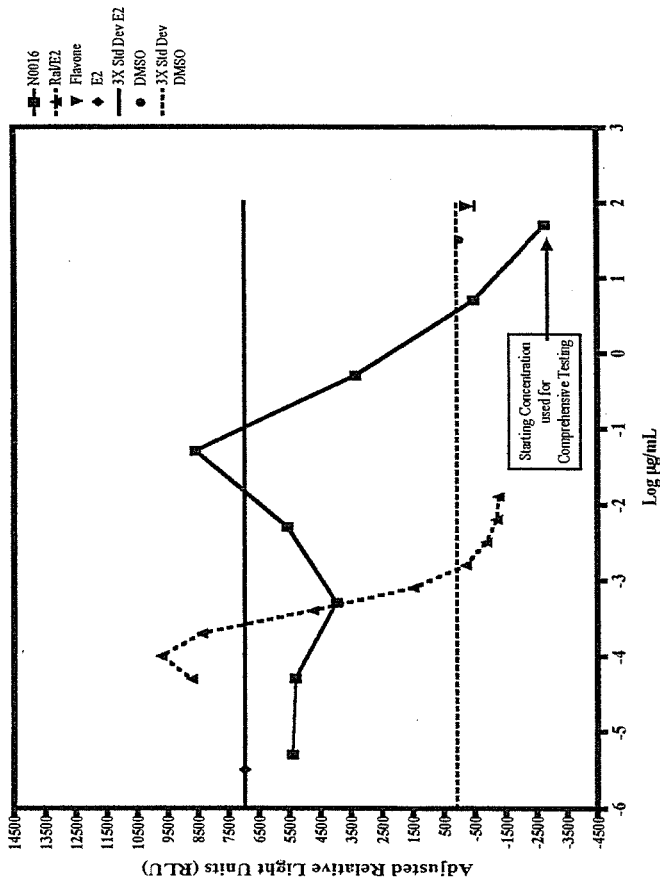
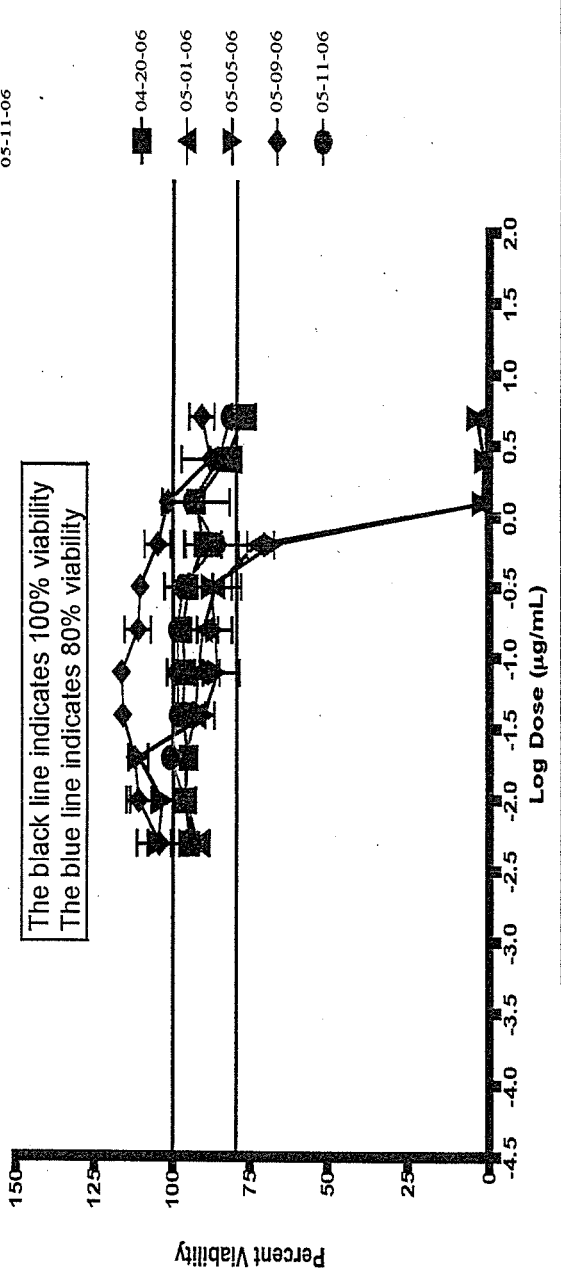
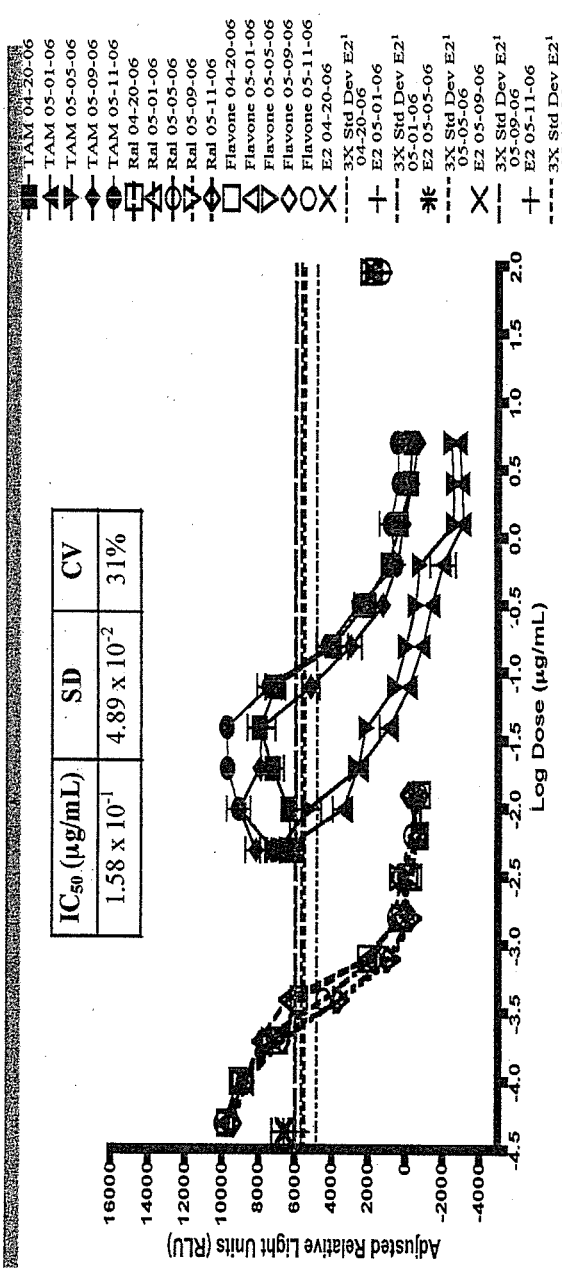


Range Finder Data for N0016-Tamoxifen

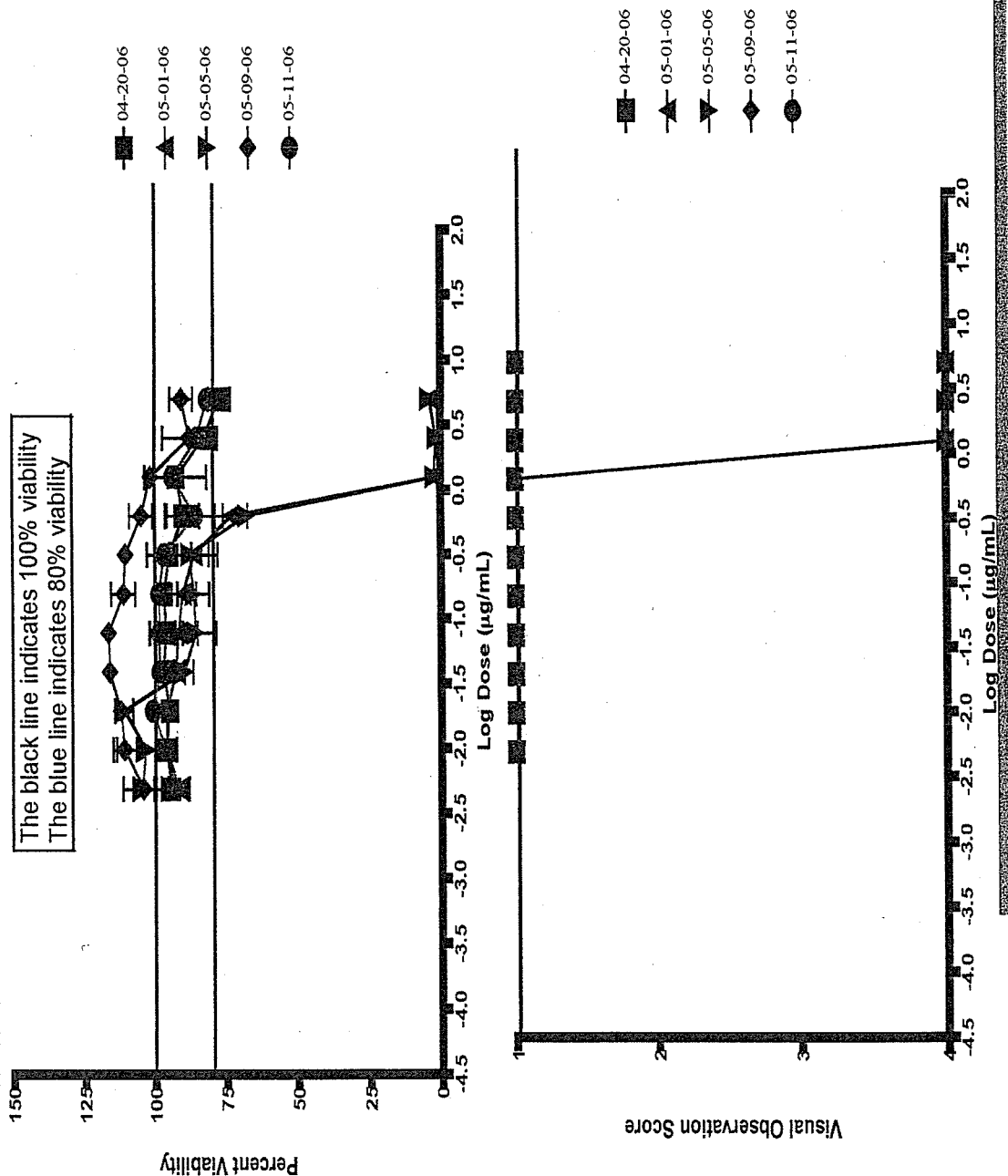


Concentration µg/mL	CellTiterGlo®	Visual Observation Score ¹
5.00 x 10 ⁺¹	5%	4
5.00 x 10 ⁺⁰	90%	1
5.00 x 10 ⁻¹	99%	1
5.00 x 10 ⁻²	108%	1
5.00 x 10 ⁻³	103%	1
5.00 x 10 ⁻⁴	106%	1
5.00 x 10 ⁻⁵	109%	1
5.00 x 10 ⁻⁶	105%	1

Antagonist and Viability Results for N0016-Tamoxifen



Comparison of CellTiterGlo[®] and Visual Observations for N0016-Tamoxifen



Problems Encountered During Testing of Coded Substances



Problems Encountered During Testing of Coded Substances

- Early in the study, cells that were being cultured for use in the assay exhibited decreased viability or did not perform to previously established historical norms
 - A series of qualifying experiments indicated that the likely cause of these cell culture problems were a combination of factors including contaminated lots of gentamycin, 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



Problems Encountered During Testing of Coded Substances (cont.)

- 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
- After the first comprehensive test of N0016 - tamoxifen, the laboratory shifted the concentration-response curve. Two experiments were conducted using the new concentrations. The new concentration-response curves did not achieve saturation, so the laboratory did two additional experiments at the original concentrations. They did not communicate this series of events to the project coordinators until after the study had been completed and data analysis initiated.

Concordance of Testing Results with ICCVAM Published Data

Concordance of Testing Results with ICCVAM Published Data (cont.)

- Estrogenic activity for substances tested using the standardized agonist protocol exhibited 100% concordance with ICCVAM published data
- Relative activity of ER agonists, based on calculated EC₅₀ values was in agreement with ICCVAM reported median activity
- Estrogen antagonist activity for substances tested using the standardized agonist protocol exhibited 75% concordance with ICCVAM published data.
 - o Four substances (butylbenzyl phthalate, flavone, genistein, and tamoxifen) were correctly classified as ER antagonists and two (dibenzo[a,h]anthracene and progesterone) as negative for ER antagonism

Concordance of Testing Results with ICCVAM Published Data (cont.)

- o Two substances (nonylphenol and *o,p'*-DDT) classified as ER antagonists in the ICCVAM published data were classified as negative in the LUMI-CELL[®] protocol standardization study
- Although these substances caused a significant decrease in ER TA activity in this test method, they also caused a significant decrease in cell viability over the same concentration range
- These two substances were classified as cytotoxic rather than as estrogenic antagonists

Summary of EC₅₀ and IC₅₀ Values

Substance Name	Agonist EC ₅₀ (µg/mL)	Antagonist	
		Relative ¹ IC ₅₀ (µg/mL) (GraphPad Prism®)	Absolute ² IC ₅₀ (µg/mL)
Atrazine	Negative		
Bisphenol A	8.76 x 10 ⁻²		
Bisphenol B	5.16 x 10 ⁻²		
Corticosterone	Negative		
Diethylstilbestrol	1.26 x 10 ⁻⁵		
EE	3.87 x 10 ⁻⁶		
Flavone	6.88 x 10 ⁺⁰	Positive	
<i>o,p'</i> -DDT	3.83 x 10 ⁻¹	Negative	
BBP		Negative	
DBA		Positive	
Genistein		Positive	
<i>p,n</i> -nonylphenol		Negative	
Progesterone		Negative	
Tamoxifen		1.58 x 10 ⁻¹	

¹ Relative IC₅₀ - calculated from fitted curve using Hill equation in GraphPad Prism®

² Absolute IC₅₀ - defined as the concentration giving exactly a 50% response of the maximal control level

Summary of the Protocol Standardization Effort

Summary of the Protocol Standardization Effort

- Reference standards and negative and positive controls were selected and standardized for both agonist and antagonist protocols
- Historical databases were developed for both protocols to establish quality control measures for subsequent experiments
- Quantitative and qualitative evaluations of cell viability were conducted throughout the protocol standardization effort



Summary of Protocol Standardization

Effort (cont.)

- Eight coded substances covering a range of ER agonist and antagonist activities were each tested in three independent experiments for both agonist and antagonist protocols
 - Bisphenol A, bisphenol B, *o,p'*-DDT, diethylstilbestrol, 17 α -ethinyl estradiol, and flavone were reproducibly classified as estrogenic agonists while atrazine and corticosterone were negative for ER TA
 - Tamoxifen, flavone, and genistein were reproducibly classified as estrogenic antagonists while dibenzo [a,h] anthracene, progesterone, nonylphenol and *o,p'*-DDT were negative for ER antagonism
- Data obtained from this study had a high degree of correlation with ICCVAM published data

Summary of Protocol Standardization

Effort (cont.)

- Cell viability data obtained during evaluation of reference standards indicating that a significant decrease in ER TA response occurred when reduction of ATP levels (as measured with CellTiterGlo[®]) exceeded 20% was confirmed during testing of coded substances
 - Concentrations of substance that caused reduction in cell viability below 80% were classified as cytotoxic and were not included in data analyses
- There was a high degree of correlation between the visual observation and CellTiterGlo[®] methods of assessing cell viability for all substances tested



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AGONIST PROTOCOL

**LUMI-CELL® ESTROGEN RECEPTOR TRANSCRIPTIONAL ACTIVATION TEST
METHOD FOR IDENTIFYING ESTROGEN AGONISTS AND ANTAGONISTS**

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

**Developed by:
Xenobiotic Detection Systems, Inc.
1601 E. Geer St., Suite S
Durham, NC 27704**

07 November 2006

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118 **LIST OF ACRONYMS AND ABBREVIATIONS**

119	13 mm test tube	13 x 100 mm glass test tubes
120	Absolute EC ₅₀ value	Concentration of a substance that increases the measured
121		activity in an agonist assay to 50% of the maximum activity
122		induced by the reference substance
123	DMEM	Dulbecco's Modification of Eagle's Medium
124	DMSO	Dimethyl Sulfoxide
125	DMSO control	1% v/v dilution of DMSO in tissue culture media used as a
126		vehicle control
127	E2	17β-estradiol
128	E2 reference standard	10 Point Serial Dilution of 17β-estradiol reference standard
129		for the LUMI-CELL® ER agonist assay
130	ER	Estrogen Receptor
131	Estrogen-free DMEM	DMEM (phenol red free) supplemented with 1%
132		Penicillin/Streptomycin, 2% L-Glutamine, and 5%
133		Charcoal-dextran treated FBS
134	FBS	Fetal Bovine Serum
135	G418	Gentamycin
136	Methoxychlor	<i>p,p'</i> -Methoxychlor
137	Methoxychlor control	3.13 µg/mL Methoxychlor Positive Control for the LUMI-
138		CELL® ER Agonist Assay
139	Relative EC ₅₀ value	Concentration that produces a half-maximal response as
140		calculated using the four parameter Hill function.
141	RPMI	RPMI 1640 growth medium
142	TA	Transcriptional Activation
143	T25	25 cm ² tissue culture flask