone = 25 µg/mL Flavone + 2.5 x 10^{-5} µM 17β–estradiol; E2 = 2.5 x 10^{-5} µg/mL 17β–estradiol; DMSO = dimethyl sulfoxide.

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.

Dashed horizontal line represents the mean of three DMSO control replicates plus three times the standard deviation of the DMSO control mean.

Figure 12-7 Antagonist Range Finder for N0015 – *o,p'*-DDT

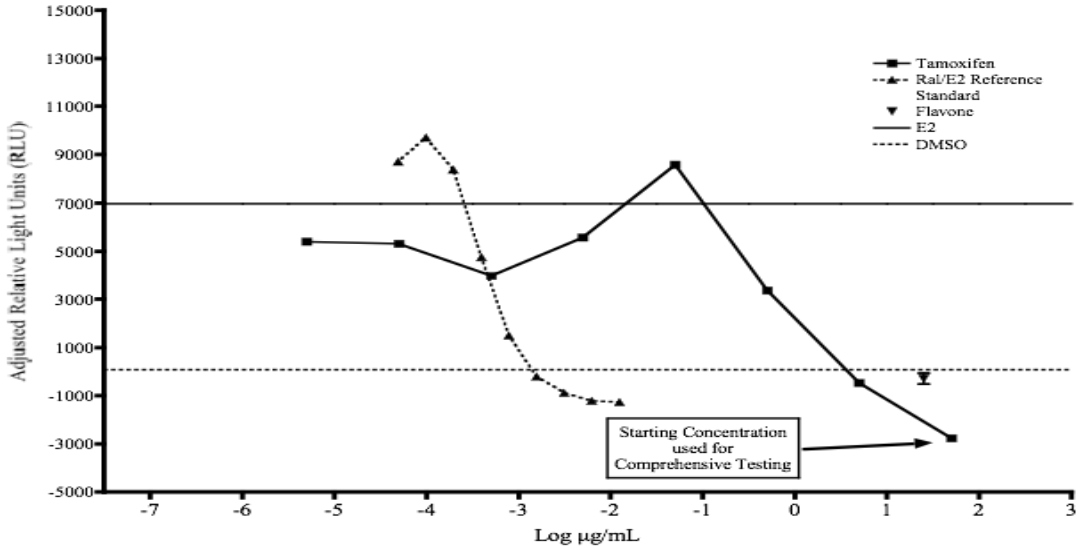


Abbreviations: *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane; Ral/E2 Reference Standard = varying concentrations of raloxifene with a fixed concentration of 2.5 x 10^{-5} µg/mL 17β–estradiol; Flavone = 25 µg/mL Flavone + 2.5 x 10^{-5} µM 17β–estradiol; E2 = 2.5 x 10^{-5}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

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.

Dashed horizontal line represents the mean of three DMSO control replicates plus three times the standard deviation of the DMSO control mean.

Figure 12-8 Antagonist Range Finder for N0016 – Tamoxifen



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

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.

Dashed horizontal line represents the mean of three DMSO control replicates plus three times the standard deviation of the DMSO control mean.

Visual observations for cell viability were conducted for all experimental plates just prior to BG1Luc ER TA evaluation. Cell viability testing (i.e., CellTiter-Glo®) was conducted in parallel plates on the same day. Comparisons of cell viability data from CellTiter-Glo® assays and visual observations are shown in **Table 12-3**.

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

Substance	Concentration (µg/mL)	CellTiter-Glo®	Visual Observation Score[1]
N0009 - BBP	50	111%	1
	5	102%	1
	0.5	116%	1
	5.00×10^{-2}	83%	1
	5.00×10^{-3}	96%	1
	5.00×10^{-4}	99%	1
	5.00×10^{-5}	98%	1
	5.00×10^{-6}	113%	1
N0010 - DBA	50	103%	1
	5	104%	1
	0.5	105%	1
	5.00×10^{-2}	120%	1
	5.00×10^{-3}	104%	1
	5.00×10^{-4}	85%	1
	5.00×10^{-5}	88%	1
	5.00×10^{-6}	92%	1
N0011 - Genistein	50	85%	1
	5	104%	1
	0.5	103%	1
	5.00×10^{-2}	112%	1
	5.00×10^{-3}	109%	1
	5.00×10^{-4}	123%	1
	5.00×10^{-5}	117%	1
	5.00×10^{-6}	81%	1
N0012 – Flavone	**50[2]**	**65%**	**2**
	5	89%	1
	0.5	93%	1
	5.00×10^{-2}	96%	1
	5.00×10^{-3}	93%	1
	5.00×10^{-4}	95%	1
	5.00×10^{-5}	100%	1
	5.00×10^{-6}	103%	1
N0013 -Nonylphenol	**50**	**8%**	**4**
	5	104%	1
	0.5	111%	1
	5.00×10^{-2}	101%	1
	5.00×10^{-3}	101%	1
	5.00×10^{-4}	100%	1
	5.00×10^{-5}	111%	1
	5.00×10^{-6}	107%	1

Table 12-3 CellTiter-Glo® and Visual Observation Data for Antagonist Range Finder Experiments (cont'd)

Substance	Concentration (µg/mL)	CellTiter-Glo®	Visual Observation Score[1]
N0014 - Progesterone	**50**	**45%**	**3**
	5	103%	1
	0.5	105%	1
	5.00×10^{-2}	117%	1
	5.00×10^{-3}	112%	1
	5.00×10^{-4}	101%	1
	5.00×10^{-5}	101%	1
	5.00×10^{-6}	104%	1
N0015 - *o,p'*-DDT	**50**	**23%**	**3**
	5	99%	1
	0.5	107%	1
	5.00×10^{-2}	108%	1
	5.00×10^{-3}	111%	1
	5.00×10^{-4}	107%	1
	5.00×10^{-5}	115%	1
	5.00×10^{-6}	93%	1
N0016 - Tamoxifen	**50**	**5%**	**4**
	5	90%	1
	0.5	99%	1
	5.00×10^{-2}	108%	1
	5.00×10^{-3}	103%	1
	5.00×10^{-4}	106%	1
	5.00×10^{-5}	109%	1
	5.00×10^{-6}	105%	1

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

[1] Visual observations are scored using the scale provide in **Table 7-1**

[2] Bolded text indicates substances and concentrations that caused a decrease in cell viability below 80%

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

12.2 Antagonist Comprehensive Testing

12.2.1 N0009 – BBP

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

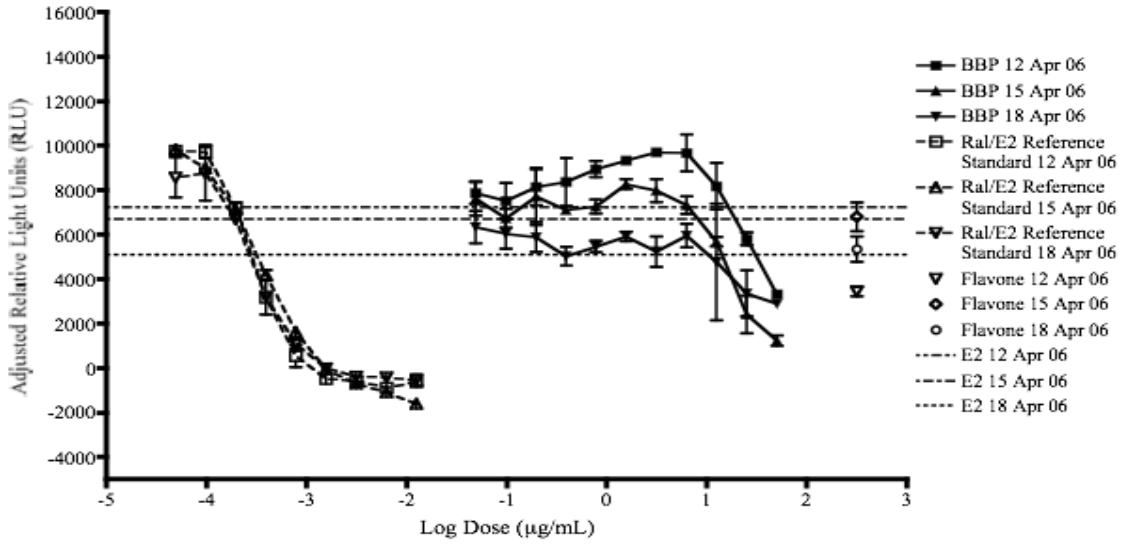
Table 12-4 Concentrations of N0009 – BBP Used in Comprehensive Testing

N0009 – BBP (µg/mL)		
50	3.13	0.2
25	1.56	9.77×10^{-2}
12.5	0.78	4.88×10^{-2}
6.25	0.39	

Abbreviations: BBP = butylbenzyl phthalate

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

Figure 12-9 Antagonist Comprehensive Testing for N0009 – BBP: Individual Experiments



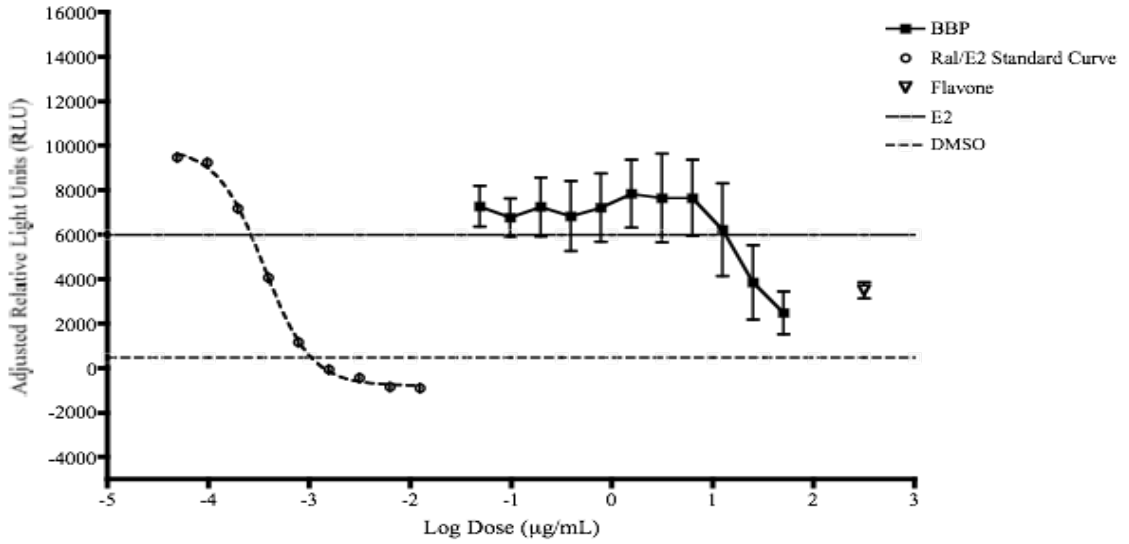
Abbreviations: BBP = butylbenzyl phthalate; Ral/E2 Reference Standard = concentrations of raloxifene with a fixed concentration of 2.5×10^{-5} µg/mL 17β–estradiol; Flavone = 25 µg/mL flavone control; E2 = 17ß-estradiol.

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.

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.

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

Figure 12-10 Antagonist Comprehensive Testing for N0009 – BBP: Averaged Experiments



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

Historical mean and standard deviation of the Ral/E2 reference standard.

Historical mean and standard deviation of the flavone control.

Historical mean and standard deviation of the E2 control.

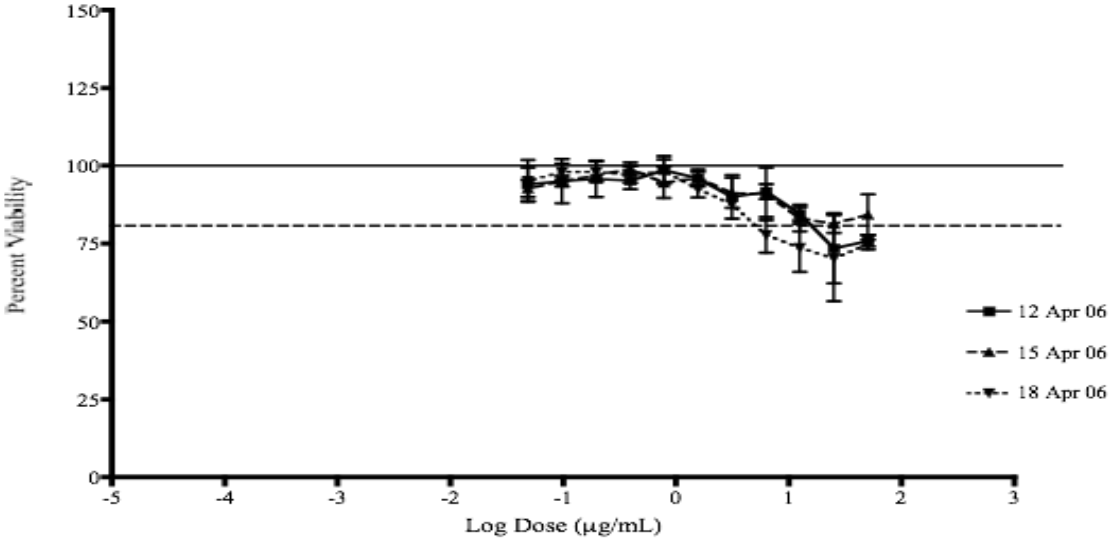
Solid horizontal line represents the historical mean of the E2 control minus three times the standard deviation of the E2 control mean. Values must be below the line without any significant decreases in cell viability in order to be considered positive for antagonism.

Dashed horizontal line represents the historical mean of the DMSO control minus three times the standard deviation of the DMSO control mean.

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.

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

Figure 12-11 CellTiter-Glo® Viability Assessment for N0009 – BBP

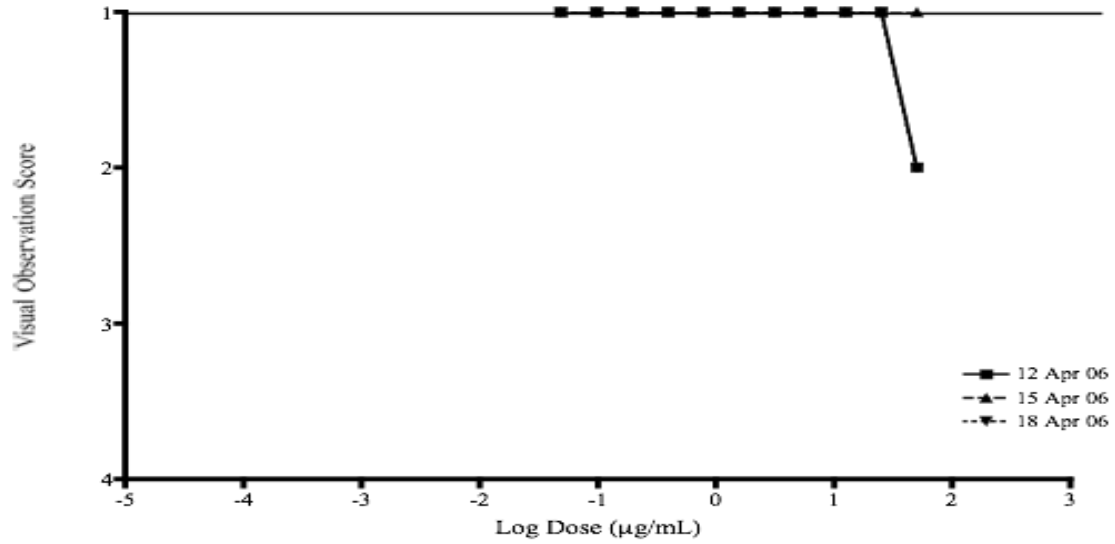


Abbreviations: BBP = Butylbenzyl phthalate

Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

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.

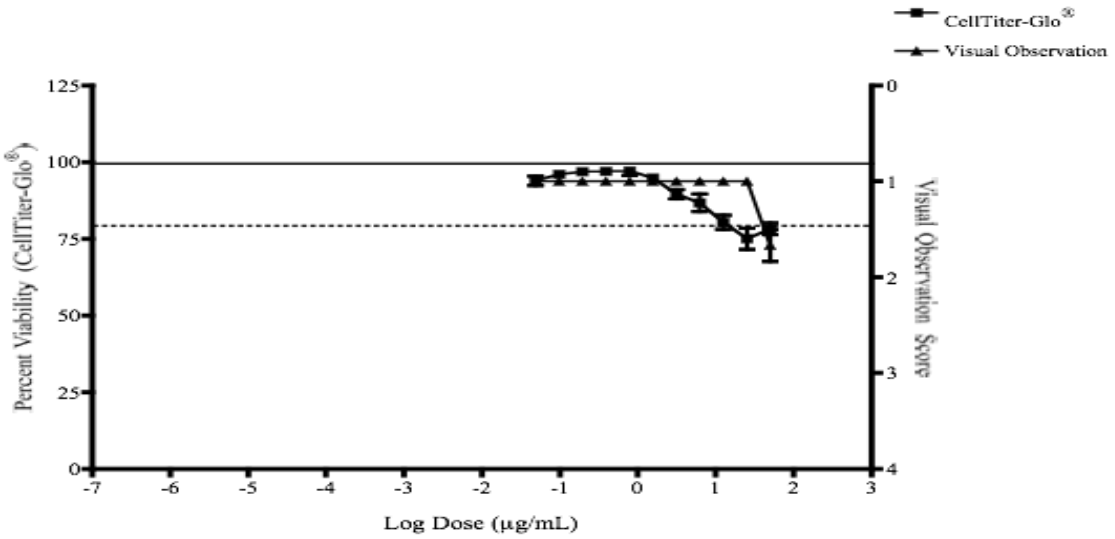
Figure 12-12 Visual Observation Viability Assessment for N0009 – BBP



Abbreviations: BBP = Butylbenzyl phthalate

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 that are exposed to concentrations resulting in visual observation scores ≥ 2 are considered cytotoxic and are not included in the evaluation of antagonist activity.

Figure 12-13 Combined Qualitative and Quantitative Viability Assessments for N0009 – BBP



Abbreviations: BBP = Butylbenzyl phthalate

Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

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.

12.2.2 N0010 – DBA

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

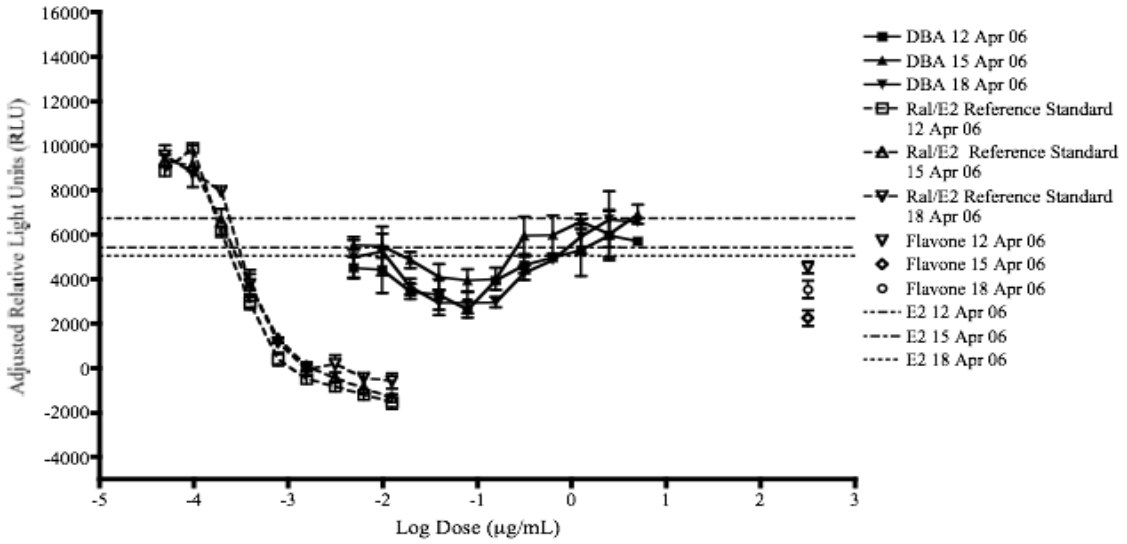
Table 12-5 Concentrations of N0010 – DBA Used in Comprehensive Testing

N0010 – DBA (µg/mL)		
5	0.31	1.95×10^{-2}
2.5	0.16	9.77×10^{-3}
1.25	7.81×10^{-2}	4.88×10^{-3}
0.63	3.91×10^{-2}	

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

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

Figure 12-14 Antagonist Comprehensive Testing for N0010 – DBA: Individual Experiments



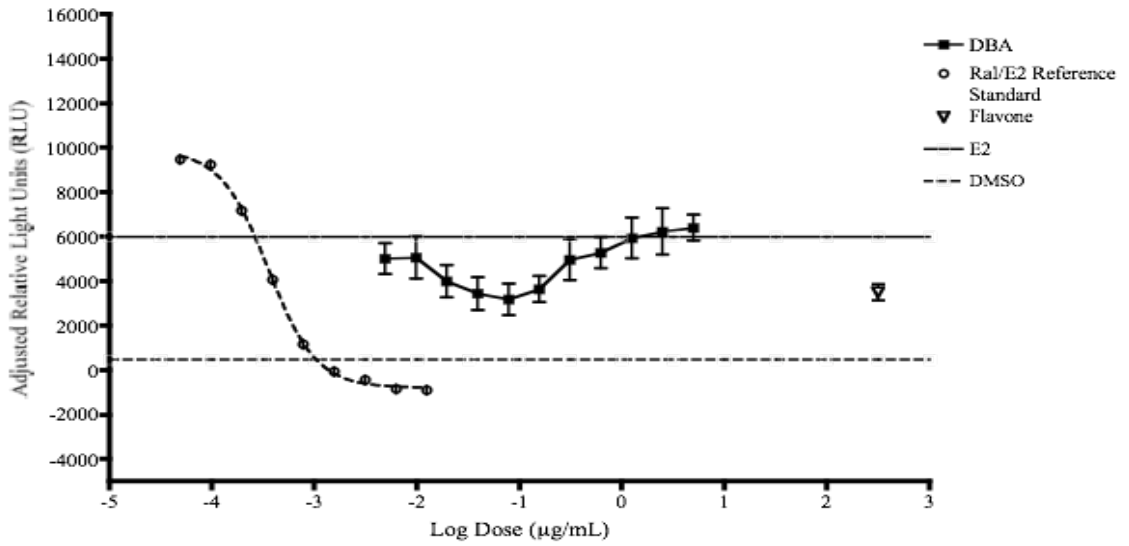
Abbreviations: DBA = dibenzo[*a*,*h*]anthracene; Ral/E2 reference standard = concentrations of raloxifene with a fixed concentration of 2.5 x 10^{-5} µg/mL 17β–estradiol; Flavone = 25 µg/mL flavone control; E2 = 17ß-estradiol.

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

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.

DBA at concentrations between 1.95 x 10^{-2} and 7.81 x 10^{-2} µg/mL showed a decrease below the E2 line. However, the concentration-response curve for DBA was biphasic and therefore an IC_{50} value could not be calculated. Results of averaged antagonist experiments for DBA are shown in **Figure 12-15**.

Figure 12-15 Antagonist Comprehensive Testing for N0010 – DBA: Averaged Experiments



Abbreviations: DBA = Dibenzo[*a,h*]anthracene; Ral/E2 reference standard = concentrations of raloxifene with a fixed concentration of 2.5 x 10^{-5} µg/mL 17β–estradioll; Flavone = 25 µg/mL flavone control; E2 = 17ß-estradiol; DMSO = dimethyl sulfoxide.

Historical mean and standard deviation of the Ral/E2 reference standard.

Historical mean and standard deviation of the flavone control

Historical mean and standard deviation of the E2 control.

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.

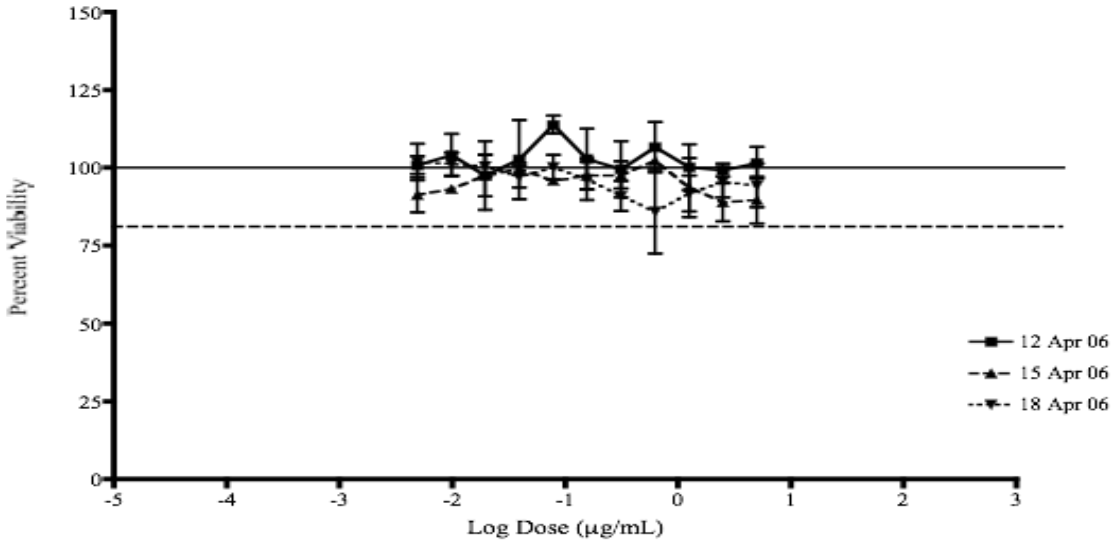
Dashed horizontal line represents the historical mean of the DMSO control minus three times the standard deviation of the DMSO control mean.

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.

DBA at concentrations between 1.95 x 10^{-2} and 7.81 x 10^{-2} µg/mL showed a decrease below the E2 line. However, the concentration-response curve for DBA was biphasic and an IC_{50} value could not be calculated.

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

Figure 12-16 CellTiter-Glo® Viability Assessment for N0010 – DBA

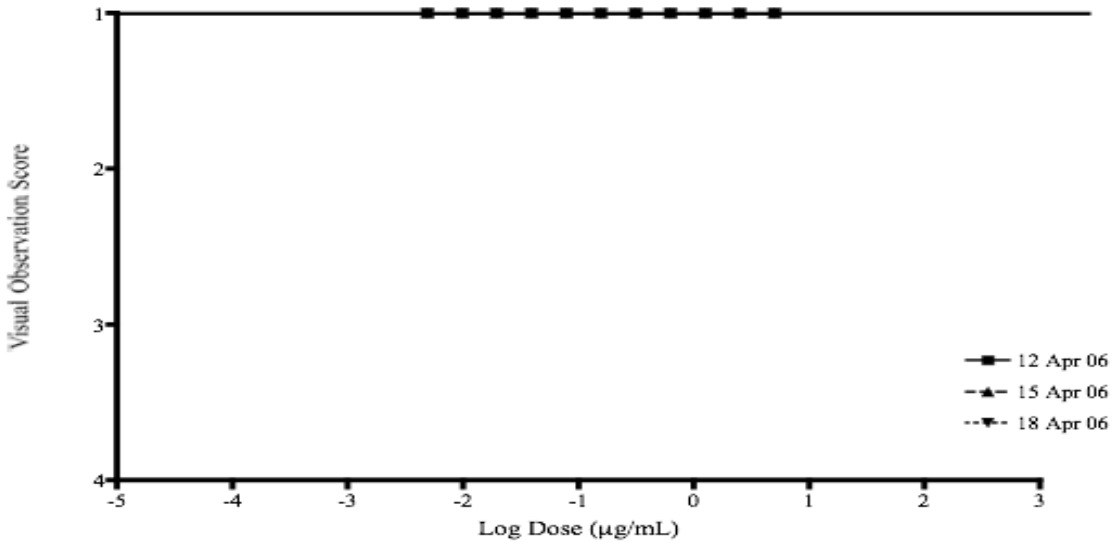


Abbreviations: DBA = Dibenzo[*a,h*]anthracene

Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

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.

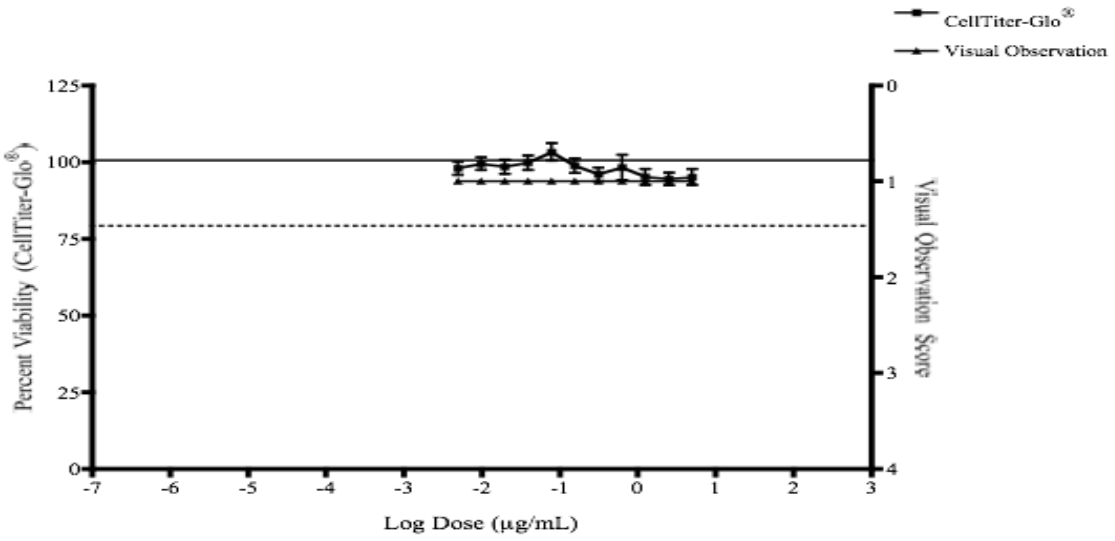
Figure 12-17 Visual Observation Viability Assessment for N0010 – DBA



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

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 ≥ 2 are considered cytotoxic and are not included in the evaluation of antagonist activity.

Figure 12-18 Combined Qualitative and Quantitative Viability Assessments for N0010 – DBA



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

Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

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.

12.2.3 N0011 – Genistein

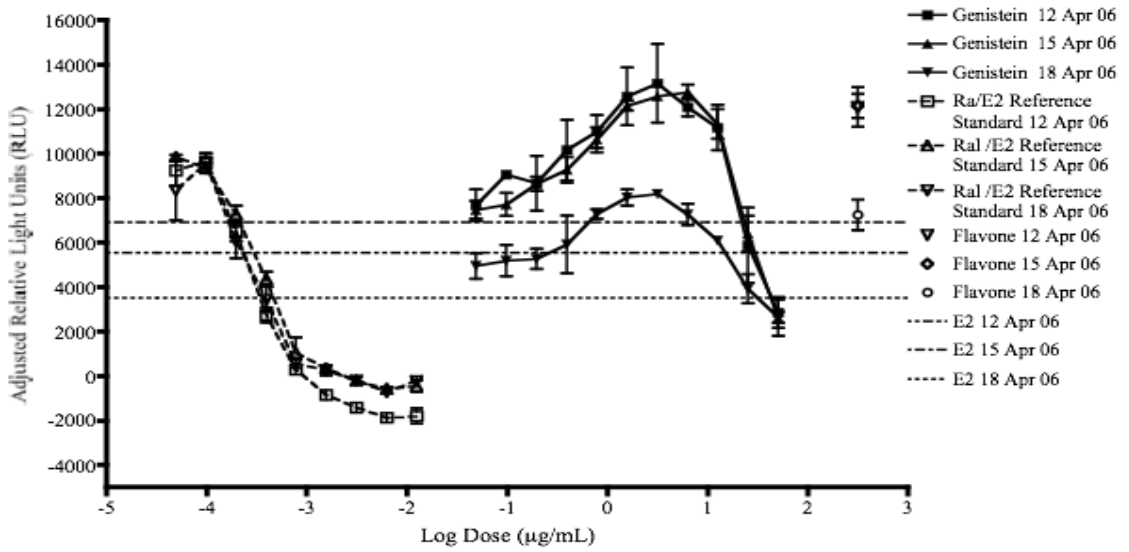
Genistein was selected for antagonist testing because it was listed in the ICCVAM Guidelines (ICCVAM 2003, 2006) as positive for ER antagonist activity in the one assay in which it was tested, and because of its potential problems with solubility in aqueous media. 50 µg/mL was selected as the starting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

Table 12-6 Concentrations of N0011 – Genistein Used in Comprehensive Testing

N0011 – Genistein (µg/mL)		
50	3.13	0.2
25	1.56	9.77×10^{-2}
12.5	0.78	4.81×10^{-2}
6.25	0.39	

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

Figure 12-19 Antagonist Comprehensive Testing for N0011 – Genistein: Individual Experiments



Abbreviations: Ral/E2 Reference Standard = concentrations of raloxifene with a fixed concentration of 2.5 x 10^{-5} µg/mL 17β–estradiol; Flavone = 25 µg/mL flavone control; E2 = 17ß-estradiol.

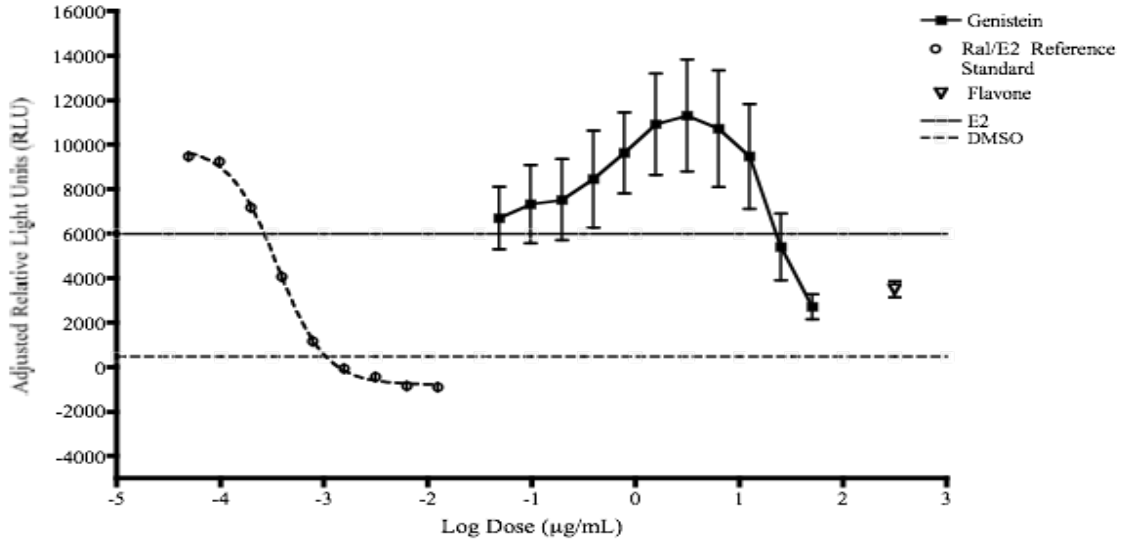
Horizontal lines represent the mean of three E2 control replicates minus three times the standard deviation of the E2 control mean. Values must be below the line without any significant decreases in cell viability in order to be considered positive for antagonism.

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.

Genistein showed potential antagonist activity at the highest concentration tested (50 µg/mL).

Results of averaged antagonist experiments for genistein are shown in **Figure 12-20**.

Figure 12-20 Antagonist Comprehensive Testing for N0011 – Genistein: Averaged Experiments



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

Historical mean and standard deviation of the Ral/E2 reference standard.

Historical mean and standard deviation of the flavone control

Historical mean and standard deviation of the E2 control.

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.

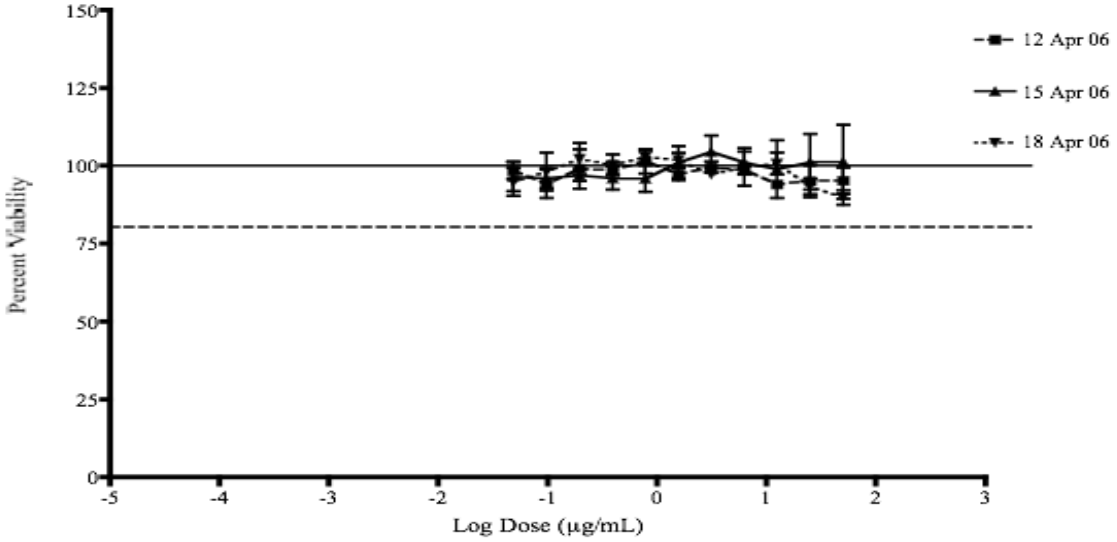
Dashed horizontal line represents the historical mean of the DMSO control minus three times the standard deviation of the DMSO control mean.

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.

Genistein showed antagonist activity at the highest concentration tested (50 μg/mL). An IC_{50} value could not be calculated because genistein did not reach saturation at the highest concentrations tested.

Genistein did not cause a decrease in cell viability at any of the concentrations tested in either the range finder or during comprehensive testing (**Figures 12-21**, **12-22**, and **12-23**).

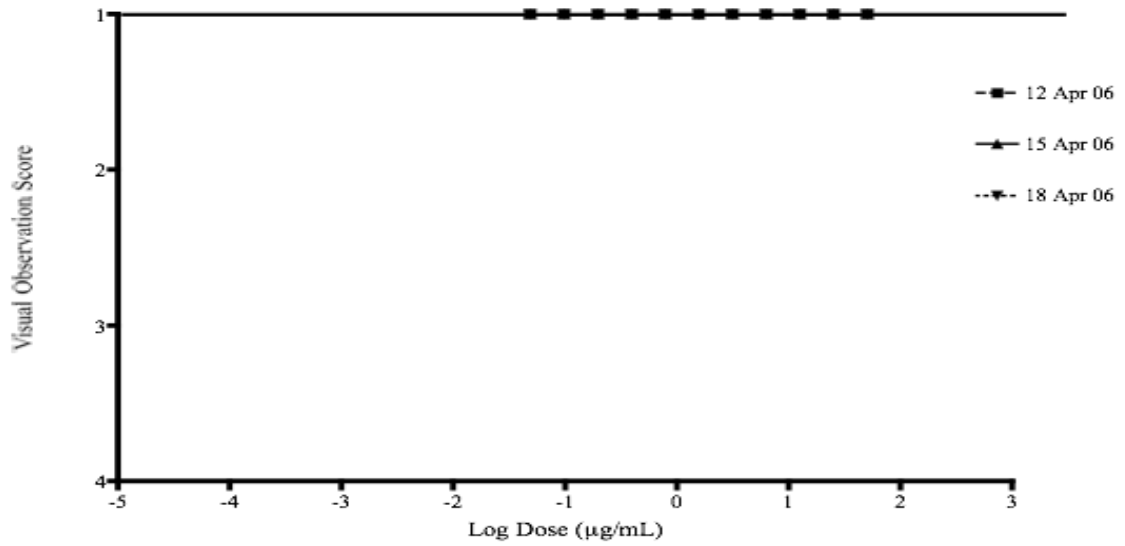
Figure 12-21 CellTiter-Glo® Viability Assessment for N0011 – Genistein



Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

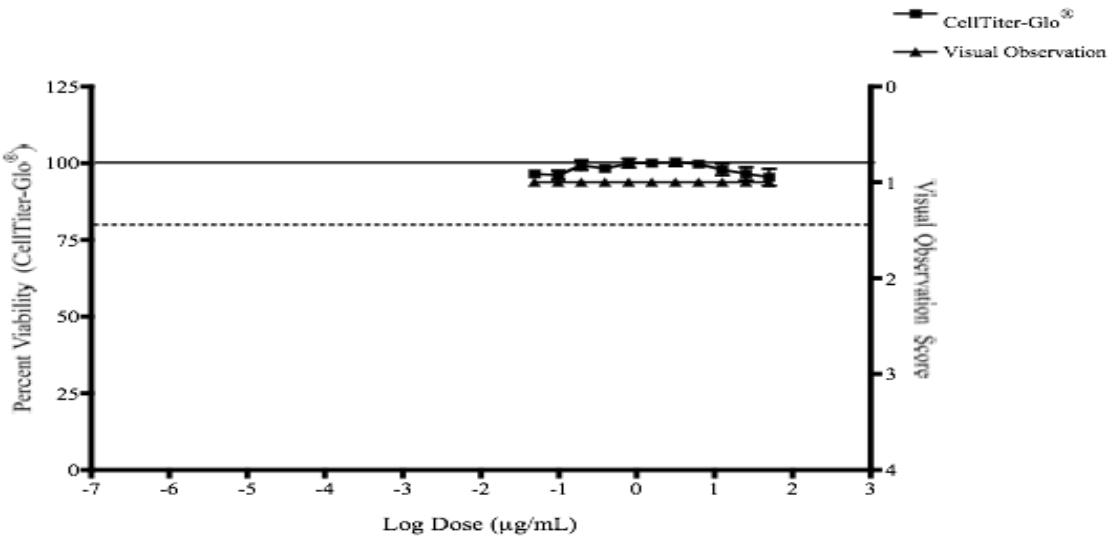
Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability below this line are considered cytotoxic and are not included in the evaluation of antagonist activity.

Figure 12-22 Visual Observation Viability Assessment for N0011 – Genistein



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 ≥ 2 are considered cytotoxic and are not included in the evaluation of antagonist activity.

Figure 12-23 Combined Qualitative and Quantitative Viability Assessments for N0011 – Genistein



Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

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.

12.2.4 N0012 – Flavone

Flavone was selected for antagonist testing because it was listed in the ICCVAM Guidelines (ICCVAM 2003, 2006) as uniformly positive for ER antagonist activity in multiple assays. 50 µg/mL was selected as

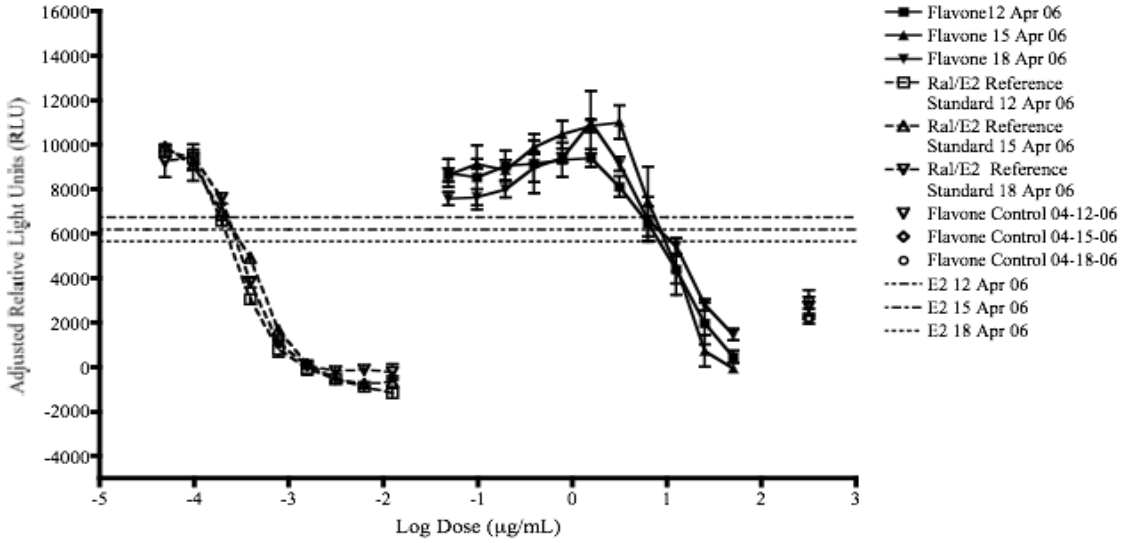
the starting concentration for the double serial dilution used for comprehensive testing because it gave the lowest adjusted RLU value during range finder testing. The concentrations of flavone tested are listed in **Table 12-7**.

Table 12-7 Concentrations of N0012 – Flavone Used in Comprehensive Testing

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

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

Figure 12-24 Antagonist Comprehensive Testing for N0012 – Flavone: Individual Experiments

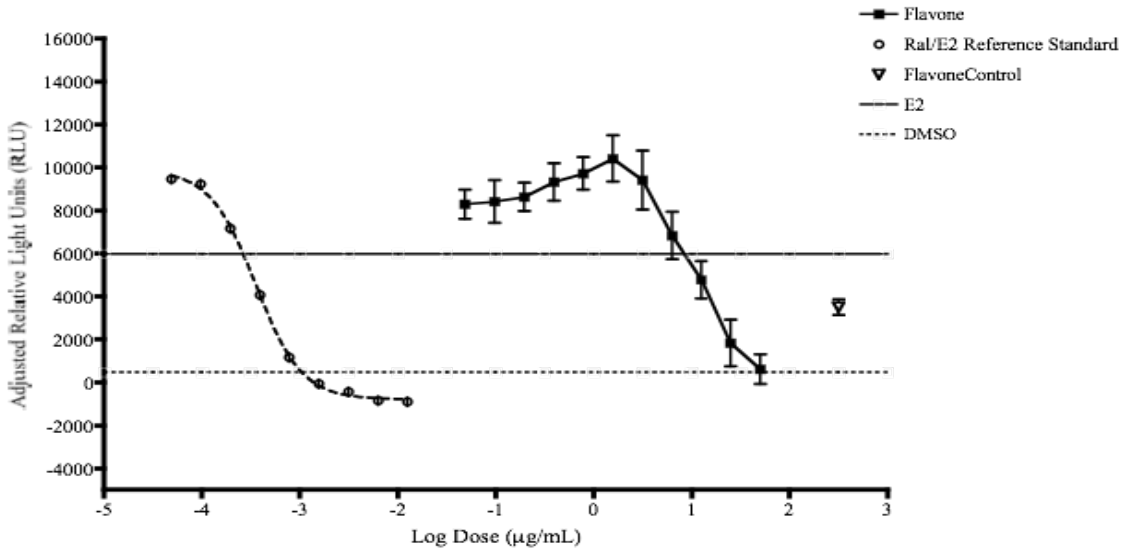


Abbreviations: Ral/E2 Reference Standard = concentrations of raloxifene with a fixed concentration of 2.5×10^{-5} µg/mL 17β–estradiol; Flavone Control = 25 µg/mL flavone control; E2 = 17ß-estradiol.

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.[2]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.

Flavone showed antagonist activity at the three highest concentrations tested (12.5, 25, and 50 µg/mL). Results of averaged antagonist experiments for flavone are shown in **Figure 12-25**.

Figure 12-25 Antagonist Comprehensive Testing for N0012 – Flavone: Averaged Experiments



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

Historical mean and standard deviation of the Ral/E2 reference standard.

Historical mean and standard deviation of the flavone control

Historical mean and standard deviation of the E2 control.

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.

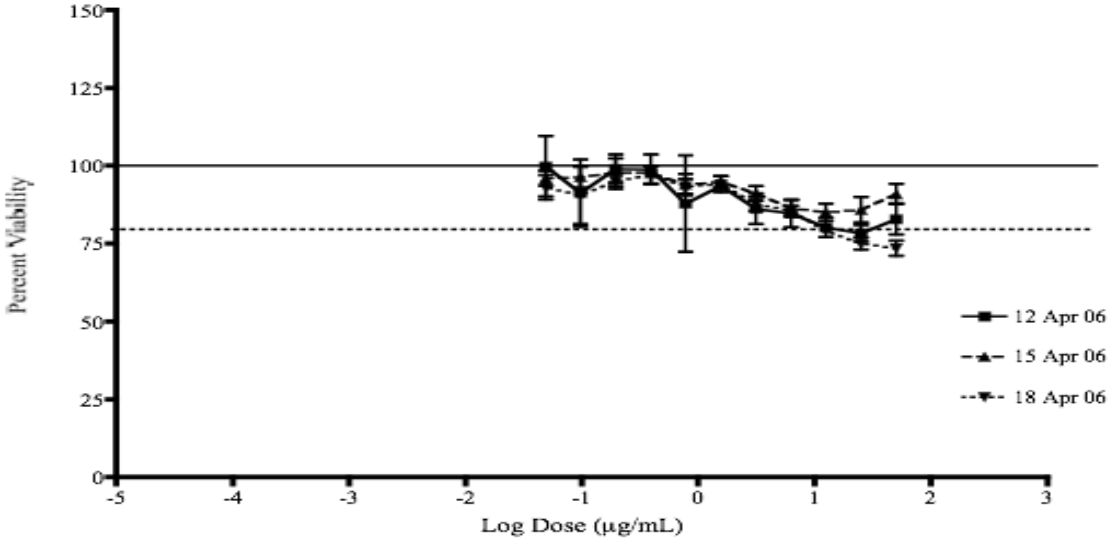
Dashed horizontal line represents the historical mean of the DMSO control minus three times the standard deviation of the DMSO control mean.

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.

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

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

Figure 12-26 CellTiter-Glo® Viability Assessment for N0012 – Flavone

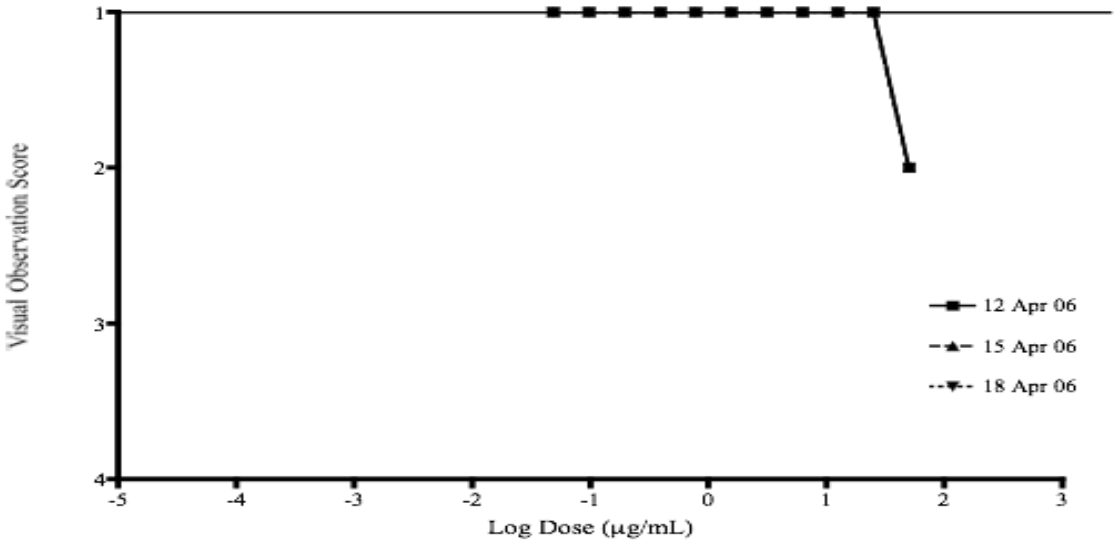


Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

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.

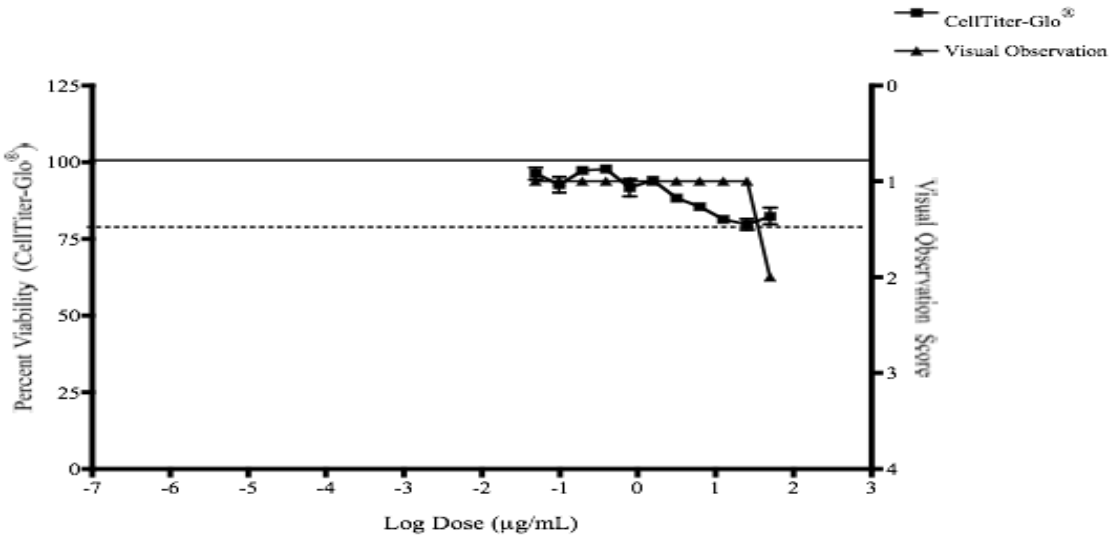
However, the results of the visual observation scoring (**Figure 12-27**) did not agree with those seen in CellTiter-Glo®. The visual observation scores indicated that flavone had a toxicity score of 2, indicating that cells were damaged. A comparison of CellTiter-Glo® data and visual observation scores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

Figure 12-27 Visual Observation Viability Assessment for N0012 – Flavone



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 that are exposed to concentrations resulting in visual observation scores ≥ 2 are considered cytotoxic and are not included in the evaluation of antagonist activity.

Figure 12-28 Combined Qualitative and Quantitative Viability Assessments for N0012 – Flavone



Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

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.

12.2.5 N0013 – Nonylphenol

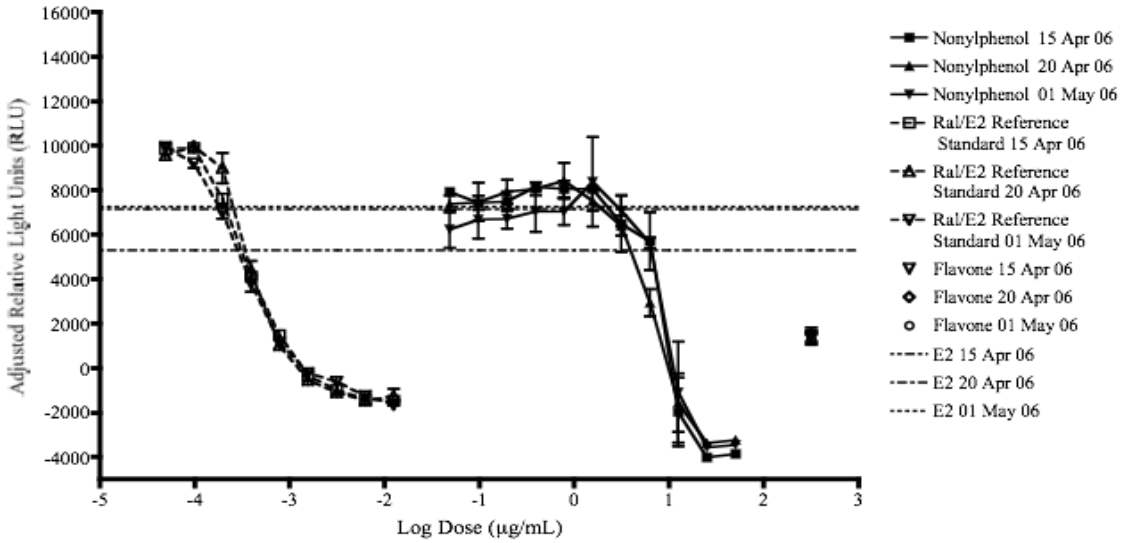
Nonylphenol was selected for antagonist testing because it was listed in the ICCVAM Guidelines (ICCVAM 2003, 2006) as positive for ER antagonist activity in the one assay in which it was tested. 50 µg/mL was selected as the starting concentration for the double serial dilution used for comprehensive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

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

N0013 – Nonylphenol (µg/mL)		
50	3.13	0.2
25	1.56	9.77×10^{-2}
12.5	0.78	4.81×10^{-2}
6.25	0.39	

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

Figure 12-29 Antagonist Comprehensive Testing for N0013 – Nonylphenol: Individual Experiments



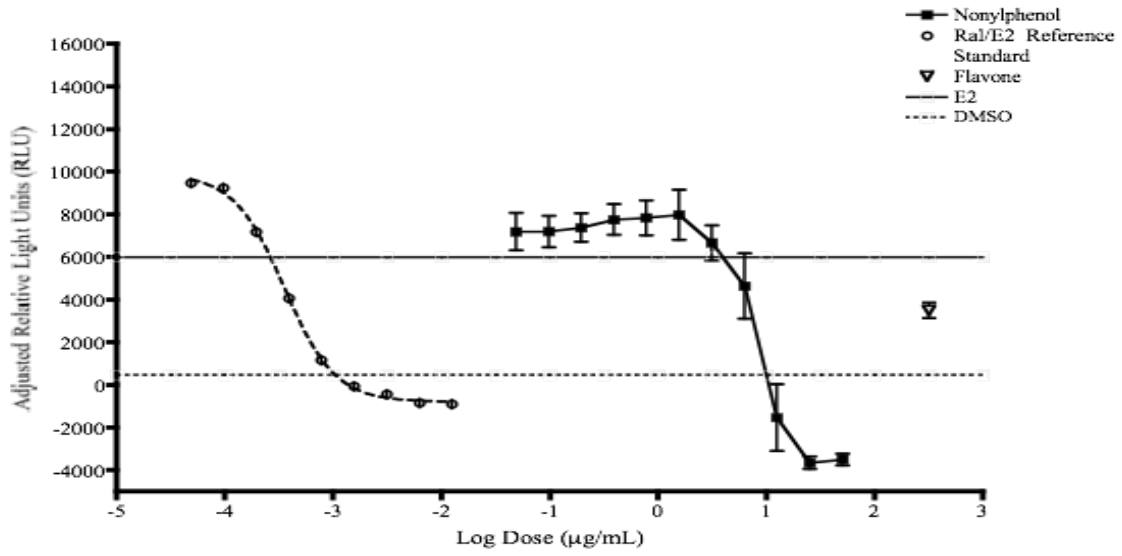
Abbreviations: Ral/E2 Reference Standard = concentrations of raloxifene with a fixed concentration of 2.5 x 10^{-5} µg/mL 17β–estradiol; Flavone = 25 µg/mL flavone control; E2 = 17ß-estradiol; DMSO = dimethyl sulfoxide.

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.

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.

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

Figure 12-30 Antagonist Comprehensive Testing for N0013 – Nonylphenol: Averaged Experiments



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

Historical mean and standard deviation of the Ral/E2 reference standard.

Historical mean and standard deviation of the flavone control

Historical mean and standard deviation of the E2 control.

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.

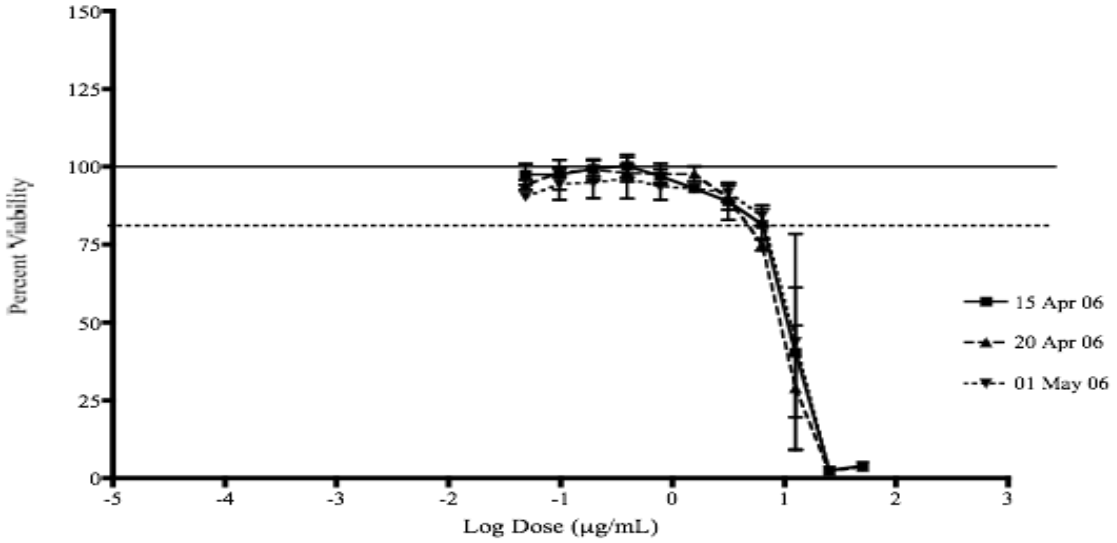
Dashed horizontal line represents the historical mean of the DMSO control minus three times the standard deviation of the DMSO control mean.

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.

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

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

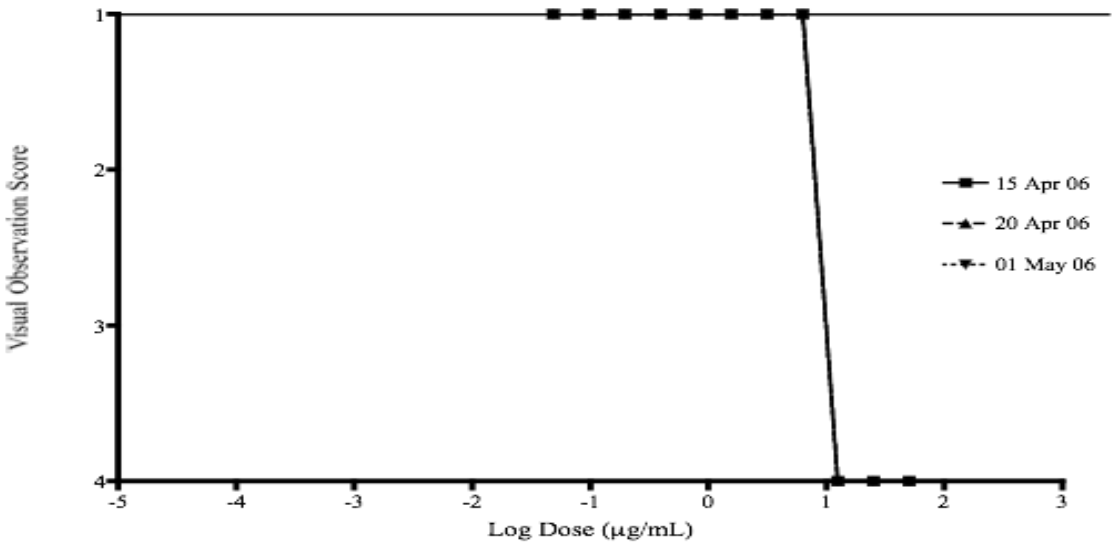
Figure 12-31 CellTiter-Glo® Viability Assessment for N0013 – Nonylphenol



Solid horizontal line indicates 100% cell viability as measured in DMSO control.

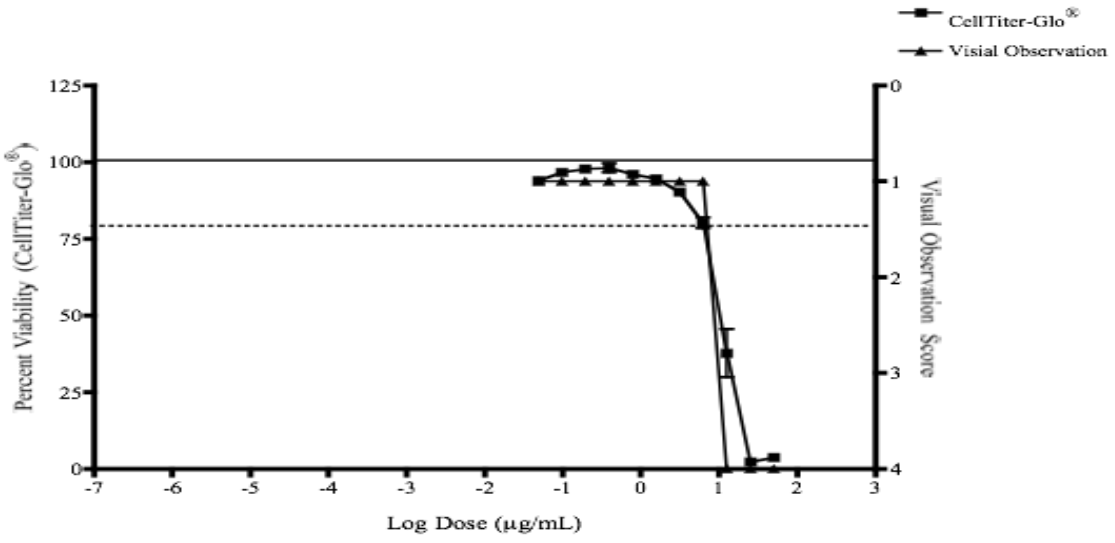
Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability below this line are considered cytotoxic and are not included in the evaluation of antagonist activity.

Figure 12-32 Visual Observation Viability Assessment for N0013 – Nonylphenol



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

Figure 12-33 Combined Qualitative and Quantitative Viability Assessments for N0013 – Nonylphenol



Solid horizontal line indicates 100% cell viability as measured in DMSO control.

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.

12.2.6 N0014 – Progesterone

Progesterone was selected for antagonist testing because it was listed as negative for ER antagonist activity in the ICCVAM Guidelines (ICCVAM 2003, 2006). 50 µg/mL was selected as the starting

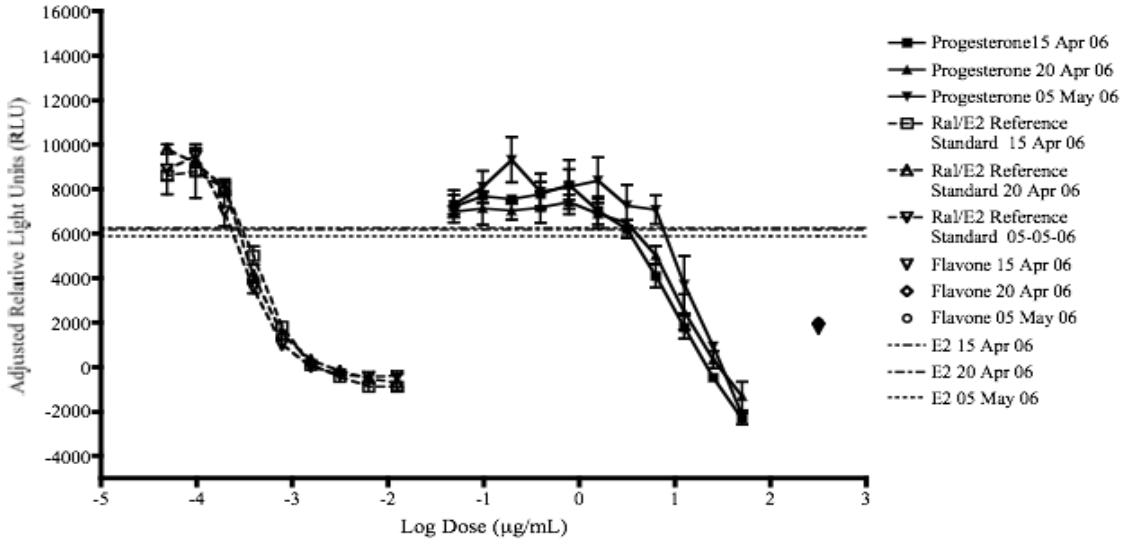
concentration for the double serial dilution used for comprehensive testing because it gave the lowest adjusted RLU value during range finder testing. The concentrations of progesterone tested are listed in **Table 12-9**.

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

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

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

Figure 12-34 Antagonist Comprehensive Testing for N0014 – Progesterone: Individual Experiments



Abbreviations: Ral/E2 Reference Standard = concentrations of raloxifene with a fixed concentration of 2.5×10^{-5} µg/mL 17β–estradiol; Flavone = 25 µg/mL flavone control; E2 = 17ß-estradiol.

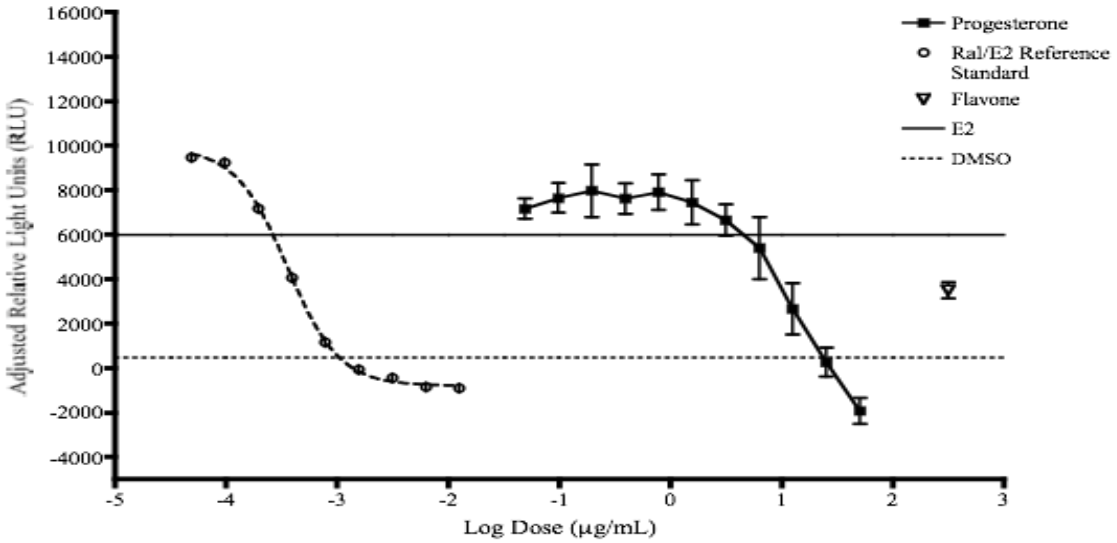
Horizontal lines represent the mean of three E2 control replicates minus three times the standard deviation of the E2 control mean. Values must be below the line without any significant decreases in cell viability in order to be considered positive for antagonism.

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.

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

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

Figure 12-35 Antagonist Comprehensive Testing for N0014 – Progesterone: Averaged Experiments



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

Historical mean and standard deviation of the Ral/E2 reference standard.

Historical mean and standard deviation of the flavone control

Historical mean and standard deviation of the E2 control.

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.

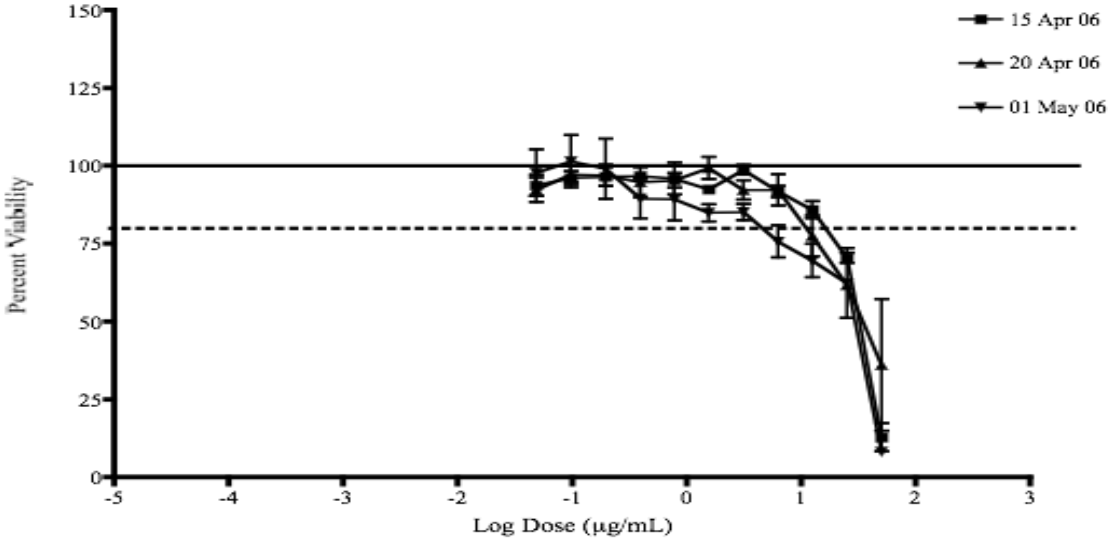
Dashed horizontal line represents the historical mean of the DMSO control minus three times the standard deviation of the DMSO mean.

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.

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

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

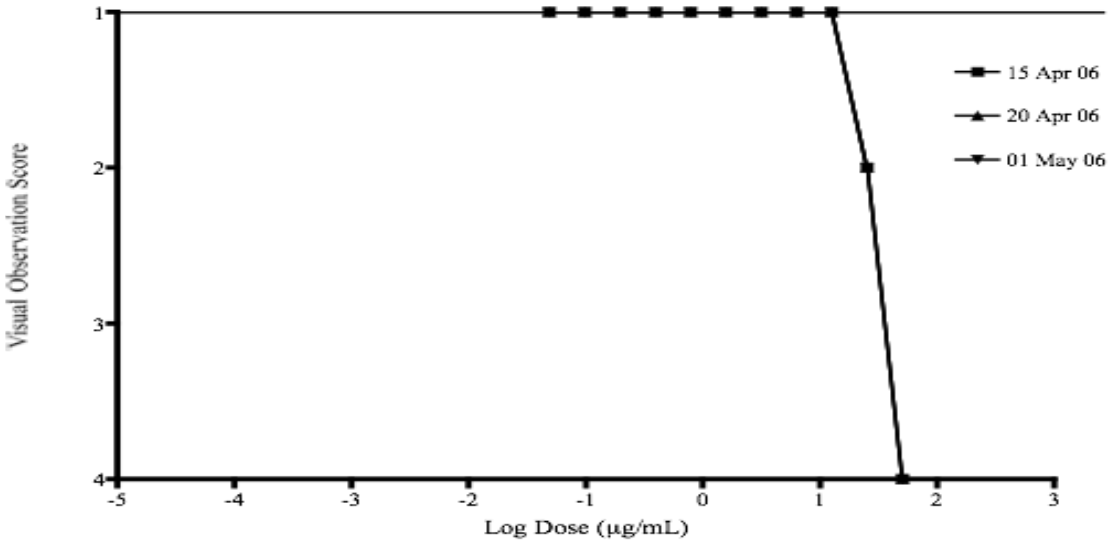
Figure 12-36 CellTiter-Glo® Viability Assessment for N0014 – Progesterone



Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

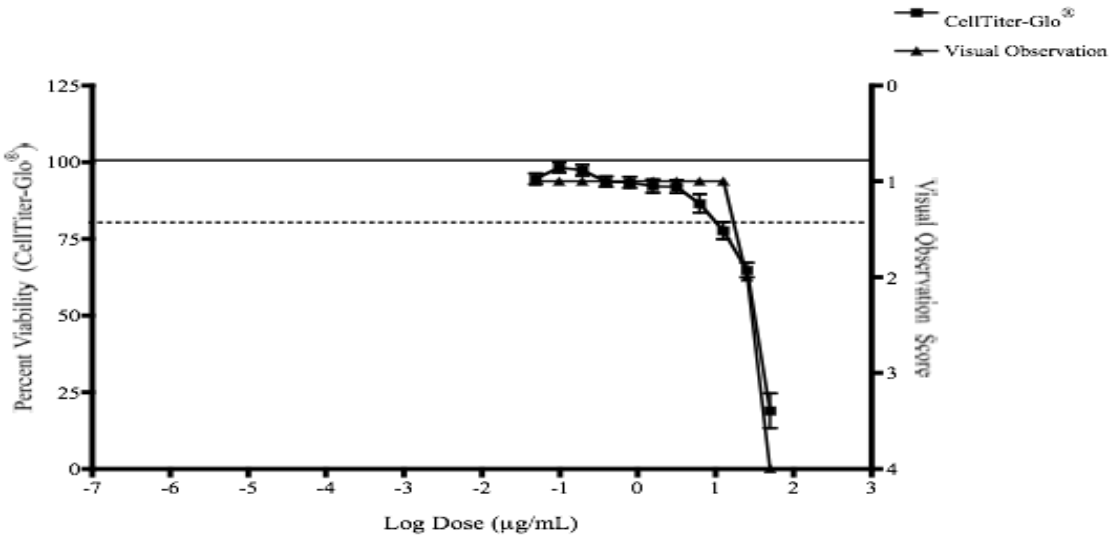
Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability below this line are considered cytotoxic and are not included in the evaluation of antagonist activity.

Figure 12-37 Visual Observation Viability Assessment for N0014 – Progesterone



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 ≥ 2 are considered cytotoxic and are not included in the evaluation of antagonist activity.

Figure 12-38 Combined Qualitative and Quantitative Viability Assessments for N0014 – Progesterone



Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

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.

12.2.7 N0015 – *o,p'-DDT*

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

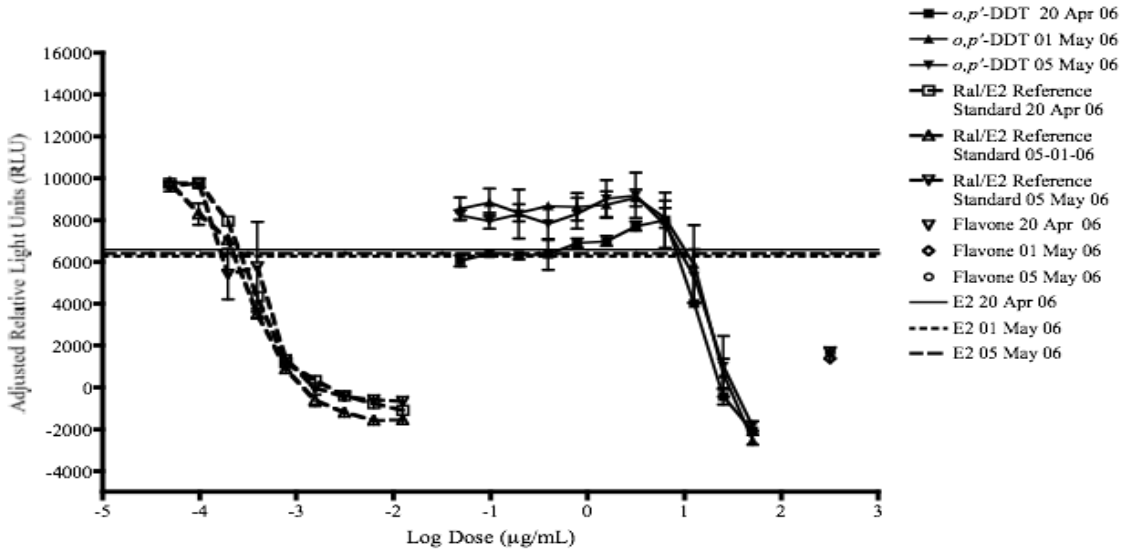
Table 12-10 Concentrations of N0015 - *o,p'*-DDT Used in Comprehensive Testing

N0015 – *o,p'*-DDT (µg/mL)		
50	3.13	0.2
25	1.56	9.77×10^{-2}
12.5	0.78	4.81×10^{-2}
6.25	0.39	

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

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

Figure 12-39 Antagonist Comprehensive Testing for N0015 – *o,p'*-DDT: Individual Experiments



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 x 10^{-5} µg/mL 17β–estradiol; Flavone = 25 µg/mL flavone control; E2 = 17ß-estradiol.

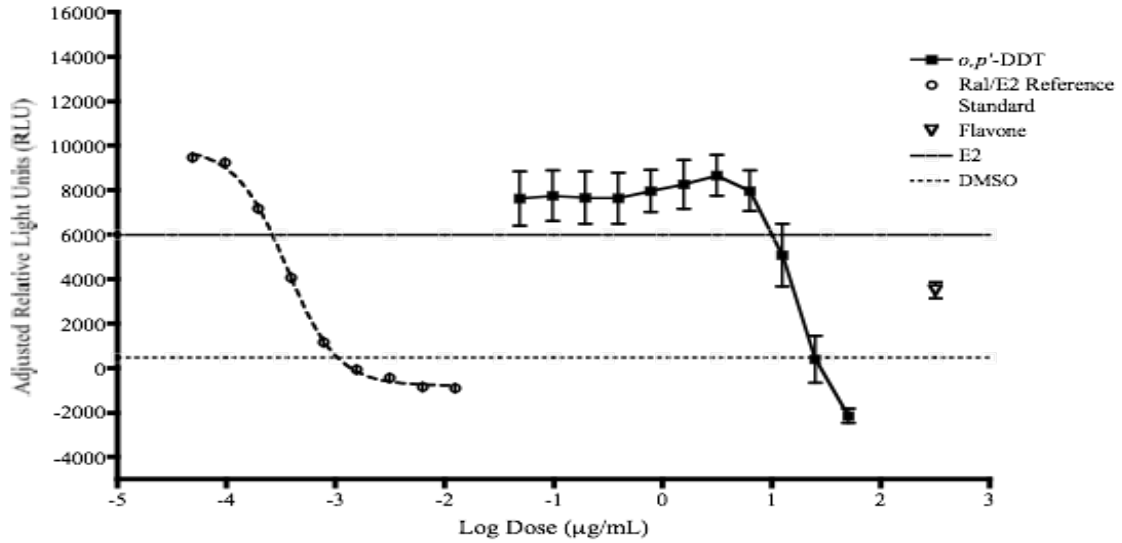
Horizontal lines represent the mean of three E2 control replicates minus three times the standard deviation of the E2 control mean. Values must be below the line without any significant decreases in cell viability in order to be considered positive for antagonism.

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.

o,p'-DDT showed potential antagonist activity at the three highest concentrations tested (12.5, 25, and 50 µg/mL).

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

Figure 12-40 Antagonist Comprehensive Testing for N0015 – *o,p'*-DDT: Averaged Experiments



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 x 10^{-5} µg/mL 17β–estradiol; Flavone = 25 µg/mL flavone control; E2 = 17ß-estradiol; DMSO = dimethyl sulfoxide.

Historical mean and standard deviation of the Ral/E2 reference standard.

Historical mean and standard deviation of the flavone control

Historical mean and standard deviation of the E2 control.

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.

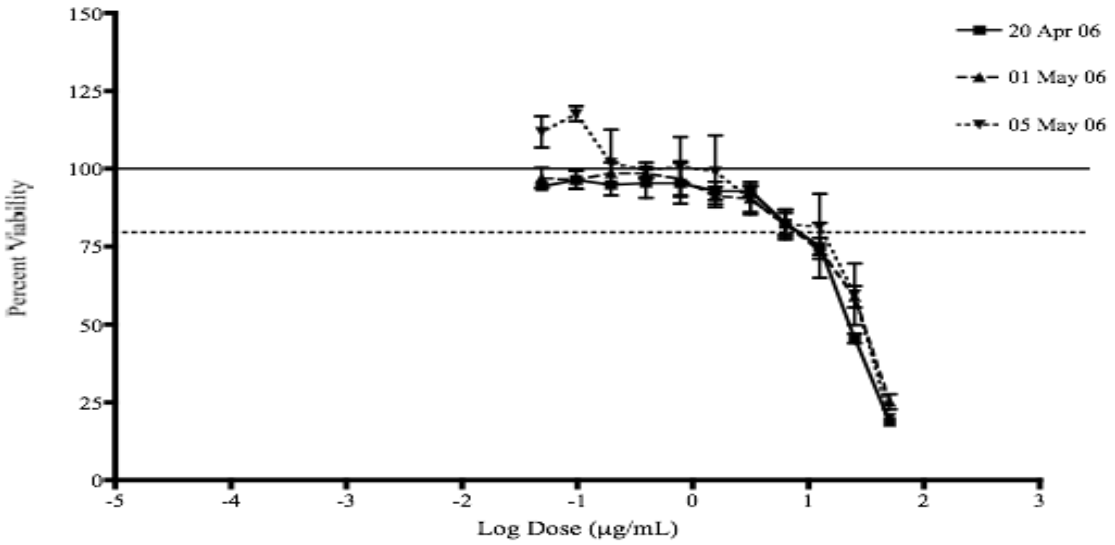
Dashed horizontal line represents the historical mean of the DMSO control minus three times the standard deviation of the DMSO mean.

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.

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

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

Figure 12-41 CellTiter-Glo® Viability Assessment for N0015 – *o,p'*-DDT

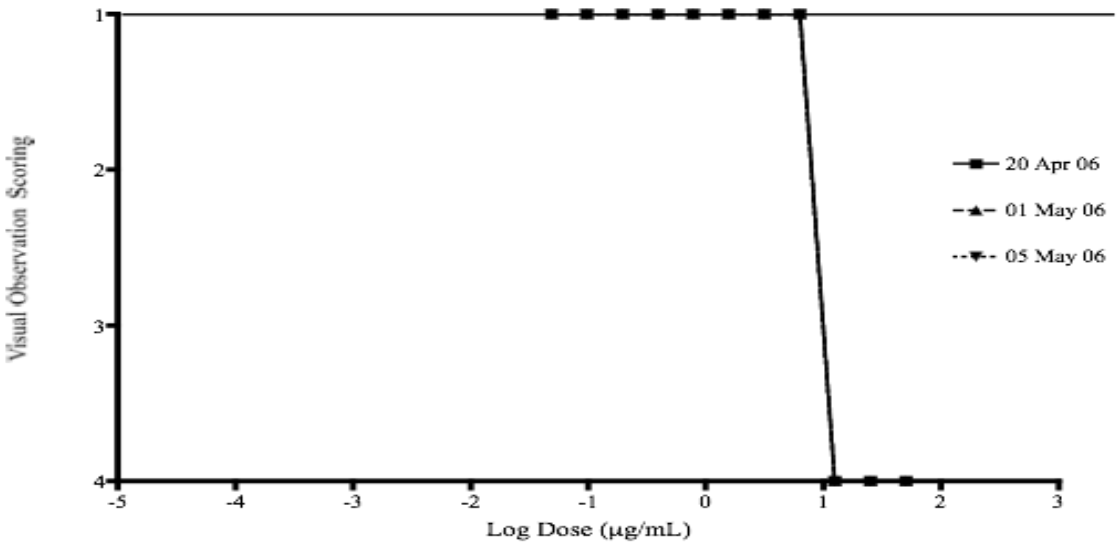


Abbreviations: *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane.

Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

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.

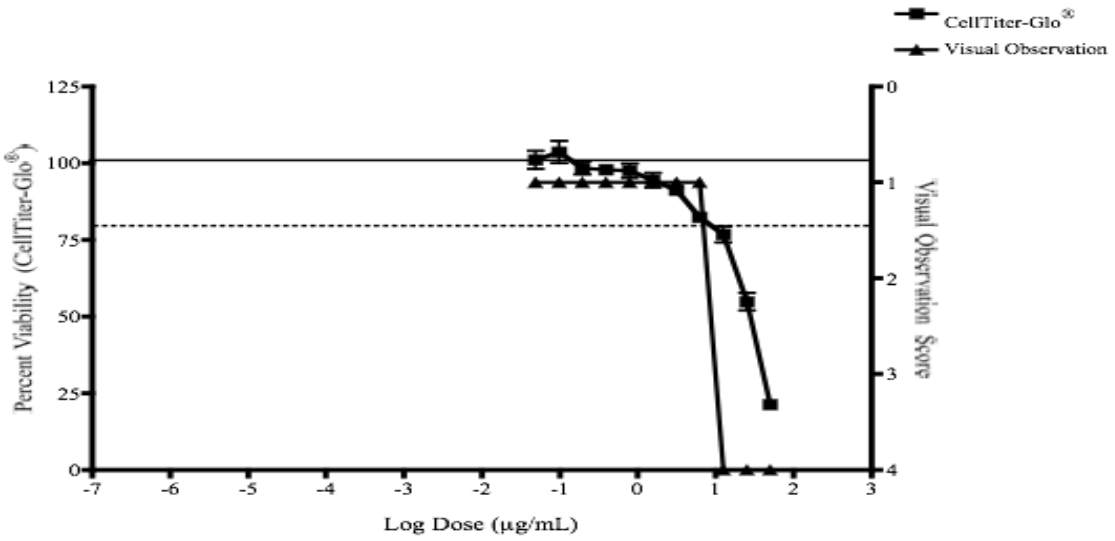
Figure 12-42 Visual Observation Viability Assessment for N0015 – *o,p'*-DDT



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

Wells containing cells that exhibit normal morphology and density are given a visual observation score of 1; 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 ≥ 2 are considered cytotoxic and are not included in the evaluation of antagonist activity.

Figure 12-43 Combined Qualitative and Quantitative Viability Assessments for N0015 – *o,p'*-DDT



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

Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

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.

12.2.8 N0016 – Tamoxifen

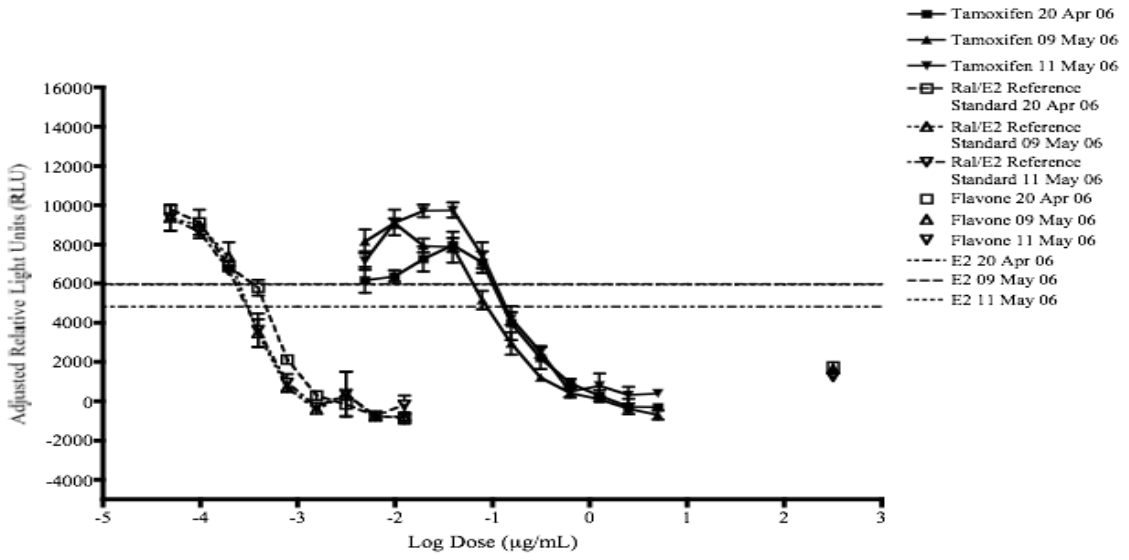
Tamoxifen was selected for antagonist testing because it was listed in the ICCVAM Guidelines (ICCVAM 2003, 2006) as uniformly positive for ER antagonist activity in multiple assays. 5 μg/mL was selected as the starting concentration for the double serial dilution used for comprehensive testing because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

Table 12-11 Concentrations of N0016 – Tamoxifen Used in Comprehensive Testing

N0016 – Tamoxifen (μg/mL)		
5	0.31	1.95×10^{-2}
2.5	0.16	9.77×10^{-3}
1.25	7.81×10^{-2}	4.81×10^{-3}
0.63	3.91×10^{-2}	

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

Figure 12-44 Antagonist Comprehensive Testing for N0016 – Tamoxifen: Individual Experiments



Abbreviations: Ral/E2 Reference Standard = concentrations of raloxifene with a fixed concentration of 2.5×10^{-5} μg/mL 17β–estradiol; Flavone = 25 μg/mL flavone control; E2 = 17ß-estradiol.

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.

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.

Tamoxifen showed antagonist activity at the majority of concentrations tested. IC_{50} values for tamoxifen experiments are reported in **Table 12-12**.

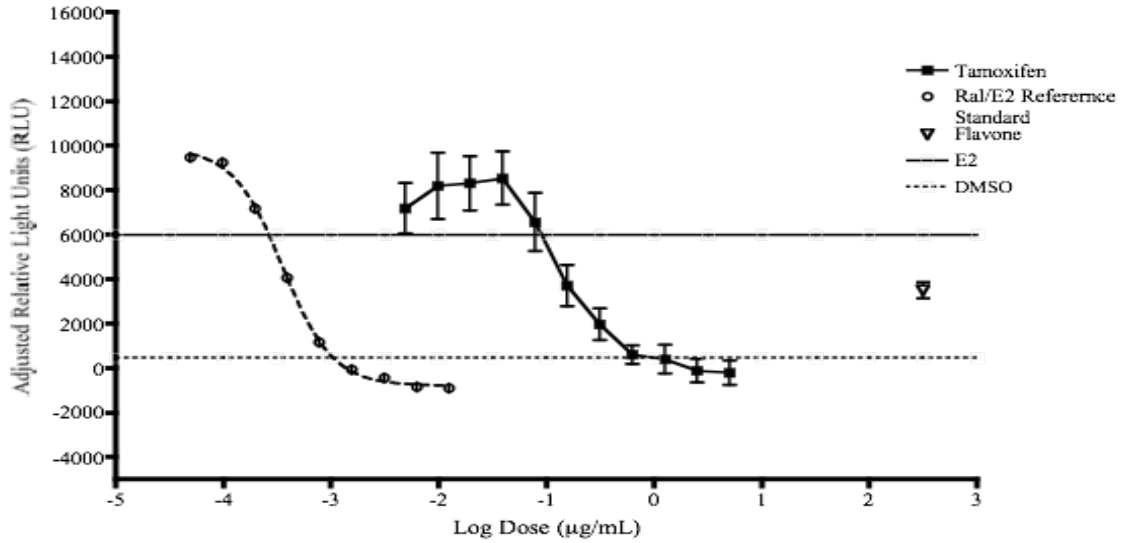
Table 12-12 Individual IC_{50} Values for N0016 – Tamoxifen

Experiment Date	IC_{50} (µg/mL)
20 April 06	0.21
9 May 06	0.11
11 May 06	0.15

Abbreviations: IC_{50} = concentration of test substance that inhibits the reference estrogen response by 50%

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

Figure 12-45 Antagonist Comprehensive Testing for N0016 – Tamoxifen: Averaged Experiments



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

Historical mean and standard deviation of the Ral/E2 reference standard.

Historical mean and standard deviation of the flavone control

Historical mean and standard deviation of the E2 control.

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.

Dashed horizontal line represents the historical mean of the DMSO control minus three times the standard deviation of the DMSO mean.

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.

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

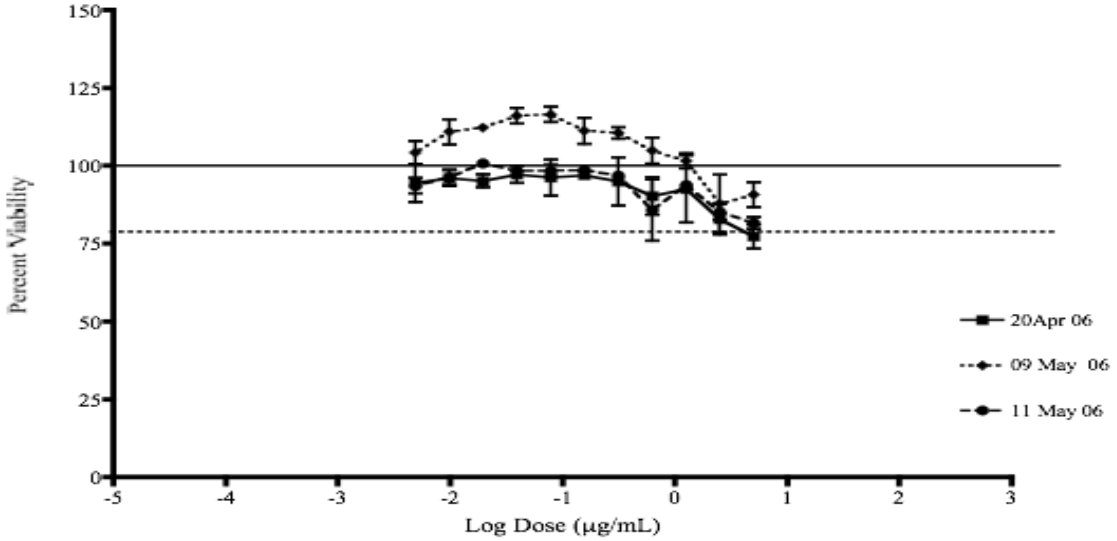
Table 12-13 Averaged IC_{50} Value for N0016 –Tamoxifen

IC_{50} (µg/mL)[1,2]	STD DEV	CV
0.16	4.89×10^{-2}	31%

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

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

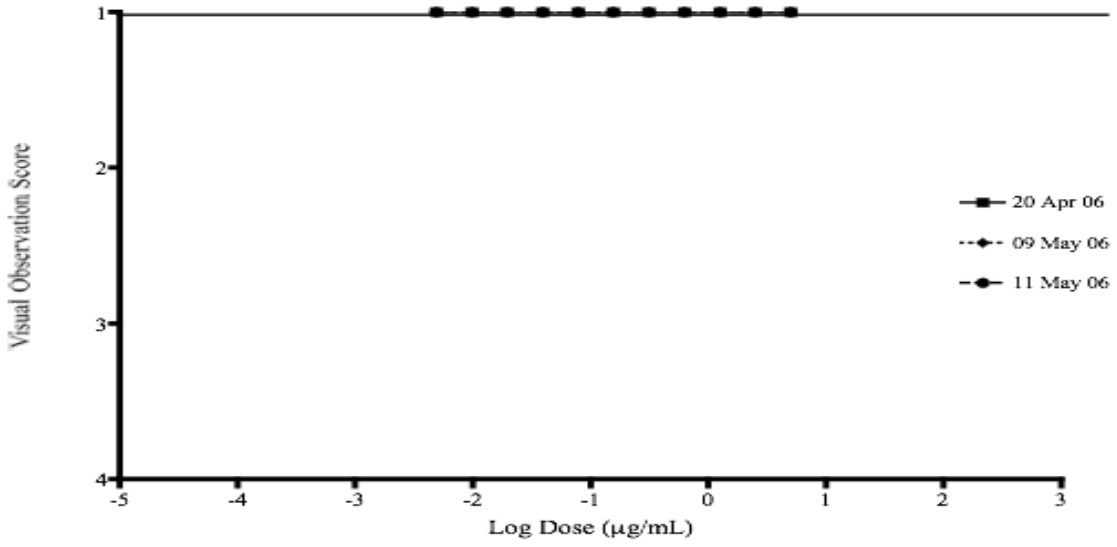
Figure 12-46 CellTiter-Glo® Viability Assessment for N0016 – Tamoxifen



Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

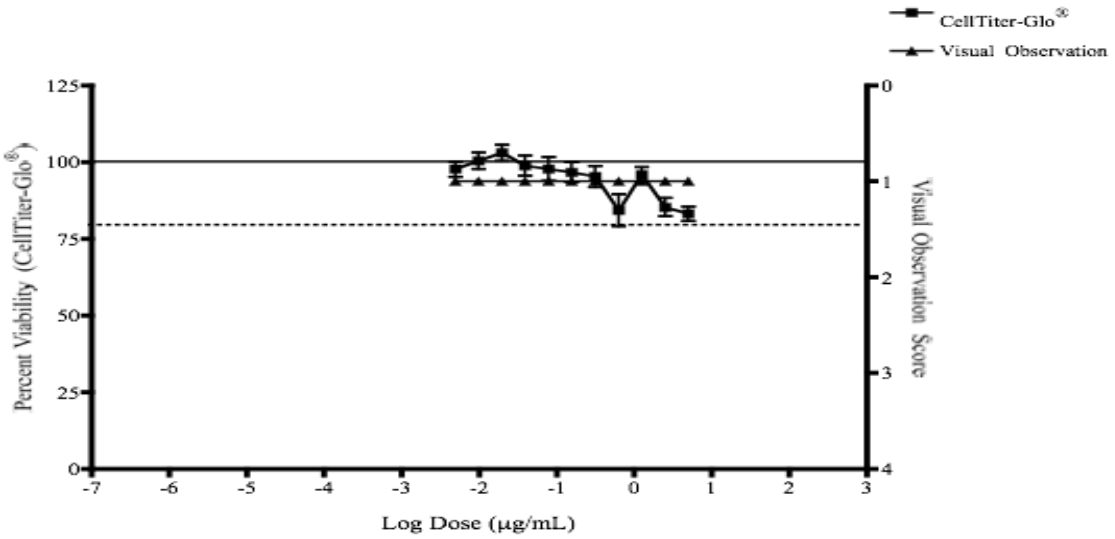
Dashed horizontal line indicates 80% cell viability. Concentrations that cause a decrease in cell viability below this line are considered cytotoxic and are not included in the evaluation of antagonist activity.

Figure 12-47 Visual Observation Viability Assessment for N0016 – Tamoxifen



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 ≥ 2 are considered cytotoxic and are not included in the evaluation of antagonist activity.

Figure 12-48 Combined Qualitative and Quantitative Viability Assessments for N0016 – Tamoxifen



Solid horizontal line indicates 100% cell viability as measured in dimethyl sulfoxide control.

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.

13.0 Evaluation of Reference Standard and Control Data

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

13.1 Agonist Reference Standards and Controls

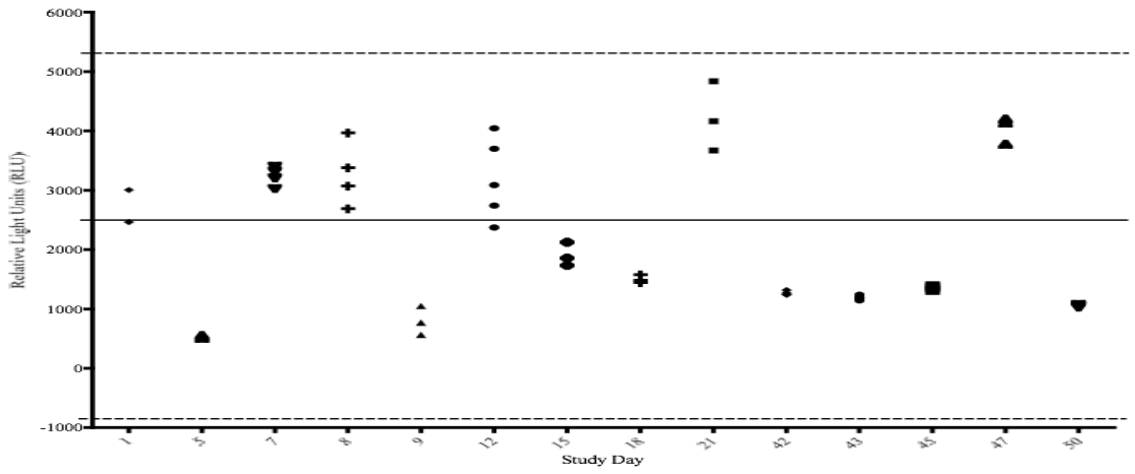
To determine whether agonist reference standard, control, and induction values changed over time, a linear regression analysis was performed with PRISM®, using a least squares method. The analysis was conducted using averaged reference standard, control, and induction values for all experiments conducted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

13.1.1 DMSO Control

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

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

Figure 13-1 DMSO Control Values for Experiments Conducted During Test Substance Agonist Range Finder and Comprehensive Testing



Abbreviations: DMSO = dimethyl sulfoxide

Values are not adjusted before analysis, and are expressed as relative light units (RLUs).

Each symbol represents the DMSO control value for each experiment performed on a given day.

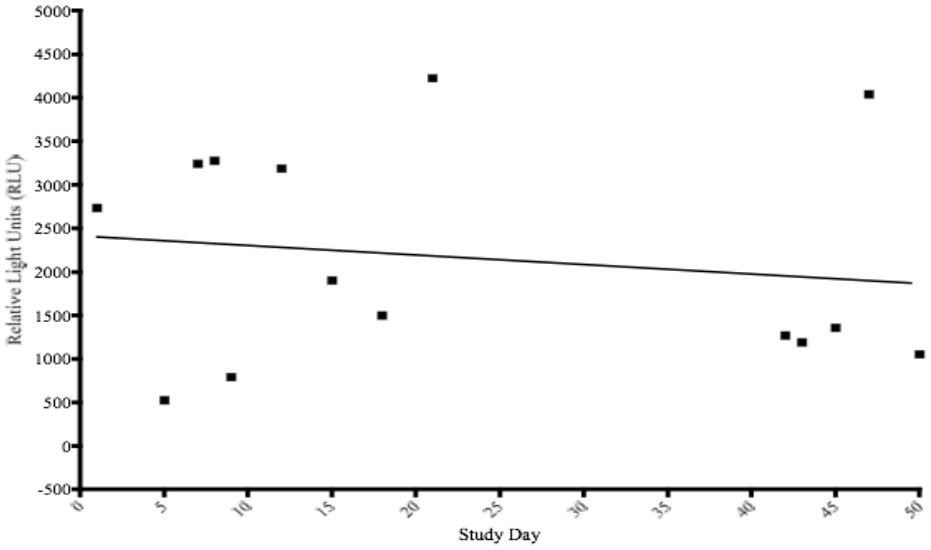
The solid line across the figure represents the mean of historical DMSO control values across all experiments.

The dashed lines across the figure represent the historical mean plus and minus 2.5 times the standard deviation from that mean.

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

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

Figure 13-2 Linear Regression of DMSO Control Values Against Time for Agonist Experiments



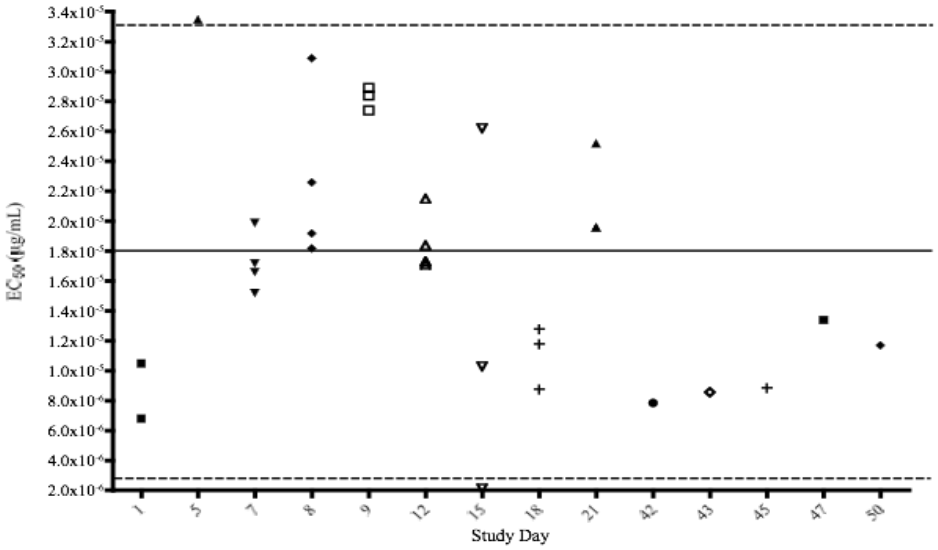
Abbreviations: DMSO = dimethyl sulfoxide
DMSO control values are not adjusted before analysis, and are expressed as relative light units (RLUs).
Each symbol represents the mean DMSO control value for experiments performed on a given day.

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

13.1.2 EC_{50} Value

Figure 13-3 shows the E2 reference standard EC_{50} values (µg/mL) for range finder and comprehensive testing, which were calculated for each experiment using the Hill function.

Figure 13-3 E2 Reference Standard EC_{50} Values for Experiments Conducted During Test Substance Range Finder and Comprehensive Testing

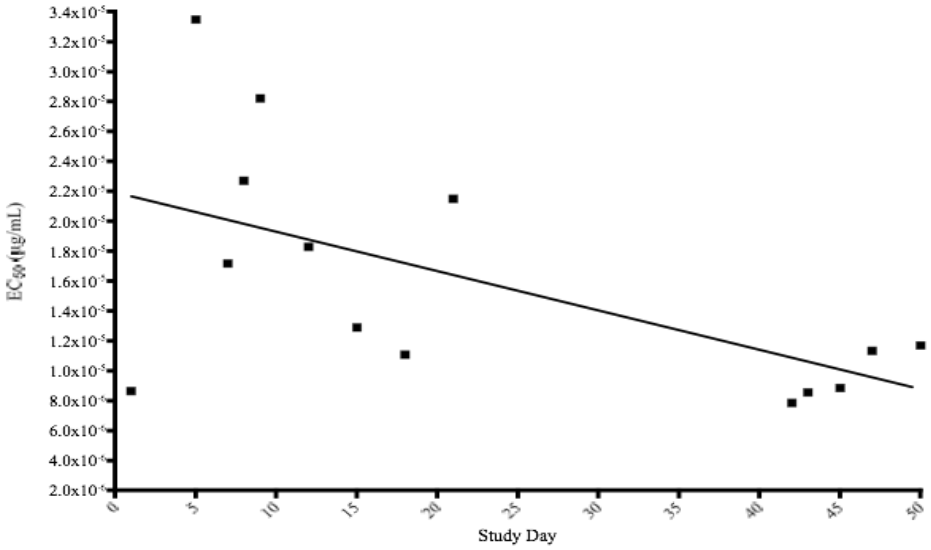


Abbreviations: E2 = 17ß-estradiol; EC_{50} = half-maximal effect concentration.
Each symbol represents the E2 reference standard EC_{50} value for each experiment performed on a given day.
The solid line across the figure represents the mean of historical experiments.
The dashed lines across the figure represent the historical mean plus and minus 2.5 times the Standard deviation from that mean.

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

Each point on the regression line represents the averaged E2 reference standard EC_{50} value for all experiments performed on a given day. **Figure 13-4** shows the linear regression of EC_{50} values over time.

Figure 13-4 Linear Regression of E2 Reference Standard EC_{50} Values Over Time



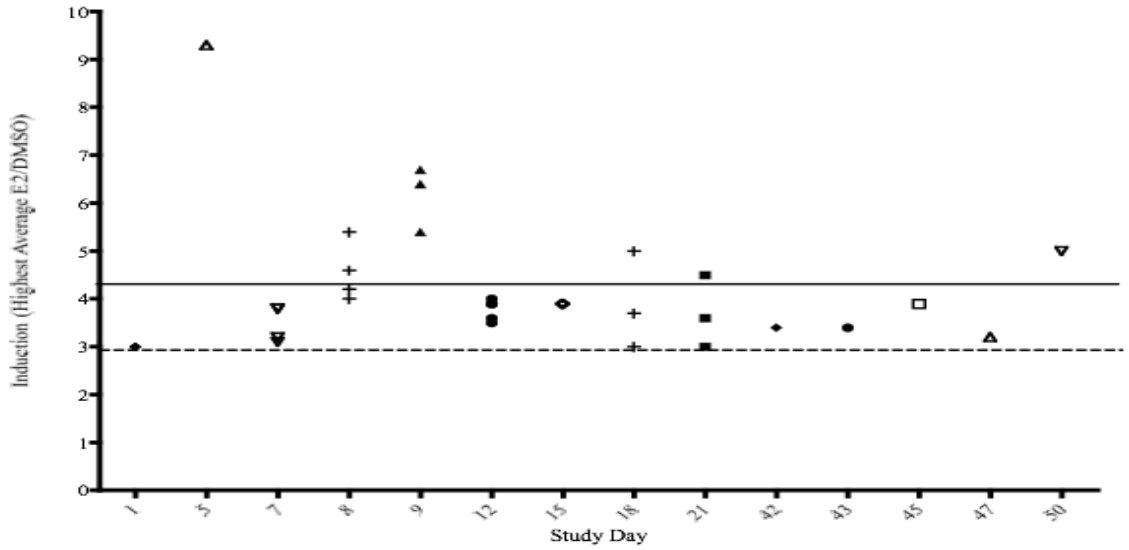
Abbreviations: E2 = 17ß-estradiol; EC_{50} = half-maximal effect concentration.
Each symbol represents the mean E2 reference standard EC_{50} value for experiments performed on a given day.

The slope of the linear regression of E2 reference standard EC_{50} values was significantly (p=0.03) different from zero, showing that EC_{50} values varied significantly over time.

13.1.3 Induction

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

Figure 13-5 Induction Values for Experiments Conducted During Test Substance Range Finder and Comprehensive Testing



Induction values are calculated as the averaged highest non-adjusted 17ß-estradiol relative light unit (RLU) value divided by the averaged non-adjusted dimethyl sulfoxide control RLU value for each experiment.

Each symbol represents the induction value for each experiment performed on a given day.

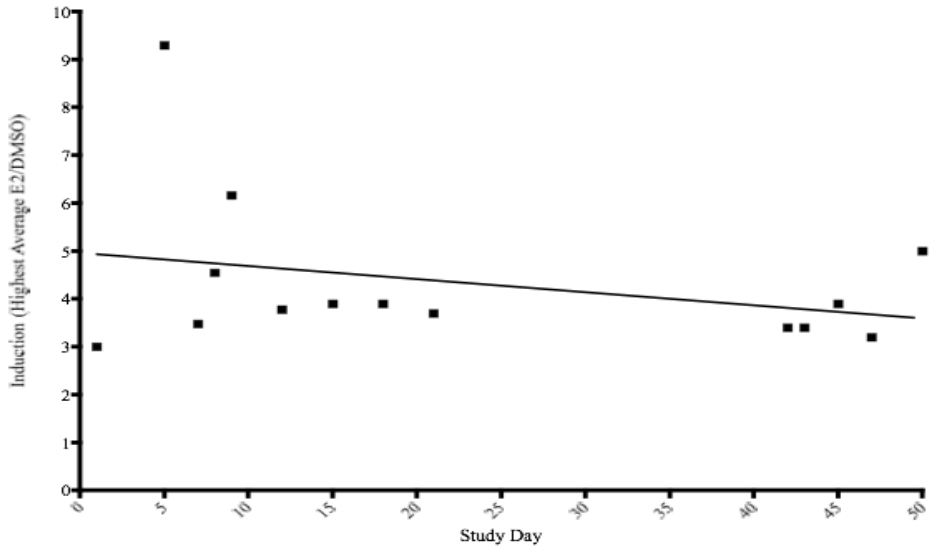
The solid line across the figure represents the mean of historical experiments.

The dashed line represents a value of "3". All values must be at or above this line for an experiment to be included in data analysis.

Each point represents the induction value for each experiment conducted on a given day. The number of experiments per day ranged from one to five. The solid line on the figure represents the historical mean of induction values, and the dashed line represents an induction value of three. All induction values obtained during range finder and comprehensive testing had to be greater than or equal to three for the experiment to be accepted.

Each point on the regression line represents the averaged induction value for all experiments performed on a given day. **Figure 13-6** shows the linear regression of induction values over time.

Figure 13-6 Linear Regression of Induction Values Against Time



Induction control values are expressed as the averaged highest non-adjusted 17ß-estradiol relative light unit (RLU) value divided by the averaged non-adjusted dimethyl sulfoxide control RLU value for each experiment.

Each symbol represents the mean induction value for experiments performed on a given day.

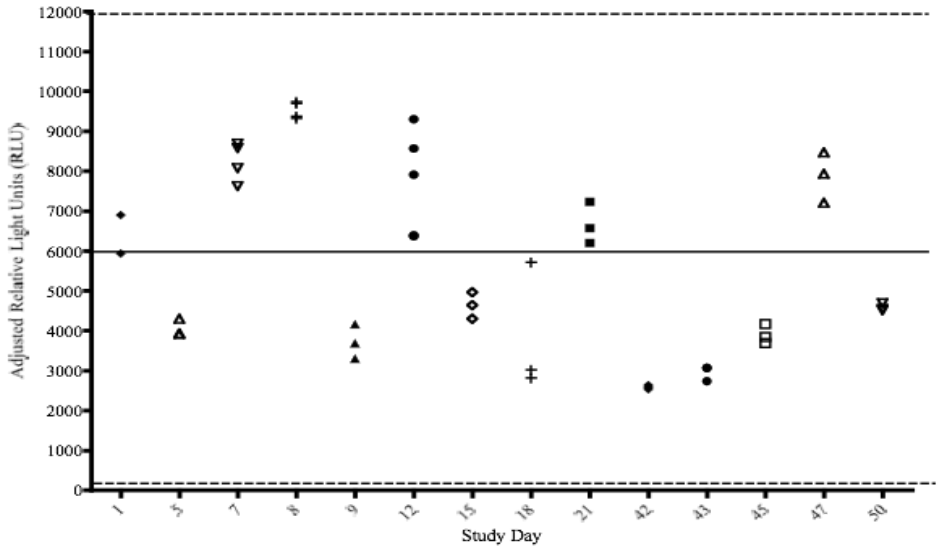
The slope of the linear regression of induction was not significantly (p=0.29) different from zero, showing that induction values did not vary significantly over time.

13.1.4 Methoxychlor Control

Methoxychlor values used for tracking of experimental data over time are presented as adjusted RLUs.

Figure 13-7 shows the methoxychlor control values for range finder and comprehensive testing.

Figure 13-7 Methoxychlor Control Values for Experiments Conducted During Range Finder and Comprehensive Testing

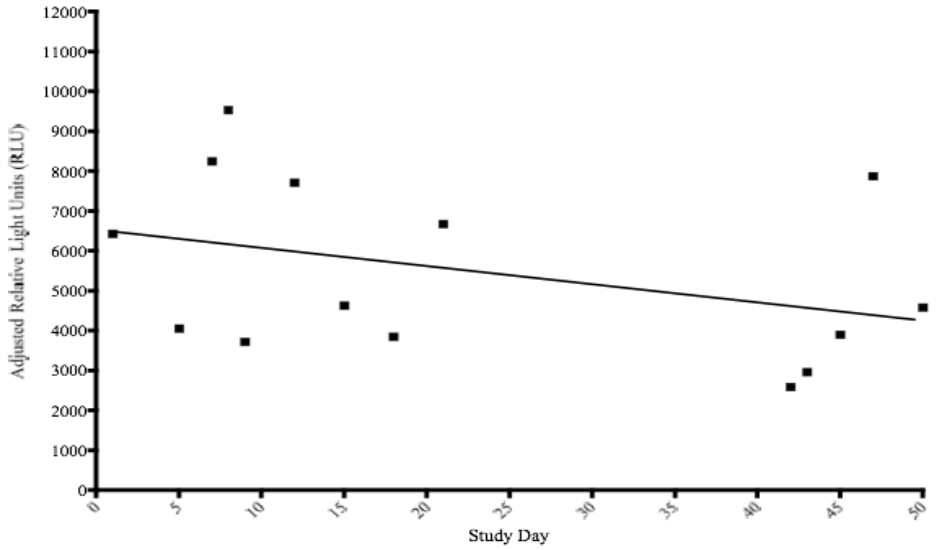


Methoxychlor control values are expressed as adjusted relative light units.
Each symbol represents the methoxychlor value for each experiment performed on a given day.
The solid line across the figure represents the mean of historical experiments.
The dashed lines across the figure represent the historical mean plus and minus 2.5 times the Standard deviation from that mean.

Each point represents the methoxychlor control value for each experiment conducted on a given day. The number of experiments per day ranged from one to five. The lines on the figure represent the historical mean, and the mean plus or minus 2.5 times the standard deviation of the methoxychlor control. All methoxychlor control values obtained during range finder and comprehensive testing had to fall within these limits for the experiment to be accepted.

The linear regression tracks the averaged experimental methoxychlor control values for each day of the study. Each point represents the averaged methoxychlor value for all experiments performed on a given day. **Figure 13-8** shows the linear regression of averaged, methoxychlor control adjusted RLU values over time.

Figure 13-8 Linear Regression of Methoxychlor Control Values Against Time



Methoxychlor control values are expressed as adjusted relative light units.
Each symbol represents the mean methoxychlor control value for experiments performed on a given day.

The slope of the linear regression of the methoxychlor control was not significantly (p=0.19) different from zero, showing that methoxychlor control values did not vary significantly over time.

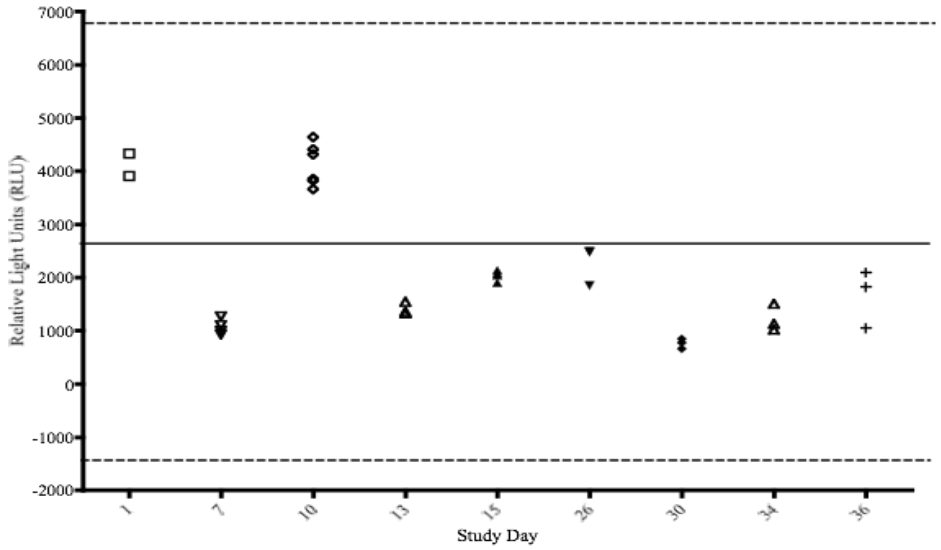
13.2 Antagonist Reference Standards and Controls

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

13.2.1 DMSO Control

DMSO control values used for tracking of experimental data over time are presented as non-adjusted RLUs. Adjusted RLUs are not used because the first step in adjustment is to control for the experimental background by subtracting the average experimental DMSO control value from each sample on the experimental plate. This practice leads to adjusted DMSO control values that are either zero or are extremely small. **Figure 13-9** shows the DMSO control values for range finder and comprehensive testing.

Figure 13-9 DMSO Control Values for Experiments Conducted During Test Substance Antagonist Range Finder and Comprehensive Testing

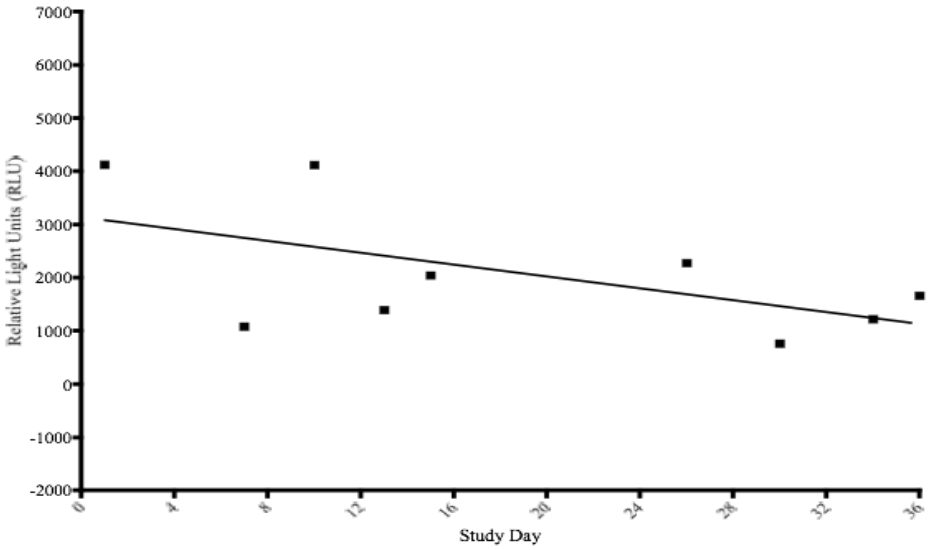


Abbreviations: DMSO = dimethyl sulfoxide
Control values are not adjusted before analysis, and are expressed as relative light units (RLUs).
Each symbol represents the DMSO control value for each experiment performed on a given day.
The solid line across the figure represents the mean of historical experiments.
The dashed lines across the figure represent the historical mean plus and minus 2.5 times the Standard deviation from that mean.

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

Each point on the regression line represents the averaged DMSO control value for all experiments performed on a given day. **Figure 13-10** shows the linear regression of averaged, non-adjusted DMSO control RLU values over time.

Figure 13-10 Linear Regression of DMSO Control Values Against Time for Antagonist Experiments



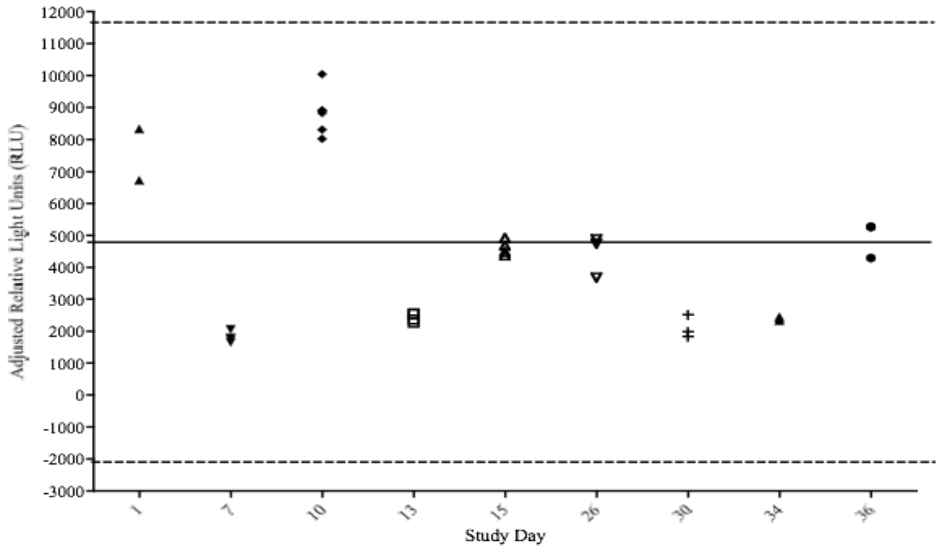
Abbreviations: DMSO = dimethyl sulfoxide
DMSO control values are not adjusted before analysis, and are expressed as relative light units (RLUs).
Each symbol represents the mean DMSO control value for experiments performed on a given day.

The slope of the linear regression of the DMSO control was not significantly different from zero (p = 0.11), showing that DMSO control values did not vary significantly over time.

13.2.2 E2 Control

E2 control values used for tracking of experimental data over time are presented as adjusted RLUs. **Figure 13-11** shows the E2 control values for range finder and comprehensive testing.

Figure 13-11 E2 Control Values for Experiments Conducted During Range Finder and Comprehensive Testing

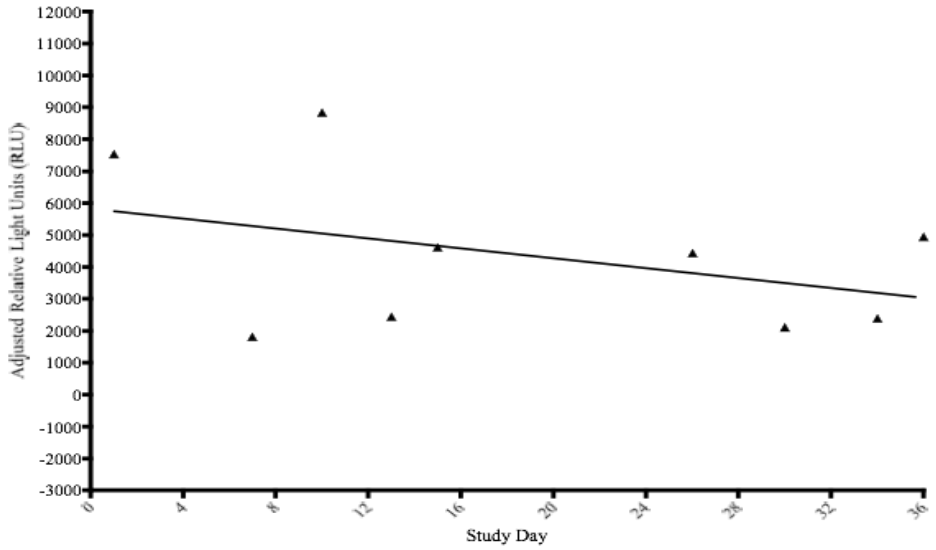


Abbreviations: E2 = 17β–estradiol
E2 values are expressed as adjusted relative light units.
Each symbol represents the E2 control value for each experiment performed on a given day.
The solid line across the figure represents the mean of historical experiments.
The dashed lines across the figure represent the historical mean plus and minus 2.5 times the standard deviation from that mean.

Each point represents the E2 control value for each experiment conducted on a given day. The number of experiments per day ranged from one to six. The lines on the figure represent the historical mean, and the mean plus and minus 2.5 times the standard deviation of the E2 control. All E2 control values obtained during range finder and comprehensive testing had to fall within these limits for the experiment to be accepted.

The linear regression tracks the averaged experimental E2 control values for each day of the study. Each point represents the averaged E2 control value for all experiments performed on a given day. **Figure 13-12** shows the linear regression of averaged adjusted E2 control RLU values over time.

Figure 13-12 Linear Regression of E2 Control Values Against Time



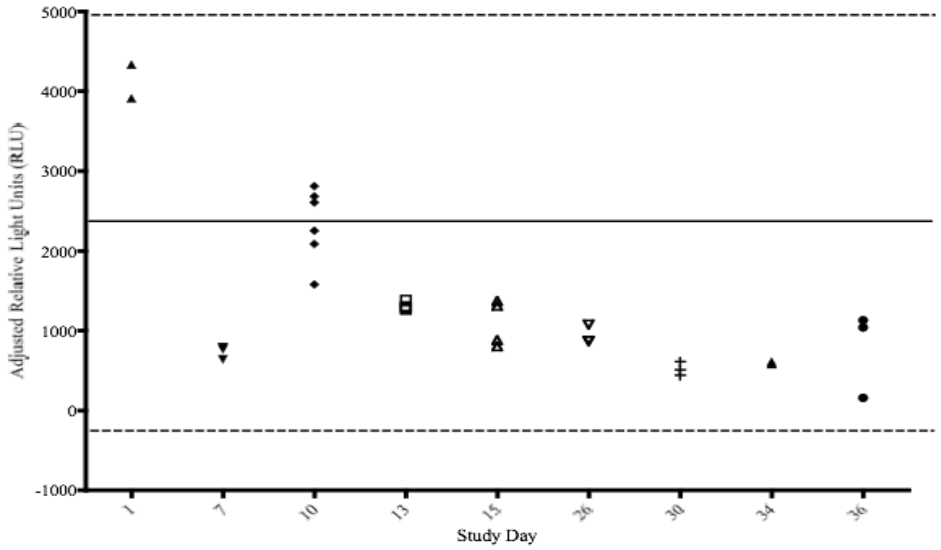
Abbreviations: E2 = 17β–estradiol
E2 control values are expressed as adjusted relative light units.
Each symbol represents the mean E2 control value for experiments performed on a given day.

The slope of the linear regression of the E2 control was not significantly (p=0.29) different from zero, showing that the E2 control values did not vary significantly over time.

13.2.3 Flavone Control

Flavone values used for tracking of experimental data over time are presented as adjusted RLUs. **Figure 13-13** shows the flavone control values for range finder and comprehensive testing.

Figure 13-13 Flavone Control Values for Experiments Conducted During Range Finder and Comprehensive Testing

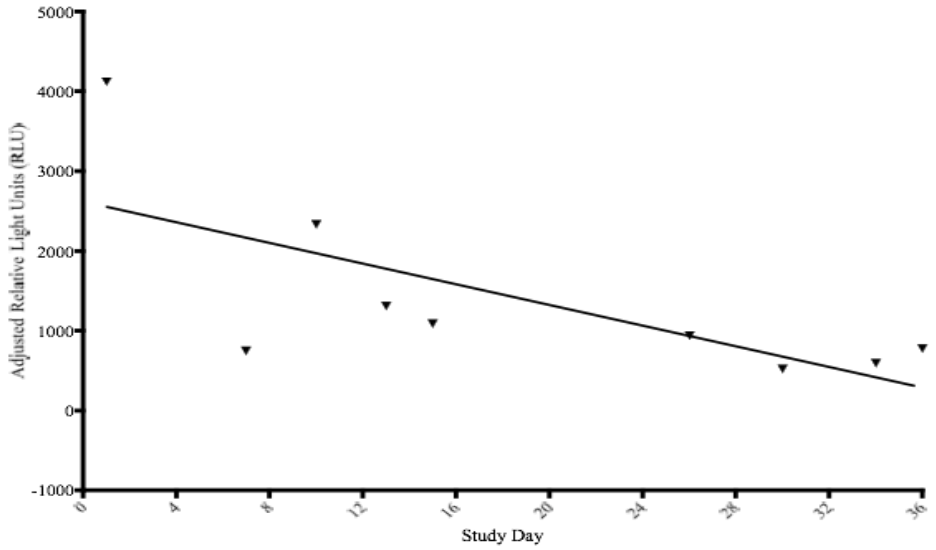


Flavone control values are expressed as adjusted relative light units.
Each symbol represents the flavone control value for each experiment performed on a given day.
The solid line across the figure represents the mean of historical experiments.
The dashed lines across the figure represent the historical mean plus and minus 2.5 times the Standard deviation from that mean.

Each point represents the flavone control value for each experiment conducted on a given day. The number of experiments per day ranged from one to six. The lines on the figure represent the historical mean, and the mean plus and minus 2.5 times the standard deviation of the flavone control. All flavone control values obtained during range finder and comprehensive testing had to fall within these limits for the experiment to be accepted.

Each point on the regression line represents the averaged flavone control value for all experiments performed on a given day. **Figure 13-4** shows the linear regression of averaged adjusted flavone control RLU values over time.

Figure 13-14 Linear Regression of Flavone Control Values Against Time



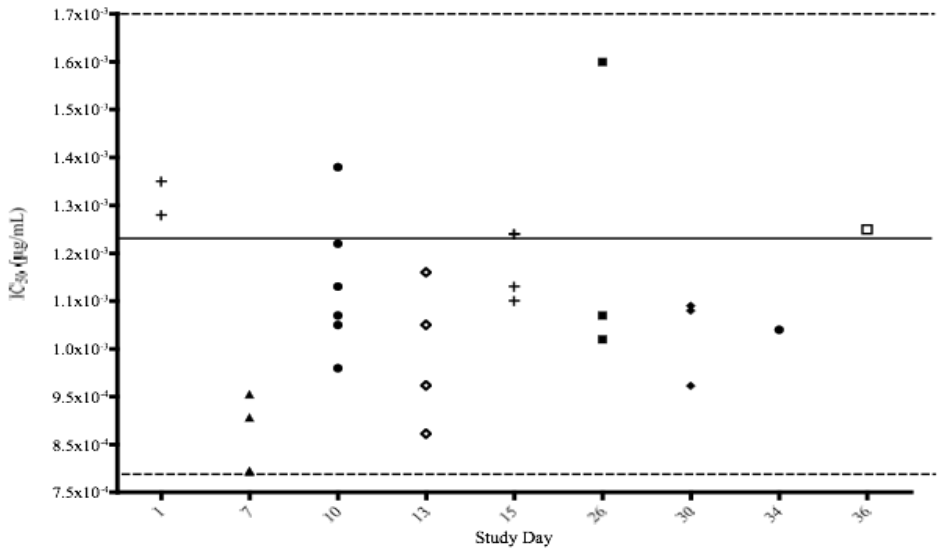
Flavone control values are expressed as adjusted relative light units.
Each symbol represents the mean flavone control value for experiments performed on a given day.

The slope of the linear regression of the flavone control was significantly different from zero ($p = 0.03$), showing that flavone control values decreased significantly over time.

13.2.4 IC_{50} Value

Ral/E2 reference standard IC_{50} values are calculated for each experiment using the PRISM® Hill function and are presented in µg/mL. **Figure 13-15** shows the Ral/E2 reference standard IC_{50} values for range finder and comprehensive testing.

Figure 13-15 Ral/E2 Reference Standard IC_{50} Values for Experiments Conducted During Range Finder and Comprehensive Testing



Abbreviations: Ral/E2 Reference Standard = concentrations of raloxifene with a fixed concentration of 2.5 x 10^{-5} µg/mL; IC_{50} = concentration of test substance that inhibits the reference estrogen response by 50%.

Each symbol represents the Ral/E2 reference standard IC_{50} value for each experiment performed on a given day.

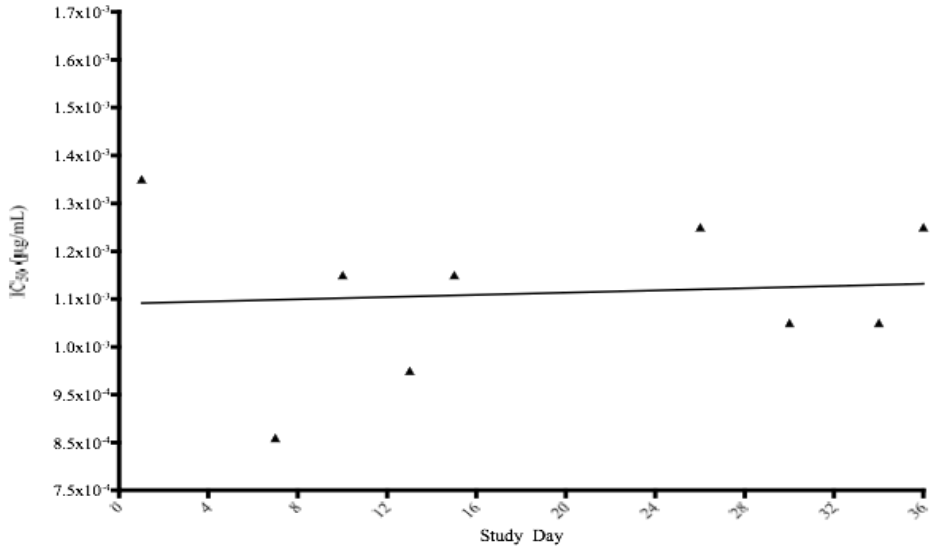
The solid line across the figure represents the mean of historical experiments.

The dashed lines across the figure represent the historical mean plus and minus 2.5 times the standard deviation from that mean.

Each point represents the Ral/E2 reference standard IC_{50} value for each experiment conducted on a given day. The number of experiments per day ranged from one to six. The lines on the figure represent the historical mean, and the mean plus and minus 2.5 times the standard deviation of the IC_{50} control. All IC_{50} values obtained during range finder and comprehensive testing had to fall within these limits for the experiment to be accepted.

Each point on the regression line represents the averaged Ral/E2 reference standard IC_{50} value for all experiments performed on a given day. **Figure 13-16** shows the linear regression of IC_{50} values over time.

Figure 13-16 Linear Regression of IC_{50} Values Against Time



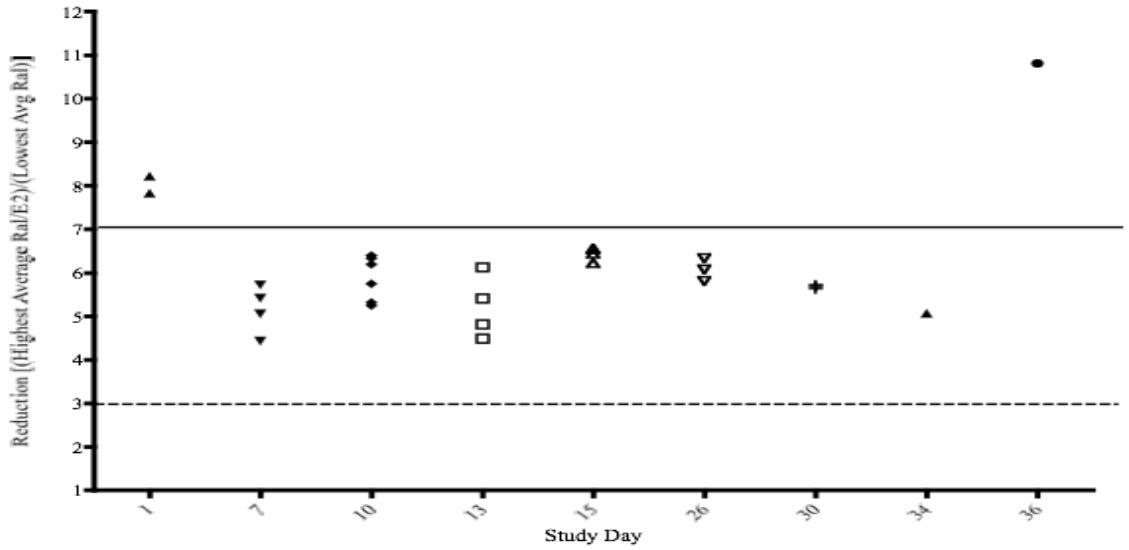
IC_{50} = concentration of test substance that inhibits the reference estrogen response by 50%
Each symbol represents the mean IC_{50} value for experiments performed on a given day.

The slope of the linear regression of the IC_{50} control was not significantly (p=0.82) different from zero, showing that the IC_{50} control data did not vary significantly over time.

13.2.5 Reduction

Reduction is a measure of the degree of responsiveness of the cells and is calculated by dividing the averaged highest non-adjusted Ral/E2 reference standard value by the averaged lowest non-adjusted Ral/E2 control value. **Figure 13-17** shows the reduction values (presented as a ratio) for range finder and comprehensive testing.

Figure 13-17 Reduction Values for Experiments Conducted During Range Finder and Comprehensive Testing



Reduction values are calculated as the averaged highest non-adjusted Ral/E2 (concentrations of raloxifene with a fixed concentration of 2.5 x 10^{-5} µg/mL) values divided by the averaged non-adjusted Ral/E2 values for each experiment.

Each symbol represents the reduction value for each experiment performed on a given day.

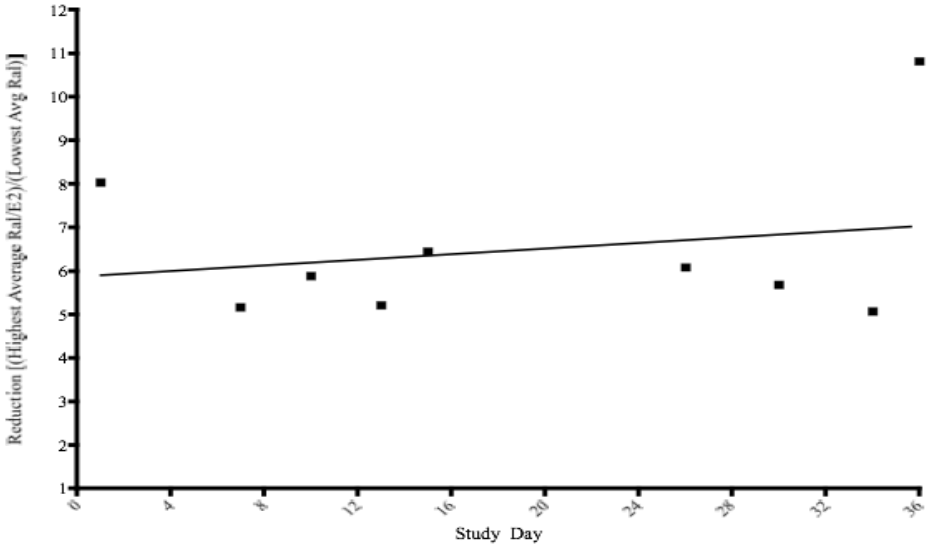
The solid line across the figure represents the mean of historical experiments.

The dashed lines across the figure represent the historical mean plus and minus 2.5 times the standard deviation from that mean.

Each point represents the reduction value for each experiment conducted on a given day. The number of experiments per day ranged from one to six. The line on the figure represents the historical mean of reduction values, and the dashed line represents a reduction value of three. All reduction values obtained during range finder and comprehensive testing had to be greater than or equal to three for the experiment to be accepted.

Each point on the regression line represents the averaged reduction value for all experiments performed on a given day. **Figure 13-18** shows the linear regression of reduction values over time.

Figure 13-18 Linear Regression of Reduction Values Against Time



Reduction control values are expressed as the averaged highest non-adjusted Ral/E2 (concentrations of raloxifene with a fixed concentration of 2.5 x 10^{-5} µg/mL) values divided by the averaged non-adjusted Ral/E2 values for each experiment.

Each symbol represents the mean reduction value for experiments performed on a given day.

The slope of the linear regression of reduction was not significantly (p=0.56) different from zero, showing that the reduction values did not vary significantly over time.

13.3 Summary of Results for Agonist and Antagonist Reference Standards and Controls

Agonist and antagonist results for reference standards, controls, induction or reduction for range finder and comprehensive testing are summarized in **Table 13-1**.

Table 13-1 Mean Values for Agonist and Antagonist Reference Standards and Controls

	Control	Mean	Standard Deviation	N[1]
Agonist Controls	DMSO Control[2]	2386	1213	33
	EC_{50} Value[3]	1.74×10^{-5}	7.87×10^{-6}	33
	Induction[4]	4.2	1.3	33
	Methoxychlor Control[5]	6218	2306	33
Antagonist Controls	DMSO Control[2]	2252	1304	28
	E2 Control[6]	4664	2751	28
	Flavone Control[6]	1149	808	28
	IC_{50} Value[3]	1.14×10^{-3}	2.25×10^{-4}	28
	Reduction[6]	6.06	1.36	28

Abbreviations: DMSO = dimethyl sulfoxide; E2 = 17β–estradiol; EC_{50} = half-maximal effect concentration; IC_{50} = concentration of test substance that inhibits the reference estrogen response by 50%.

[1]N = Number of experiments. Fewer experiments were conducted for antagonist testing than for agonist testing.

[2]Values are expressed as unadjusted relative light units.

[3]Values are expressed as μM.

[4]Induction is expressed as the ratio of the averaged highest unadjusted RLU value for the E2 reference standard in each experiment over the averaged DMSO control value.

[5]Values are expressed as adjusted relative light units.

[6]Reduction is expressed as the ratio of the averaged highest unadjusted RLU value for the Ral/E2 reference standard in each experiment over the averaged lowest unadjusted RLU value for the Ral/E2 reference standard.

Agonist and antagonist linear regression results for reference standards, controls, induction or reduction for range finder and comprehensive testing are summarized in **Table 13-2**.

Table 13-2 Linear Regression Analysis of Agonist and Antagonist Reference Standards and Controls

	Control	Slope	P-value (Slope)	r^2	y-intercept
Agonist Controls	DMSO Control	-11.09	0.58*	0.03	2420
	EC_{50} Value	-2.63×10^{-2}	0.03	0.35	2.2×10^{-5}
	Induction	-0.03	0.29*	0.09	4.97
	Methoxychlor Control	-45.70	0.19*	0.14	6539
Antagonist Controls	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_{50} Value	1.18×10^{-6}	0.82*	0.01	1.14×10^{-3}
	Reduction	0.03	0.56*	0.05	5.86

Abbreviations: DMSO = dimethyl sulfoxide; E2 = 17β–estradiol; EC_{50} = half-maximal effect concentration; IC_{50} = concentration of test substance that inhibits the reference estrogen response by 50%.

Each experiment was assigned a number based on the order in which testing occurred, without respect to the time lapsing between tests.

* The slope of the linear regression across experiments is not statistically different from zero.

14.0 Comparison of CellTiter-Glo® Versus Visual Observation Methods of Assessing Cell Viability

As part of the BG1Luc ER TA protocol standardization study, XDS evaluated the use of the CellTiter-Glo® (Promega Corporation) quantitative cell viability assay. Cell viability is measured by a luminescent signal that is proportional to the amount of adenosine triphosphate (ATP) in viable cells. Separate plates must be used for CellTiter-Glo® and BG1Luc ER TA as both assays use luminescence platforms. CellTiter-Glo® assays were conducted for all agonist and antagonist experiments during the BG1Luc ER TA protocol standardization study. A qualitative method using visual observation to assess cell viability was also conducted for all agonist and antagonist experiments during the protocol standardization study. Criteria for assessing and scoring cell viability using XDS's visual observation method are provided in **Table 7-1**.

A comparison of E2 reference standard data from the LUMI-CELL® and CellTiter-Glo® assays indicated that no decrease in response in the BG1Luc ER TA occurred when cell viability was at least 80% in the CellTiter-Glo® assay. In addition, CellTiter-Glo® values of 80% or above corresponded with a score of 1 in the visual observation method. Therefore, concentrations of test substance that caused a reduction in cell viability below 80% using CellTiter-Glo® or that had viability scores of 2 or more in the visual observation method were classified as cytotoxic and these data were not used to assess ER activity in the BG1Luc ER TA protocol standardization study.

A critical consideration in standardizing BG1Luc ER TA protocols is the efficacy of limiting the assessment of cell viability to visual observation. This would greatly reduce the effort and cost of cell viability assessment by eliminating the need for running concurrent parallel plates required when using the CellTiter-Glo® method. In the protocol standardization study, CellTiter-Glo® results from the testing of substances (eight for agonism and eight for antagonism) were compared to results from the XDS visual observation method.

14.1 Agonist Range Finder Testing

Cytotoxicity was only observed at the highest concentration tested (100 µg/mL) for coded test substances during agonist range finder testing. All substances tested were classified as cytotoxic at this concentration except for atrazine (N0001). Classification of cell viability agreed between the two methods (i.e., CellTiter-Glo® and visual observation) for all substances except for corticosterone (N0004), which was classified as "not cytotoxic" using the CellTiter-Glo® method (80% viability) but as "cytotoxic" using the visual observation method (score of "2") (see . **Table 14-2** and **Figure 14-1**).

Table 14-2 Cell Viability for Agonist Range Finder Testing at 100 µg/mL

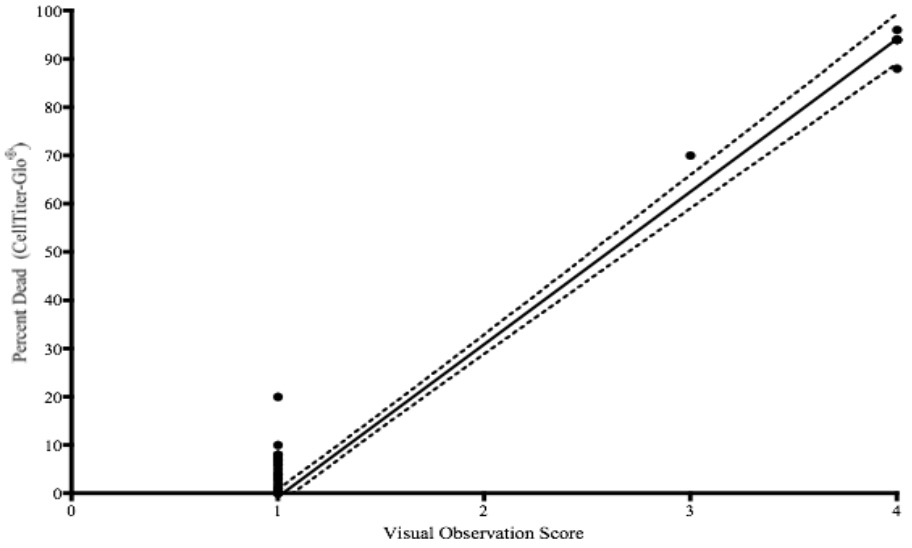
Code	Substance Name	% Cell Viability[1]	Visual Observation[2]
N0001	Atrazine	93%	1
N0002	Bisphenol A	6%	4
N0003	Bisphenol B	6%	4
N0004	Corticosterone	80%	2
N0005	*o,p'*-DDT	12%	4
N0006	Diethylstilbestrol	6%	4
N0007	17α-ethinyl estradiol	30%	3
N0008	Flavone	12%	4

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

[1] Cell viability as measured by CellTiter-Glo®.

[2] See **Table 7-1** for a description of the visual observation scores.

Figure 14-1 CellTiter-Glo® Versus Visual Observation Scores for Agonist Range Finder Testing



Each point on the figure represents a single replicate well for a single test substance.

The solid line represents the linear regression as calculated by PRISM®.

The dashed lines represent the 95% confidence limits of the linear regression as calculated by PRISM®.

14.2 Agonist Comprehensive Testing

Cytotoxicity was not observed at any of the concentrations used in the comprehensive testing for agonism. Classification of cell viability agreed between the two methods with all concentrations tested scoring at 87% or above using the CellTiter-Glo® method and at "1" using the visual observation method.

14.3 Antagonist Range Finder Testing

Cytotoxicity was only observed at the highest concentration tested (50 µg/mL) for five of the eight coded test substances during agonist range finder testing. The remaining three substances, BBP (N0009), DBA (N0010) and genistein (N0011), were not classified as cytotoxic at any concentration tested. Classification of cell viability agreed between the two methods and results are presented in **Table 14-3** and **Figure 14-**2.

Table 14-3 Cell Viability for Antagonist Range Finder Testing at 50 µg/mL

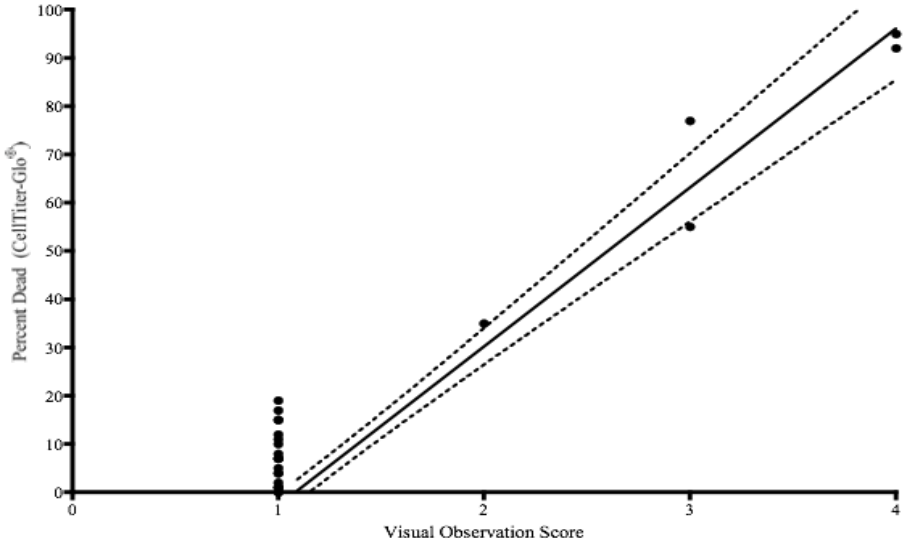
Code	Substance Name	% Cell Viability[1]	Visual Observation[2]
N0009	Butylbenzyl phthalate	111%	1
N0010	Dibenzo[*a*,*h*]anthracene	103%	1
N0011	Genistein	85%	1
N0012	Flavone	65%	2
N0013	*p*-n-nonylphenol	8%	4
N0014	Progesterone	45%	3
N0015	*o*,*p*'-DDT	23%	3
N0016	Tamoxifen	5%	4

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

[1] Cell viability as measured by CellTiter-Glo®.

[2] See **Table 7-1** for a description of the visual observation scores.

Figure 14-2 CellTiter-Glo® Versus Visual Observation Scores for Antagonist Range Finder Testing



Each point on the figure represents a single replicate well for a single test substance.

The solid line represents the linear regression as calculated by PRISM®.

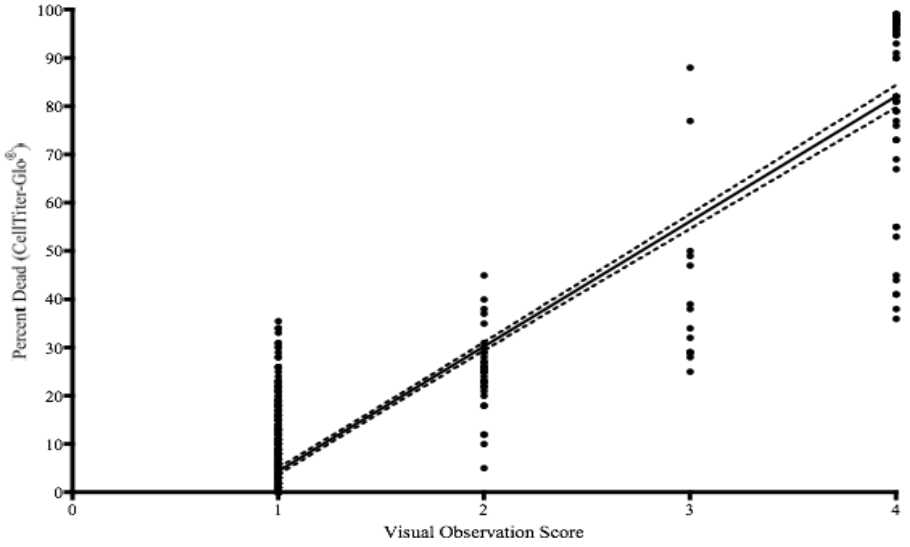
The dashed lines represent the 95% confidence limits of the linear regression as calculated by PRISM®.

14.4 Antagonist Comprehensive Testing

Cytotoxicity was observed at various concentrations used in the comprehensive testing of five of the eight coded antagonist test substances. The remaining three substances, DBA (N0010), genistein (N0011), and tamoxifen (N0016), were not classified as cytotoxic at any concentration tested.

An assessment of cell viability is especially important when testing for antagonism in order to determine whether reduction of luminescence is based on cytotoxicity or reduced ER mediated transcriptional activity. Therefore, BG1Luc ER TA results must be considered when comparing methods for assessing cell viability. For this comparison, BG1Luc ER TA results are expressed as percent reduction of E2 and is defined as the ability of a given concentration of test substance to reduce the ER TA activity induced by the E2 control (2.5×10^{-5} µg/mL, a concentration of E2 that induces 80-90% of maximum ER TA in the test system). BG1Luc ER TA results for the five substances that showed cytotoxicity are compared to scores from CellTiter-Glo® and visual observation methods in **Sections 14.4.1** through **14.4.6** below. The data presented and discussed does not include all concentrations tested in each experiment but is limited to those concentrations that were classified as cytotoxic in one or more experiments and focuses on visual observation scores that did not correspond with CellTiter-Glo® cell viability values (i.e., cell viability of 80% or above should correspond to a visual observation score of "1"). Comparison of CellTiter-Glo® data and visual observation scores for all concentrations of substances tested for antagonism are presented graphically in **Figure 14-3**.

Figure 14-3 CellTiter-Glo® Versus Visual Observation Scores for Antagonist Comprehensive Testing



Each point on the figure represents a single replicate well for a single test substance.
The solid line represents the linear regression as calculated by PRISM®.
The dashed lines represent the 95% confidence limits of the linear regression as calculated by PRISM®.

14.4.1 N0009 - BBP

Selected BG1Luc ER TA, CellTiter-Glo®, and visual observation results for BBP are provided in **Table 14-4**.

Table 14-4 Comparison of Selected Results for N0009 - BBP

Date	Concentration (μg/mL)	% Reduction of E2[1]	% Cell Viability[2]	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

Abbreviations: BBP = butylbenzyl phthalate; E2 = 17β–estradiol

[1] 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 E2 control RLU value times 100.

[2] Cell viability as measured by CellTiter-Glo®.

A comparison of the results indicated the following:

- 12 April 06 experiment:
  - CellTiter-Glo® values and visual observation scores agreed.
  - Concentrations which reduce E2 activity were classified as cytotoxic, and were not used to assess ER activity.
  - Test substance was classified as negative for ER antagonist activity.
- 15 April /06 experiment:
  - CellTiter-Glo® values and visual observation scores agreed.
  - None of the concentrations reducing E2 activity were classified as cytotoxic by either CellTiter-Glo® or visual observation, so they were used to assess ER activity.
  - Test substance was classified as positive for ER antagonist activity.
- 18 April 06 experiment:
  - CellTiter-Glo® values and visual observation scores did not correspond at the 25 and 12.5 μg/mL concentrations.
  - The concentration (25 μg/mL) which reduced E2 activity was classified as cytotoxic by CellTiter-Glo® and was not used to assess ER activity.
  - The 25 μg/mL concentration would have been classified as positive using visual observations.

Butylbenzyl phthalate was classified as negative for ER antagonist activity when cell viability was measured using CellTiter-Glo®, but it would have been classified as positive if using visual observations.

14.4.2 N0012 - Flavone

Selected BG1Luc ER TA, CellTiter-Glo®, and visual observation results for flavone are provided in **Table 14-5**.

Table 14-5 Comparison of Selected Results for N0012 - Flavone

Date	Concentration (µg/mL)	% Reduction of E2[1]	% Cell Viability[2]	Visual Observation
12 April 06	50	93	83	2
	25	72	78	1
	12.5	38	78	1
	6.25	9	85	1
15April 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

Abbreviations: E2 = 17β–estradiol

[1] 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 E2 control RLU value times 100.

[2] Cell viability as measured by CellTiter-Glo®. Concentrations of test substances that cause a decrease in cell viability to below 80% are considered to be cytotoxic and are not included in data analyses.

A comparison of the results indicated the following:

- 12 April 06 experiment:
  - — CellTiter-Glo® values and visual observation scores did not agree.
  - — Two of the concentrations which reduced E2 activity (25 and 12.5 µg/mL) were classified as cytotoxic by CellTiter-Glo® and were not used to assess ER activity.
  - — These concentrations would have been classified as positive if using visual observations.
- 15 April 06 experiment:
  - — CellTiter-Glo® values and visual observation scores did not agree at 50 µg/mL.
  - — Using CellTiter-Glo®, the two concentrations (50 and 25 µg/mL) causing a reduction in E2 activity were not cytotoxic and were used to assess ER activity.
  - — Using visual observations, the 50 µg/mL concentration would have been classified as cytotoxic.
  - — Flavone would have been classified as positive for ER antagonist activity using CellTiter-Glo® and negative using visual observations.
- 18 April 06 experiment:
  - — CellTiter-Glo® values and visual observation scores did not correspond at the 25 and 12.5 µg/mL concentrations.
  - — Two of the concentrations reducing E2 activity (25 and 12.5 µg/mL) were classified as cytotoxic by CellTiter-Glo® and were not used to assess ER activity.
  - — These concentrations would have been classified as positive for ER antagonist activity using visual observations.

Flavone was classified as positive for ER antagonist activity when cell viability was measured using CellTiter-Glo®, but it would have been classified as negative if using visual observations only.

14.4.3 N0013 - Nonylphenol

Selected BG1Luc ER TA, CellTiter-Glo®, and visual observation results for nonylphenol are provided in **Table 14-6**.

Table 14-6 Comparison of Selected Results for N0013 - Nonylphenol

Date	Concentration (µg/mL)	% Reduction of E2[1]	% Cell Viability[2]	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

Abbreviations: E2 = 17β–estradiol

[1] 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 E2 control RLU value times 100.

[2] Cell viability as measured by CellTiter-Glo®.

A comparison of the results indicated the following:

- 15 April 06 experiment:
  - — CellTiter-Glo® values and visual observation scores agreed.
  - — The 12.5 µg/mL concentration, which reduced E2 activity, was classified as cytotoxic and was not used to assess ER activity.
  - — Substance was classified as positive for ER antagonist activity at 6.25 µg/mL using both CellTiter-Glo® and visual observations.
- 20 April 06 experiment:
  - — CellTiter-Glo® values and visual observation scores agreed.
  - — Both concentrations reducing E2 activity were classified as cytotoxic and were not used to assess ER activity.
  - — Substance was classified as negative for ER antagonist activity using both CellTiter-Glo® and visual observations.
- 1 May 06 experiment:
  - — CellTiter-Glo® values and visual observation scores agreed.
  - — The 12.5 µg/mL concentration, which reduced E2 activity, was classified as cytotoxic and was not used to assess ER activity.

Nonylphenol was classified as positive for ER antagonist activity at 6.25 µg/mL using both CellTiter-Glo® and visual observations.

14.4.4 N0014 - Progesterone

Selected BG1Luc ER TA, CellTiter-Glo®, and visual observation results for progesterone are provided in **Table 14-7**.

Table 14-7 Comparison of Selected Results for N0014 - Progesterone

Date	Concentration (µg/mL)	% Reduction of E2[1]	% Cell Viability[2]	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

Abbreviations: E2 = 17β–estradiol

[1] 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 E2 control RLU value times 100.

[2] Cell viability as measured by CellTiter-Glo®.

A comparison of results indicated the following:

- 15 April 06 experiment:
  - — CellTiter-Glo® values and visual observation scores agreed.
  - — Neither concentration reducing E2 activity (12.5 and 6.25 µg/mL) was classified as cytotoxic with either CellTiter-Glo® or visual observations, so both concentrations were used to assess ER activity.
  - — Progesterone was classified as positive for ER antagonist activity using both CellTiter-Glo® and visual observations.
- 20 April 06 experiment:
  - — CellTiter-Glo® values and visual observation scores did not correspond at 12.5 µg/mL.
  - — The concentrations reducing E2 activity (25 and 12.5 µg/mL) were classified as cytotoxic using CellTiter-Glo®, so they were not used to assess ER activity.
  - — 12.5 µg/mL would have been considered positive for antagonism using visual observations.
- 1 May 06 experiment:
  - — CellTiter-Glo® values and visual observation scores agreed.
  - — Concentrations reducing E2 activity were classified as cytotoxic using both CellTiter-Glo® and visual observations, so they were not used to assess ER activity.

Progesterone was classified as negative for ER antagonist activity.

14.4.5 N0015 - o,p'-DDT

Selected BG1Luc ER TA, CellTiter-Glo®, and visual observation results for *o,p'*-DDT are provided in **Table 14-8**.

Table 14-8 Comparison of Selected Results for N0015 - *o,p'*-DDT

Date	Concentration (µg/mL)	% Reduction of E2[1]	% Cell Viability[2]	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

Abbreviation: *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane; E2 = 17β–estradiol

[1] 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 E2 control RLU value times 100.

[2] Cell viability as measured by CellTiter-Glo®.

A comparison of the results indicated the following:

- 20 April 06 experiment:
  - — CellTiter-Glo® values and visual observation scores agreed.
  - — Concentrations reducing E2 activity were classified as cytotoxic using both CellTiter-Glo® and visual observations, so they were not used to assess ER activity.
  - — *o,p'*-DDT was classified as negative for ER antagonist activity using both CellTiter-Glo® and visual observations.

- 1 May 06 experiment:
  - — CellTiter-Glo® values and visual observation scores agreed.
  - — Concentrations reducing E2 activity were classified as cytotoxic using both CellTiter-Glo® and visual observations, so they were not used to assess ER activity.
  - — *o,p'*-DDT was classified as negative for ER antagonist activity using both CellTiter-Glo® and visual observations.

- 5 May 06 experiment:
  - — CellTiter-Glo® values and visual observation scores did not correspond at 12.5 µg/mL.
  - — Concentrations reducing E2 activity were classified as cytotoxic by visual observation but not at 12.5 µg/mL with CellTiter-Glo®.

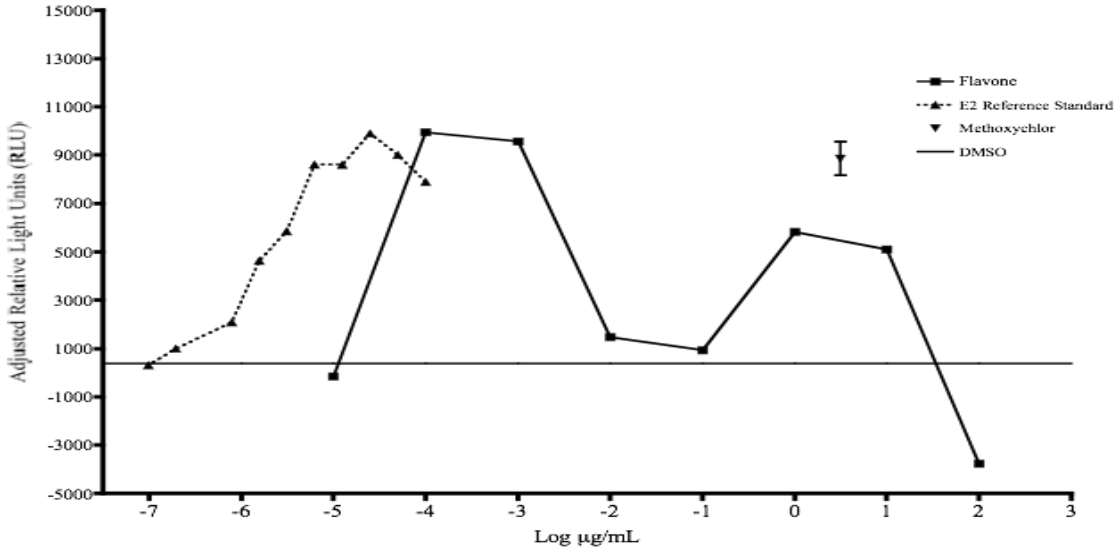
o,p'-DDT was classified as negative for ER antagonist activity when using visual observations, but would have been classified positive for antagonism at 12.5 µg/mL with CellTiter-Glo®.

15.0 Problems Encountered During the Protocol Standardization Study

15.1 Aberrant Range Finder Concentration-Response Curve for N0008 - Flavone

During protocol standardization, flavone yielded a biphasic concentration-response curve (**Figure 15-1**).

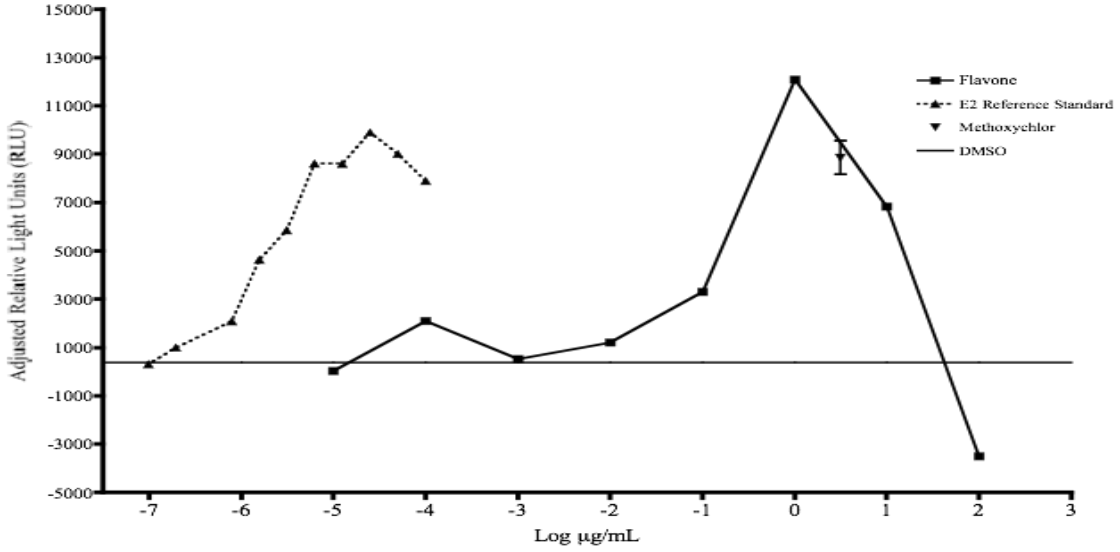
Figure 15-1 Initial Agonist Range Finder for N0008 – Flavone



Abbreviations: E2 = 17β–estradiol; methoxychlor = 3.13 µg/mL methoxychlor control; DMSO = dimethyl sulfoxide.
Line represents the mean of four DMSO replicates plus three times the standard deviation of the DMSO mean.

Due to concerns about possible experimental error, range finder testing for this substance was repeated three additional times (**Figure 15-2**).

Figure 15-2 Repeated Agonist Range Finder for N0008 – Flavone



Abbreviations: E2 = 17β–estradiol; methoxychlor = 3.13 µg/mL methoxychlor control; DMSO = dimethyl sulfoxide.
Line represents the mean of four DMSO replicates plus three times the standard deviation of the DMSO mean.

Repeat range finder testing of flavone showed that the peak occurring at the lowest concentrations of flavone did not appear consistently. It is possible that contamination was present during the creation of two of the four serial dilutions used to produce the four flavone range finder concentration-response curves, either in the batch of flavone sent to the laboratory or in the test tubes used to make the dilution.

15.2 Errors in Serial Dilutions

During protocol standardization, each serial dilution used on an experimental plate was independently diluted, such that serial dilution A is different from serial dilutions B and C. Serial dilutions were performed in this manner in order to minimize the loss of experimental data caused by experimenter error.

15.2.1 N0001 – Atrazine

The atrazine agonist experiment conducted on 04/04/06 had a portion of replicate serial dilution B omitted due to experimenter error. In dilution B, adjusted RLU values for atrazine (**Table 15-1**) were significantly higher than those obtained in either the range finder testing or the other experiments using atrazine. These values were excluded from analysis.

Table 15-1 Adjusted RLU Values for the Atrazine Experiment Conducted on 4 April 2006

Atrazine Concentration (µg/mL)	Serial Dilution A[1]	Serial Dilution B[1]	Serial Dilution C[1]
1.00×10^{-2}	***2444***[2]	867	634
5.00×10^{-3}	298	441	901
2.50×10^{-3}	428	467	513
1.25×10^{-3}	251	128	500
6.25×10^{-4}	62	224	488
3.13×10^{-4}	826	***7061***	275
1.56×10^{-4}	147	***7544***	475
7.81×10^{-5}	79	***7302***	516
3.91×10^{-5}	-97	***5416***	210
1.95×10^{-5}	24	***3844***	440
9.77×10^{-6}	***5505***	***2498***	328

Abbreviations: RLU = relative light unit

Values are presented as adjusted RLUs.

Values presented in bolded and italicized text did not pass the Q test for outliers (Zar 1984).

15.2.2 N0004 – Corticosterone

The corticosterone agonist experiment conducted on 8 April 06 had a portion of replicate serial dilution B omitted due to experimenter error. In dilution B, adjusted RLU values for corticosterone (**Table 15-2**) were significantly higher than those obtained in either the range finder testing or the other experiments using corticosterone. These values were excluded from analysis.

Table 15-2 Adjusted RLU Values for the Corticosterone Experiment Conducted on 8 April 2006

Corticosterone Concentration (µg/mL)	Serial Dilution A[1]	Serial Dilution B[1]	Serial Dilution C[1]
5.00×10^{-1}	-832	***11202***[2]	191
2.50×10^{-1}	-86	800	2519
1.25×10^{-1}	-331	-148	2347
6.25×10^{-2}	338	185	1340
3.13×10^{-2}	***13577***	835	1139
1.56×10^{-2}	***9268***	-147	276
7.81×10^{-3}	***8044***	434	327
3.91×10^{-3}	***5894***	-646	-1222
1.95×10^{-3}	***2162***	-788	-754
9.77×10^{-4}	544	-823	-422
6.10×10^{-4}	150	-495	***2788***

RLU = relative light unit

[1] Values are presented as adjusted RLUs.

[2] Values presented in bolded and italicized text did not pass the Q test for outliers (Zar 1984).

15.2.3 N0006 – Diethylstilbestrol

The diethylstilbestrol agonist experiment conducted on 17 April 06 had a portion of replicate serial dilution A omitted due to experimenter error. In this serial dilution, adjusted RLU values for diethylstilbestrol (**Table 15-3**), which had been decreasing with decreasing concentration of diethylstilbestrol suddenly increased. Since diethylstilbestrol did not exhibit a biphasic response in any other experiment, these values were excluded from analysis.

Table 15-3 Adjusted RLU Values for the Diethylstilbestrol Experiment Conducted on 17 April 2006

Diethylstilbestrol Concentration (µg/mL)	Serial Dilution A[1]	Serial Dilution B[1]	Serial Dilution C[1]
1.00×10^{-4}	9654	9640	9854
5.00×10^{-5}	10667	10105	7880
2.50×10^{-5}	8847	6428	7728
1.25×10^{-5}	4382	6239	7649
6.25×10^{-6}	3112	2964	3032
3.13×10^{-6}	***8626***[2]	2103	1710
1.56×10^{-6}	***9220***	557	2064
7.81×10^{-7}	***8530***	-370	467
3.91×10^{-7}	***7499***	570	708
1.95×10^{-7}	***5442***	-161	1083
9.77×10^{-8}	***5196***	-323	91

RLU = relative light unit

[1] Values are presented as adjusted RLUs.

[2] Values presented in bolded and italicized text did not pass the Q test for outliers (Zar 1984).

15.2.4 N0007 – EE

The EE agonist experiment conducted on 17 April 06 had the entirety of replicate serial dilution B omitted due to experimenter error. In this serial dilution, adjusted RLU values for EE (**Table 15-4**), remain stable throughout the entire concentration-response curve.

Table 15-4 Adjusted RLU Values for the EE Experiment Conducted on 17 April 2006

EE Concentration (μg/mL)	Serial Dilution A[1]	Serial Dilution B[1]	Serial Dilution C[1]
1.00×10^{-4}	8305	**9011**[3]	9148
5.00×10^{-5}	9516	**8772**	7682
2.50×10^{-5}	10280	**8154**	7573
1.25×10^{-5}	7081	**8643**	7984
6.25×10^{-6}	8773	**8955**	6288
3.13×10^{-6}	7161	**9431**	4655
1.56×10^{-6}	6064	**8689**	2577
7.81×10^{-7}	4047	**8145**	1212
3.91×10^{-7}	3590	**8840**	2223
1.95×10^{-7}	2248	**9345**	2885
9.77×10^{-8}	2406	**9015**	521

Abbreviations: RLU = relative light unit; EE = 17α-ethinyl estradiol

[1] Values are presented as adjusted RLUs.

[2] Values presented in bolded text were discarded because they did not exhibit a concentration-responsive decrease in adjusted RLUs.

16.0 Deviations From Protocol

16.1 Alteration of Testing Concentrations During Agonist Comprehensive Testing

Concentrations for comprehensive testing were selected during evaluation of range finder results. These concentrations were selected to optimize the possibility of detecting a positive result during comprehensive testing. For agonism, the starting concentration for serial dilution was selected as the concentration that was a log-dilution higher than that giving the highest adjusted RLU value during range finder testing. Selection of this concentration allowed for saturation to be reached at the highest concentrations tested. After the first experiment, two substances, bisphenol B (N0003), and flavone (N0008) did not reach saturation, so their starting concentrations were adjusted to start at a double serial dilution higher in order to generate concentration-response curves that reached saturation.

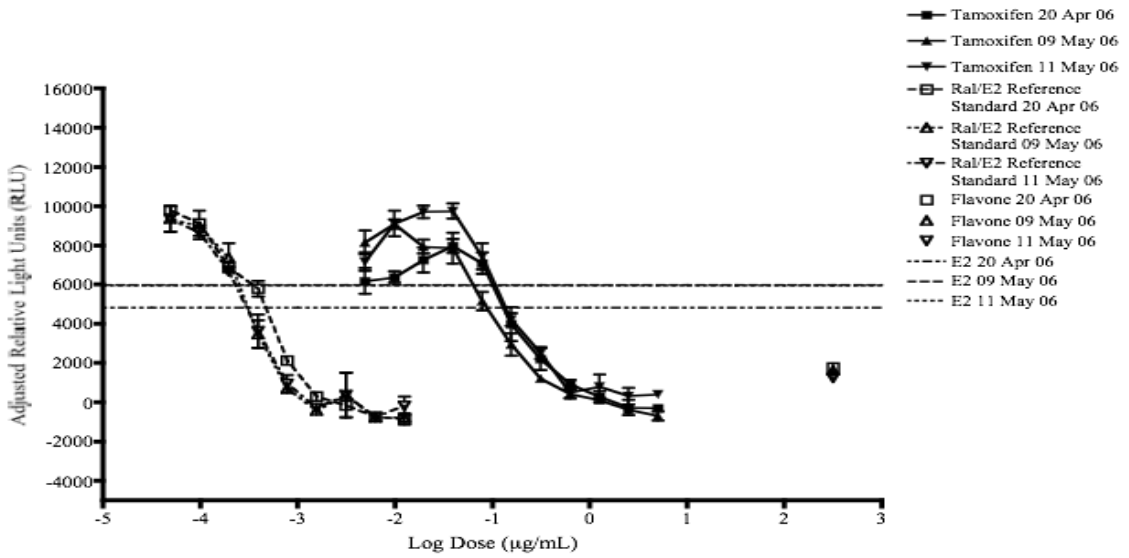
16.2 Alteration of Highest Testing Concentration for Antagonist Testing

According to the protocol, the highest antagonist concentration used for range finder testing should have been 100 μg/mL, a concentration that had previously been determined to be the limit of solubility. Upon receipt of the final report, it was noted that the highest concentration used in antagonist testing was 50 μg/mL, which contradicted the concentrations reported by XDS during antagonist range finding. Queries to XDS determined that the 50 μg/mL starting concentrations were correct and had previously been incorrectly reported because the experimenter had failed to account for the additional dilution caused by the 1:1 dilution resulting from the addition of 2.5×10^{-5} μg/mL E2 to the mixing tubes. This deviation was not relevant for BBP, flavone, nonylphenol, progesterone, and *o,p'*-DDT, because although these compounds were not tested to the limit concentration or the limit of solubility, they were tested at concentrations high enough to cause cytotoxicity. The two substances that were not cytotoxic at 50 μg/mL, DBA and genistein, were not soluble at 100 μg/mL.

16.3 Alteration of Testing Concentrations for Tamoxifen Comprehensive Testing

A starting concentration for tamoxifen comprehensive testing of 5 μg/mL was selected as the result of range finder testing (**Figure 16-1**). No cytotoxicity was observed at any concentration during this experiment (**Figure 16-2**). After the first comprehensive experiment, the starting concentration of tamoxifen was changed to 50 μg/mL to better define the high end of the concentration-response curve. However, this shift resulted in a concentration-response curve that did not reach saturation at the highest concentrations tested (**Figure 16-3**) because of excessive cytotoxicity (**Figure 16-4**). Two additional experiments (**Figure 16-1**) were performed with the original starting concentration of tamoxifen with no observable cytotoxicity at any concentration (**Figure 16-2**).

Figure 16-1 Tamoxifen Concentration-Response Curve When the Starting Concentration is 5 µg/mL

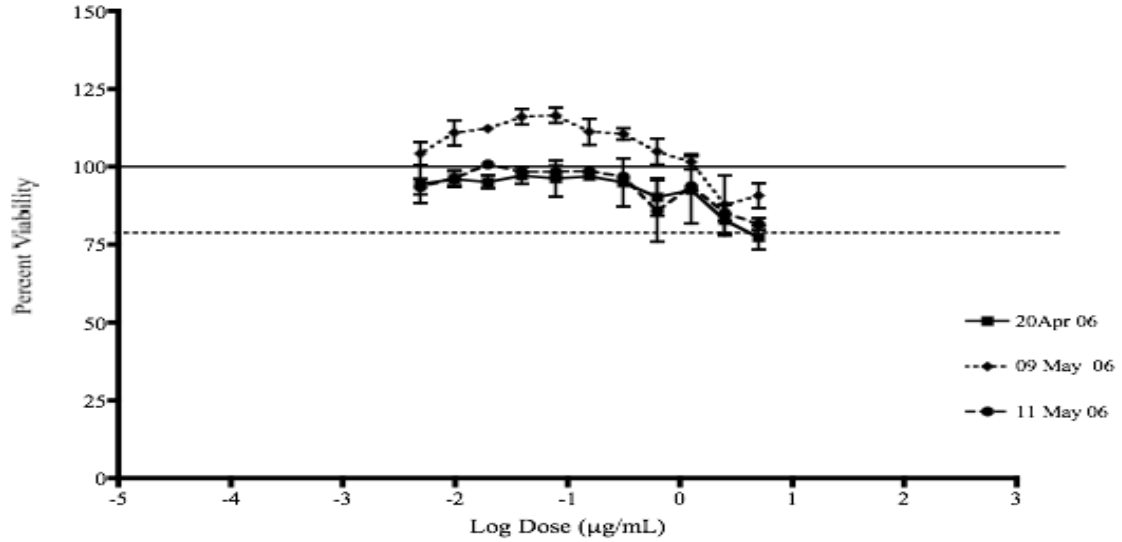


Abbreviations: Ral/E2 Reference Standard = concentrations of raloxifene with a fixed concentration of 2.5 x 10^{-5} µg/mL 17β–estradiol; Flavone = 25 µg/mL flavone control; E2 = 17ß-estradiol.

Line represents the mean of three E2 replicates minus three times the standard deviation of the E2 mean.

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.

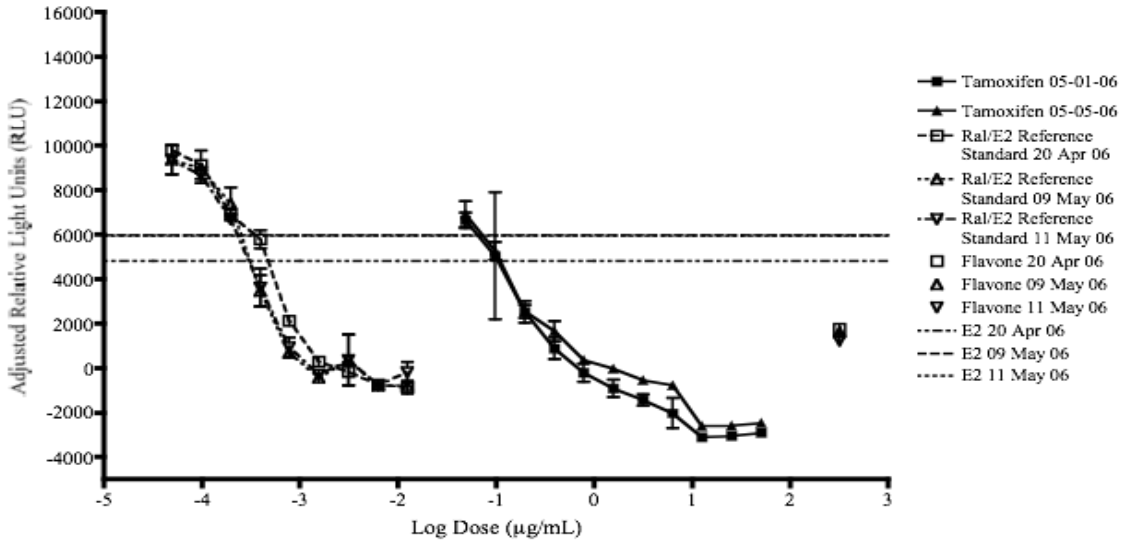
Figure 16-2 CellTiter-Glo® Viability Data for Tamoxifen Experiments When the Starting Concentration is 5 µg/mL



Solid line drawn across the graph at 100 percent viability indicates 100% viability as measured in DMSO solvent control.

Dashed line drawn across the graph at 80% viability indicates the viability limit for this assay. Points that fall below this line are not included in data analyses.

Figure 16-3 Tamoxifen Concentration-Response Curve When the Starting Concentration is 50 µg/mL

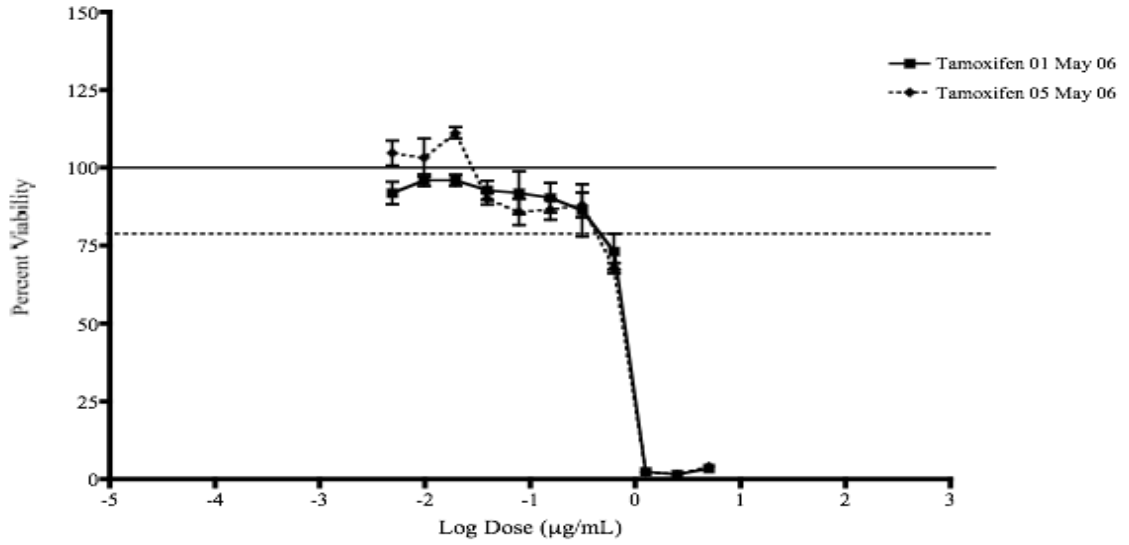


Abbreviations: Ral/E2 Reference Standard = concentrations of raloxifene with a fixed concentration of 2.5 x 10^{-5} µg/mL 17β–estradiol; Flavone = 25 µg/mL flavone control; E2 = 17ß-estradiol.

Line represents the mean of three E2 replicates minus three times the standard deviation of the E2 mean.

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.

Figure 16-4 CellTiter-Glo® Viability Data for Tamoxifen Experiments When the Starting Concentration is 50 µg/mL



Solid line drawn across the graph at 100 percent viability indicates 100% viability as measured in DMSO solvent control.

Dashed line drawn across the graph at 80% viability indicates the viability limit for this assay. Points that fall below this line are not included in data analyses.

17.0 Cell Culture Failures

During protocol standardization, there were several instances where cells that were being cultured for use in the BG1Luc ER TA did not perform to previously established historical norms, or exhibited decreased viability.

17.1 Cytotoxicity Due to G418

On 9 November 2005, a new lot (#30234193) of G418 was added to media used on the cells in growth flasks to select cells containing the luciferase reporter gene. Twenty-four hours later, cell viability was reduced by more than 50%. A new aliquot of frozen cells was thawed and subcultured and a different lot (#30234198) of G418 was added to a single flask containing the new subculture. No signs of cytotoxicity were observed in the new subculture indicating that the previous lot (#30234193) of G418 was the likely cause of the cytotoxicity. Based on this information, the BG1Luc ER TA cell culture Standard Operating Procedure (SOP) was modified to test the performance of new lots of G418 for the cell selection process.

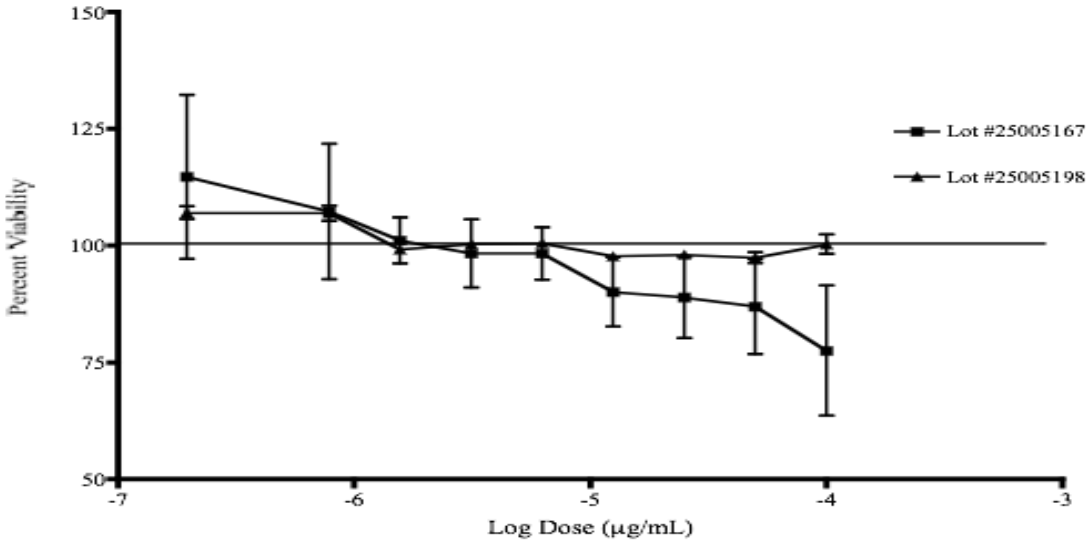
17.2 Decreased Viability and Diminished Response

During the period from 27 December 05 to 27 February 06, cells exhibited abnormal morphology, poor growth, and decreased viability at higher concentrations of the E2 reference standard. During the same period, experiments showed a shift in reference standard EC_{50} and IC_{50} values. A common feature for both agonist and antagonist assays was the cell culture media; therefore, various components of the media were investigated as the cause of the abnormal results. Several potential causes were investigated and are discussed below.

17.2.1 L-Glutamine

A new lot of L-glutamine (#25005167) had been in use since 27 December 05. A different lot (#25005198) was tested, and cells exhibited improved morphology and viability (**Figure 17-1**), indicating that the previous lot (#25005167) of L-glutamine may have contributed to the decreases in viability. Based on this information, the BG1Luc ER TA cell culture SOP was modified to test the performance of new lots of L-glutamine used for cell culture.

Figure 17-1 Increased Toxicity of E2 in the Presence of L-Glutamine Lot #25005167



Abbreviations:E2 = 17ß-estradiol
Horizontal line indicates 100% viability as measured in DMSO solvent control.

17.2.2 Stripped FBS

On 5 January 06, a new bottle of FBS was used for ongoing cell culture. Cells from this culture exhibited increased background luminescence in solvent controls and decreased induction for E2 reference standard in the BG1Luc ER TA. Background and induction improved when a different bottle of FBS was used to culture cells, indicating possible estrogenic contamination of the bottle used on 5 January 06. Based on this information, the BG1Luc ER TA cell culture SOP was modified to test the performance of new bottles of FBS used for cell culture.

17.2.3 Tissue Culture Flasks

On 27 December 05, a new lot of tissue culture flasks was used for ongoing cell culture. New flasks were purchased from a different manufacturer and cells from ongoing cultures were transferred to the new flasks. Cell morphology and viability improved, and reference standard EC_{50} and IC_{50} values return to historical norms. Based on this information, the BG1Luc ER TA cell culture SOP was specifically modified to test the performance of new lots of cell culture flasks.

18.0 Substance Concentrations Tested and the ICCVAM Recommended Limit Concentration

The ICCVAM Guidelines (ICCVAM 2003, 2006) recommend that both agonist and antagonist assays test up to a limit concentration of 1mM, within the limits of test substance solubility and toxicity. Validation studies were conducted in a blinded manner requiring test substances to be coded. Therefore, concentrations to be tested were specified on a μg/mL basis, with the limit concentration being 1000 μg/mL, in the absence of solubility or cytotoxicity constraints. However, none of the test substances could be tested to the intended limit concentration of 1 mg/mL because none were soluble at this concentration in cell culture media containing 1% DMSO, so the limit concentration for protocol standardization was set to 100 μg/mL, one log concentration lower than the recommended limit concentration.

Upon completion of testing and data analysis, molar concentrations of test substances were calculated and are presented in the sections below.

18.1 Agonist Concentrations Tested

The limit concentration for test substance used during agonist range finder testing was 100 μg/mL. This limit concentration correlated with a range of molar concentrations ranging from 282 to 463 μM (**Table 18-1**).

Table 18-1 Maximum Concentration of Test Substances Tested in the BG1Luc ER TA Agonist Protocol

Maximum Concentration Used in Agonist Testing				
	Concentration in μg/mL		Concentration in μM[1]	
Substance	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
o,p'-DDT	100	10	282	28.2
Diethylstilbestrol	100	1.00×10^{-4}	372	3.73×10^{-4}
EE	100	1.00×10^{-4}	337	3.37×10^{-4}
Flavone	100	50	450	22.5

Abbreviations: EE = 17α-ethinyl estradiol; *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane

[1] Formula weights used to calculate molarity were taken from MSDS sheets provided to NICEATM by the National Toxicology Program Substances Inventory

The highest concentrations used during comprehensive testing ranged from 1.00×10^{-6} to 50 μg/mL, corresponding to 3.37×10^{-4} to 43.8 μM.

18.2 Antagonist Concentrations Tested

Because of solubility considerations, the highest concentration of test substance used during antagonist range finder testing was 50 μg/mL, which corresponded to a range of molar concentrations ranging from 135 to 227 μM (**Table 18-2**).

Table 18-2 Maximum Concentration of Test Substances Tested in the BG1Luc ER TA Antagonist Protocol

Maximum Concentration Used in Antagonist Testing				
Substance	**Concentration in μg/mL**		**Concentration in μM[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
o,p'-DDT	50	50	141	141
Tamoxifen	50	5	135	13

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

[1] Formula weights used to calculate molarity were taken from MSDS sheets provided to NICEATM by the National Toxicology Program Substances Inventory

The highest concentrations used during comprehensive testing ranged from 5 to 50 μg/mL, correlating to 13 to 227 μM.

19.0 Overview of Results from the BG1Luc ER TA Protocol Standardization Study

19.1 Agonist Results

Of the eight test substances evaluated during agonist testing, six were positive (bisphenol A, bisphenol B, *o,p'*-DDT, diethylstilbestrol, EE, and flavone), and two were negative (atrazine and corticosterone) for agonist activity. EC_{50} values were calculated for all positive test substances and are presented in **Table 19-1**. EC_{50} values are presented as both μg/mL and μM values. A range of concentrations (in both μg/mL and μM values) at which each substance was active is also presented.

Table 19-1 EC_{50} Values Obtained in the BG1Luc ER TA Agonist Protocol

	Data Presented as μg/mL		Data Presented as μM[1]	
Substance	**EC_{50}**	**Activity Range**	**EC_{50}**	**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
o,p'-DDT	0.38	0.16 to 10	1.08	0.44 to 28.2
Diethylstilbestrol	1.26×10^{-5}	3.13×10^{-6} to 1.00×10^{-4}	4.69×10^{-5}	1.17×10^{-5} to 3.73×10^{-4}
EE	3.87×10^{-6}	7.81×10^{-7} to 1.00×10^{-4}	1.31×10^{-5}	2.64×10^{6} to 3.37×10^{-4}
Flavone	6.88	0.31 to 5	31	14.1 to 22.5

Abbreviations: EC_{50} = half-maximal effect concentration; EE = 17α-ethinyl estradiol; *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane

[1] Formula weights used to calculate molarity were taken from MSDS sheets provided to NICEATM by the National Toxicology Program Substances Inventory.

19.2 Comparison of Agonist Results with ICCVAM Meta Data

Table 19-2 compares EC_{50} values obtained during protocol standardization to the *in vitro* ER TA results compiled and published in the ICCVAM Guidelines (ICCVAM meta data) presented in the ICCVAM Guidelines (ICCVAM 2003, 2006).

Table 19-2 EC_{50} Values Obtained in BG1Luc ER TA Agonist Testing Compared to Published ICCVAM Meta Data

Substance	EC_{50}*	ICCVAM EC_{50}*
Atrazine	Negative	Negative
Bisphenol A	0.38	0.40
Bisphenol B	0.21	NR
Corticosterone	Negative	Negative
o,p'-DDT	1.08	0.66
Diethylstilbestrol	4.69×10^{-5}	1.9×10^{-5}
EE	3.87×10^{-6}	1.1×10^{-5}
Flavone	31.0	NR

Abbreviations: EC_{50} = half-maximal effect concentration; EE = 17α-ethinyl estradiol; *o,p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane; NR = Not Reported

* Values are reported in μM

The EC_{50} values obtained during protocol standardization were similar to those reported in the ICCVAM Guidelines (ICCVAM 2003, 2006), with the largest difference (one order of magnitude) between EC_{50} values for EE.

19.3 Antagonist Results

Of the eight test substances evaluated during antagonist testing, four were positive (DBA, genistein, flavone, and tamoxifen), and four were negative (BBP, nonylphenol, progesterone, and *o,p'*-DDT) for agonist activity. IC_{50} values were calculated for all positive test substances and are presented in **Table 19-3**. IC_{50} values are presented as both μg/mL and μM values. A range of concentrations (in both μg/mL and μM values) at which each substance was active is also presented.

Table 19-3 IC_{50} Values Obtained in the BG1Luc ER TA Antagonist Protocol

	Data Presented as μg/mL		Data Presented as μM	
Substance	**IC_{50}**	**Activity Range**	**IC_{50}**	**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
o,p'-DDT	Negative	Negative	Negative	Negative
Tamoxifen	0.16	0.16 to 5.00	0.43	0.42 to 13.5

Abbreviations: IC_{50} = concentration of test substance that inhibits the reference estrogen response by 50%; BBP = butylbenzyl phthalate; DBA = dibenzo[*a*,*h*]anthracene; *o*,*p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane; NC = Not Calculated

19.4 Comparison of Antagonist Results with ICCVAM Meta Data

The ICCVAM Guidelines (ICCVAM 2003, 2006) did not have IC_{50} values reported for any of test substances evaluated during protocol standardization. **Table 19-4** lists the IC_{50} values obtained during protocol standardization with the ICCVAM meta data presented in the ICCVAM Guidelines (ICCVAM 2003, 2006).

Table 19-4 IC_{50} Values Obtained in BG1Luc ER TA Antagonist Testing Compared to Published ICCVAM Meta Data

Substance	IC_{50}*	ICCVAM IC_{50}*
BBP	Negative	NR
DBA	NC	NR
Genistein	NC	NR
Flavone	NC	NR
Nonylphenol	Negative	NR
Progesterone	Negative	Negative
o,*p'*-DDT	Negative	NR
Tamoxifen	0.43	NR

Abbreviations: IC_{50} = concentration of test substance that inhibits the reference estrogen response by 50%; BBP = butylbenzyl phthalate; DBA = dibenzo[*a*,*h*]anthracene; *o*,*p'*-DDT = 1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane; NC = Not Calculated; NR = Not Reported

* Values are reported in μM

20.0 The Accuracy of the BG1Luc ER TA

There is no established "gold standard" animal or human data set to serve as a reference for determining the accuracy of *in vitro* test methods for identifying substances with estrogen activity *in vivo*. For this study, ICCVAM meta data was compared with the BG1Luc ER TA protocol standardization study results. One difficulty in using the ICCVAM meta data compilation as a reference database is the lack of agreement among published studies regarding the positive or negative responses of a number of the substances recommended by ICCVAM for *in vitro* ER TA validation studies. This lack of agreement among laboratories is largely due to the diversity of test methods and the varied decision criteria developed by different investigators to evaluate ER TA activity. Another concern with using the list of ICCVAM recommended validation substances is that the classification of some substances is based on a single test in a single laboratory using a system that may not have been well-defined or was based on theory.

20.1 Evaluation of Agonist Concordance

Using the data obtained during standardization of the agonist protocol, the accuracy statistics (i.e., concordance, sensitivity, specificity, positive and negative predictivity, and false negative and false positive rates) for the agonist protocol of the BG1Luc ER TA were calculated (see **Table 20-1**).

- Positive in BG1Luc ER TA and ICCVAM Positive: 6 substances
- Negative in BG1Luc ER TA and ICCVAM Positive: 0 substances
- Negative in BG1Luc ER TA and ICCVAM Negative: 2 substances
- Positive in BG1Luc ER TA and ICCVAM Negative: 0 substances

Table 20-1 Concordance Analysis Between BG1Luc ER TA Assay Agonist Protocol and ICCVAM Agonist Meta Data

	ICCVAM Agonist Classification			
BG1Luc ER TA Classification		Positive	Negative	Total
	Positive	6	0	6
	Negative	0	2	2
	Total	6	2	8

Concordance = 100% (8/8)

Sensitivity = 100% (6/6) False Negative Rate = 0% (0/6)

Specificity = 100% (2/2) False Positive Rate = 0% (0/2)

Positive Predictivity = 100 % (6/6) Negative Predictivity = 100% (2/2)

The classification of substances as either positive or negative for agonism using results from the BG1Luc ER TA protocol standardization study are in complete agreement with the ICCVAM meta data classification for those substances.

20.2 Evaluation of Antagonist Concordance

Using the data obtained during the standardization of the antagonist protocol, the accuracy statistics (i.e., concordance, sensitivity, specificity, positive and negative predictivity, and false negative and false positive rates) for the antagonist protocol of the BG1Luc ER TA were calculated (see **Table 20-2**).

- Positive in BG1Luc ER TA and ICCVAM Positive: 4 substances

- Negative in BG1Luc ER TA and ICCVAM Positive: 2 substances[5]
- Negative in BG1Luc ER TA and ICCVAM Negative: 2 substances
- Positive in BG1Luc ER TA and ICCVAM Negative: 0 substances

Table 20-2 Concordance Analysis Between BG1Luc ER TA Antagonist Protocol and ICCVAM Antagonist Meta Data

	ICCVAM Antagonist Classification			
BG1LUC ER TA Classification		Positive	Negative	Total
	Positive	4	0	4
	Negative	2	2	4
	Total	6	2	8

Concordance = 75% (6/8)

Sensitivity = 100% (4/4) False Negative Rate = 0% (0/4)

Specificity = 50% (2/4) False Positive Rate = 50% (2/4)

Positive Predictivity = 67% (4/6) Negative Predictivity = 100% (2/2)

The classification of substances as either positive or negative for antagonism using results from the BG1Luc ER TA protocol standardization study are in agreement with the ICCVAM meta data classification except for two substances classified as antagonists by the ICCVAM meta data but as negative in BG1Luc ER TA. However, as mentioned above, classifications of substances in the ICCVAM meta data are sometimes based on a single test in a single laboratory using a system that may not have been well-defined or were based on theory rather than experimentally obtained data. It is not known if the two substances classified as negative by the BG1Luc ER TA but positive in the ICCVAM meta data had previously been evaluated for cytotoxicity, which, unless specifically controlled for, could result in a mistaken classification as antagonists in ER TA test methods.

[5] 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 BG1Luc ER TA but positive in the ICCVAM meta data are considered negative because they caused a decrease in cell viability to below the 80% limit.

21.0 Further Considerations

21.1 Considerations on the Need for Cell Viability Evaluations During Agonist Testing

In range finder testing, the six substances that were positive for agonism all exhibited significant decreases in cell viability to below the 80% limit at the highest concentration tested (100 μg/mL). This correlated with the visual observations, which scored this concentration as having moderate to high levels of cytotoxicity. In all cases, this coincided with a decreased response in the BG1Luc ER TA.

None of the substances tested showed significant decreases in cell viability (80% limit) during comprehensive testing. This agrees with the visual observations, which scored all concentrations tested as having normal cell morphology.

21.2 Considerations on the Need for Cell Viability Evaluations During Antagonist Testing

In range finder testing, seven of the eight substances exhibited decreased ER TA activity at the highest concentration tested (50 μg/mL). Six of these substances also exhibited significant decreases in cell viability below the 80% limit at the same concentration. This agreed with the visual observations, which scored this concentration as having low to high levels of cell toxicity.

In comprehensive testing, seven of the eight substances exhibited decreased ER TA activity at the highest concentrations tested. Four of these substances also exhibited significant decreases in cell viability. Therefore, these concentrations were considered cytotoxic rather than antagonistic. Cell viability for the remaining three substances exhibiting decreased ER TA activity did not fall below the 80% limit and where therefore considered antagonists. There was a high degree of correlation between visual observation scores and CellTiter-Glo® values for all substances with the exception of flavone, which did not fall below the 80% limit, but had a visual observation score of 2 (low levels of cell toxicity) at the highest concentration tested (50 μg/mL).

21.3 Choosing Concentrations for Comprehensive Testing when Range Finder Exhibits Biphasic Response

One substance tested during agonist range finding, flavone, exhibited a biphasic concentration-response. Concentrations for comprehensive testing were selected from the higher concentrations showing activity in the range finder. In comprehensive testing flavone was positive for agonism at these concentrations. However, no evaluation of activity was conducted for those concentrations showing activity at the lowest concentrations tested in the range finder. It is recommended that in cases where range finding indicates activity in a biphasic manner, comprehensive testing should be conducted at concentrations that would allow evaluation of both phases.

21.4 Considerations for Reporting Activity Levels for Substances Which are Active, but for Which an EC_{50} or IC_{50} Value Cannot be Calculated

One of the limitations of the use of the Hill equation to calculate EC_{50} and IC_{50} values is that it requires that full concentration-response curves be generated for an EC_{50} or IC_{50} value to be obtained. Of the four substances that tested positive for antagonism (DBA, genistein, flavone, and tamoxifen), an IC_{50} value could only be calculated for tamoxifen.

22.0 Summary

NICEATM has conducted an agonist and antagonist protocol standardization study for the *in vitro* BG1Luc ER TA developed by XDS. Protocol standardization procedures were based on recommendations made in the ICCVAM Guidelines (ICCVAM 2003, 2006). Specific goals of the study were to standardize procedures and develop two GLP-compliant protocols for using the BG1Luc ER TA to identify ER agonists and antagonists, quantify cell viability, and develop historical databases for reference standards and controls for these protocols.

Reference standards and controls selected and standardized for the agonist assay were a 10-point dilution of E2 as reference standard, 1% DMSO as solvent control, and 3.13 μg/mL methoxychlor as the positive control. Reference standards and controls selected and standardized for the antagonist assay were a nine-point dilution of raloxifene with a fixed concentration of 2.5×10^{-5} μg/mL E2 as reference standard, 1% DMSO as solvent control, 2.5×10^{-5} μg/mL E2 as E2 control, and 25 μg/mL flavone with 2.5×10^{-5} μg/mL E2 as positive control.

CellTiter-Glo® (Promega Inc.) was selected and standardized for use with BG1Luc ER TA assay protocols. Assessment of cell viability was also conducted qualitatively using a method developed by XDS based on visual observations of cellular morphology.

The agonist historical database was established by conducting 10 independent experiments using the 10-point E2 reference standard run in duplicate, solvent control run in quadruplicate, and the methoxychlor positive control run in triplicate in each 96-well plate.

The antagonist historical database was established by conducting 10 independent experiments using the a nine-point Ral/E2 reference standard run in duplicate, solvent control run in triplicate, and the E2 control and flavone control run in triplicate in each 96-well plate.

The adequacy of the standardized protocols was demonstrated using eight substances covering a range of ER agonist and antagonist activities, respectively. The substances selected for agonist testing were atrazine, bisphenol A, bisphenol B, corticosterone, *o,p'*-DDT, diethylstilbestrol, EE, and flavone. These substances were selected from the subset of minimum substances recommended for validation of *in vitro* ER assays in the ICCVAM Guidelines. They were selected for their estrogen receptor agonist activity classification, including those that are negative for agonism, and for properties that might make them problematic, including limited solubility or potential cell viability.

Results obtained for estrogenic activity for each substance tested using the standardized agonist protocol exhibited 100% concordance with ICCVAM meta data. There was a high degree of correlation between visual observation scores and CellTiter-Glo® values for all substances.

The substances selected for antagonist testing were BBP, DBA, flavone, genistein, nonylphenol, progesterone, *o,p'*-DDT, and tamoxifen. These substances were selected from the subset of minimum substances recommended for validation of *in vitro* ER assays in the ICCVAM Guidelines (ICCVAM 2003, 2006). They were selected for their estrogen receptor antagonist activity classification, including those that are negative for antagonism, and for properties that might make them problematic, including limited solubility or potential cytotoxicity.

Results obtained for anti-estrogenic activity for each substance tested using the standardized antagonist protocol exhibited 75% concordance with ICCVAM meta data. 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 BG1Luc ER TA but positive in the ICCVAM meta data are considered negative because they caused a significant decrease in cell viability. There was also a high degree of correlation between the visual observation and CellTiter-Glo® methods of assessing cell viability for all substances tested.

23.0 References

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24.0 Glossary[6]

Acceptance criteria[7]: Minimum standards for the performance of experimental controls and reference standards. All acceptance criteria must be met for an experiment to be considered valid.

Accuracy[7]: (a) The closeness of agreement between a test method result and an accepted reference value. (b) The proportion of correct outcomes of a test method. It is a measure of test method performance.

Adenosine triphosphate: A nucleotide involved in energy metabolism and required for RNA synthesis; it occurs in all cells and is used to store energy in the form of high-energy phosphate bonds.

Agonist: A substance that produces a response, e.g., transcription, when it binds to a specific receptor.

Androgen: A class of steroid hormone, which includes testosterone and 5α-dihydrotestosterone, responsible for the development and maintenance of the male reproductive system.

Androgen receptor: The receptor to which androgens bind.

Antagonist: A substance that inhibits a response, e.g., transcription, when it binds to a specific receptor.

Cell density: The density of cells growing in a monolayer in a single well of a tissue culture plate.

Cell morphology: The shape and appearance of cells grown in a monolayer in a single well of a tissue culture plate. Cells that are dying often exhibit abnormal cellular morphology.

Culture medium: An aqueous solution containing vitamins, minerals and growth factors to support the growth of cells in culture.

Coded test substances: Substances labeled by code rather than name so that they can be tested and evaluated without knowledge of their identity or anticipation of test results. Coded test substances are used to avoid intentional or unintentional bias when evaluating laboratory or test method performance.

Coefficient of variation: A statistical representation of the precision of a test. It is expressed as a percentage and is calculated as follows**:**

$$\left(\frac{\text{standard deviation}}{\text{mean}}\right)\times 100$$

Comprehensive test: The test performed for determination of an EC- or IC_{50} value. Compared to the range finder test the comprehensive test uses a smaller dilution factor for the concentrations tested.

Concordance[7]: The proportion of all substances tested that are correctly classified as positive or negative. It is a measure of test method performance and it is often used interchangeably with "accuracy".

Control: Substances selected for use during the research, development, protocol standardization, and validation of a proposed test method having a known response. Controls are used to evaluate the ongoing performance of a test method. All experimental controls must fall within established historical norms for an experiment to pass "acceptance criteria" and be considered valid.

Cytotoxicity: The adverse effects resulting from interference with structures and/or processes essential for cell survival, proliferation, and/or function. For most substances, toxicity is a consequence of non-specific alternations in "basal cell functions" (i.e., via mitochondria, plasma membrane integrity, etc.).

EC_{50}: The half maximal effective concentration of a test substance.

Endocrine: Of or relating to the endocrine system, endocrine glands, or hormones.

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[6] The definitions in this Glossary are restricted to their uses with respect to endocrine mechanisms and actions.

[7] Definition used by the Interagency Coordinating Committee on the Validation of Alternative Methods.

Endocrine disruptor: Substances that interact with the endocrine system to alter normal functioning. Endocrine disruptors may act directly by interfering with receptor binding, or indirectly by altering hormone biosynthesis, transport, action or metabolism.

Fluorescence: The emission of radiation, especially of visible light.

Hill function: A four parameter logistic mathematical model relating the concentration of the test substance to the response (typically following a sigmoidal shape).

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

where Y = response (i.e., luciferase activity), X is the substance concentration producing the response, Bottom is the minimum response, Top is the maximum response, EC_{50} is the substance concentration at the response midway between Top and Bottom, and HillSlope describes the slope of the curve.

IC_{50}: The half maximal inhibitory concentration of a test substance.

***In vitro*:** Literally, in glass. Refers to assays that are carried out in an artificial system (e.g., in a test tube or Petri dish), and typically use single-cell organisms, cultured cells, cell-free extracts, or purified cellular components.

Luciferase: An enzyme present in the cells of some bioluminescent organisms that catalyzes the oxidation of luciferin and ATP to produce luminescence.

Luminescence: The emission of radiation, especially of visible light caused by chemical, or biochemical processes.

Plasmid: A circle of bacterial DNA that is self-replicating. Plasmids can be artificially constructed and used as cloning vectors.

Precipitate/precipitation: A solid substance, often in the form of crystals, separated from a solution, or the act of a solid substance separating from a solution.

Protocol[2]: The precise, step-by-step description of a test, including the listing of all necessary reagents, criteria, and procedure for the valuation of the test data.

Protocol standardization: Selection of reference standards, controls, and performance standards for a protocol prior to initiation of validation efforts.

Q test: The Q test is a simple statistical test to determine if a data point that appears to be different from the rest of the data points in a set may be discarded. The Q test is

$$Q = \frac{\text{suspected outlier} - \text{closest value}}{\text{maximum value} - \text{minimum value}}$$

The resultant value, Q, is then compared to a table of critical values (Qc). If Q is larger than Qc, the data point is an outlier and can be discarded with 90% confidence (e.g., in a data set with values 100, 2655, and 241, the Q value is 0.95. For a set of three data points, Qc is 0.94. Q [0.95] is greater than Qc [0.94], so 2655 is an outlier and can be discarded).

Receptor: A protein or protein complex, which binds to specific molecules or the purpose of transporting them elsewhere in the cell, or for producing a chemical signal.

Receptor binding assay: An assay to measure the ability of a substance to bind to a hormone receptor protein, which is typically performed by measuring the ability of the substances to displace the bound natural hormone.

Reference substance: A reference substance used to demonstrate the adequacy of a test method. 17β-estradiol is the estrogenic reference standard, and raloxifene HCl is the anti-estrogenic reference standard for the BG1Luc ER TA test method

Transfection: The process by which foreign DNA is introduced into a cell to change the cell's genotype.

Transcription: Synthesis of RNA by RNA polymerases using a DNA template.

Transcriptional activation: The initiation of mRNA synthesis in response to a specific chemical signal, such as a binding of an estrogen to the estrogen receptor.

Validated test method: An accepted test method for which validation studies have been completed to determine the accuracy and reliability of the method for a specific proposed use.

Validation: The process by which the reliability and accuracy of a procedure are established for a specific purpose.

Xenobiotic: A substance that is not produced by the organism of interest.