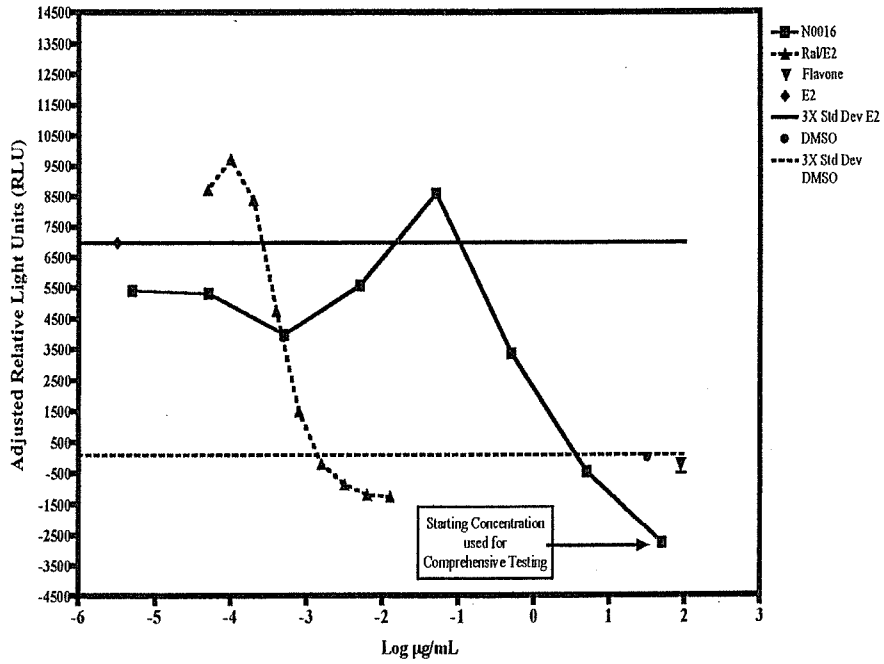
Range Finder Data for N0016-Tamoxifen

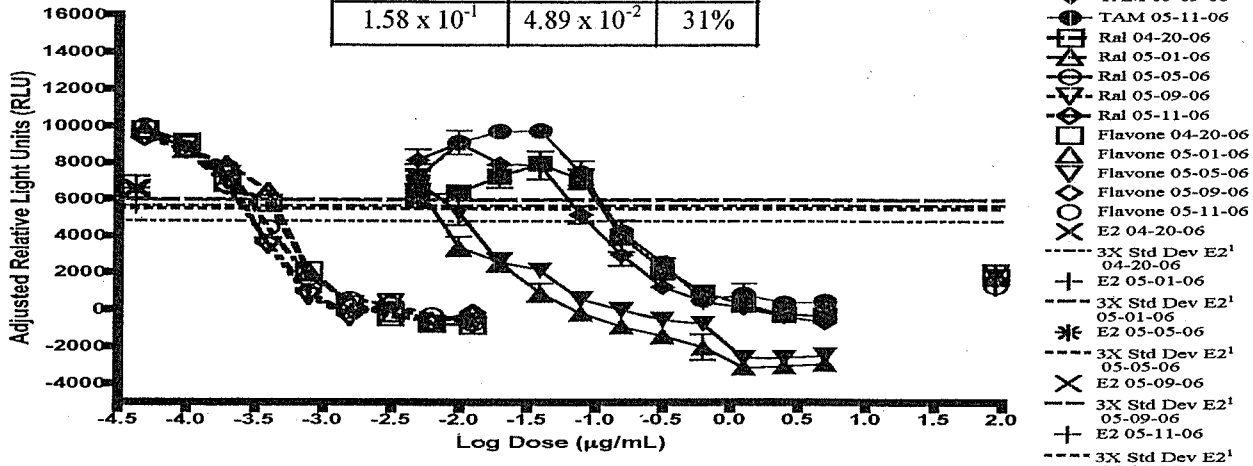


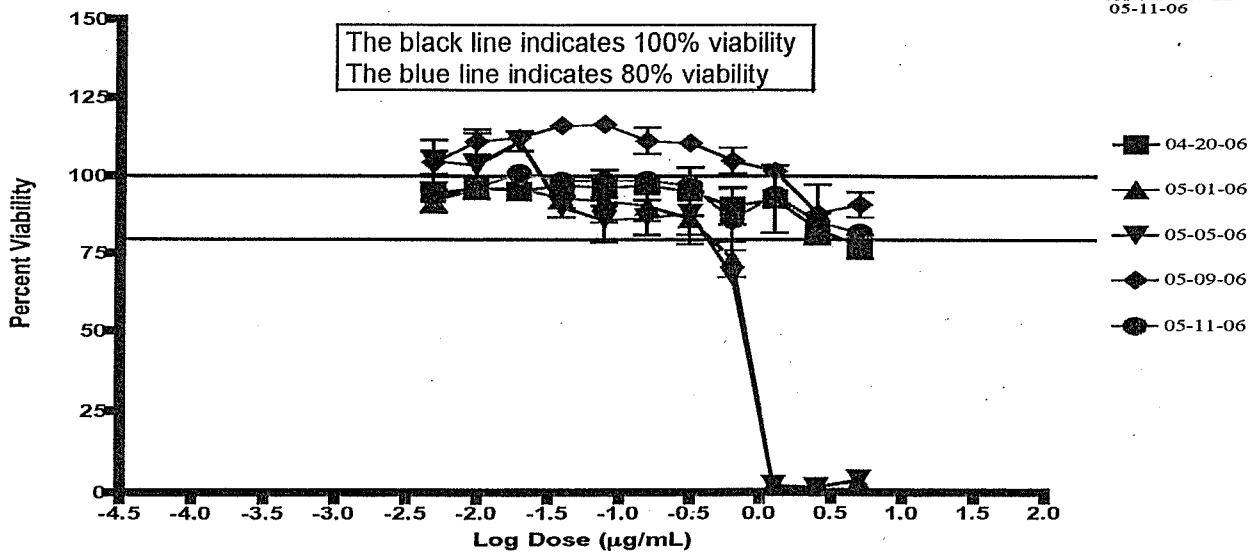
Concentration µg/mL	CellTiterGlo®	Visual Observation Score[1]
$5.00 \times 10^{+1}$	**5%**	**4**
$5.00 \times 10^{+0}$	90%	1
5.00×10^{-1}	99%	1
5.00×10^{-2}	108%	1
5.00×10^{-3}	103%	1
5.00×10^{-4}	106%	1
5.00×10^{-5}	109%	1
5.00×10^{-6}	105%	1

418

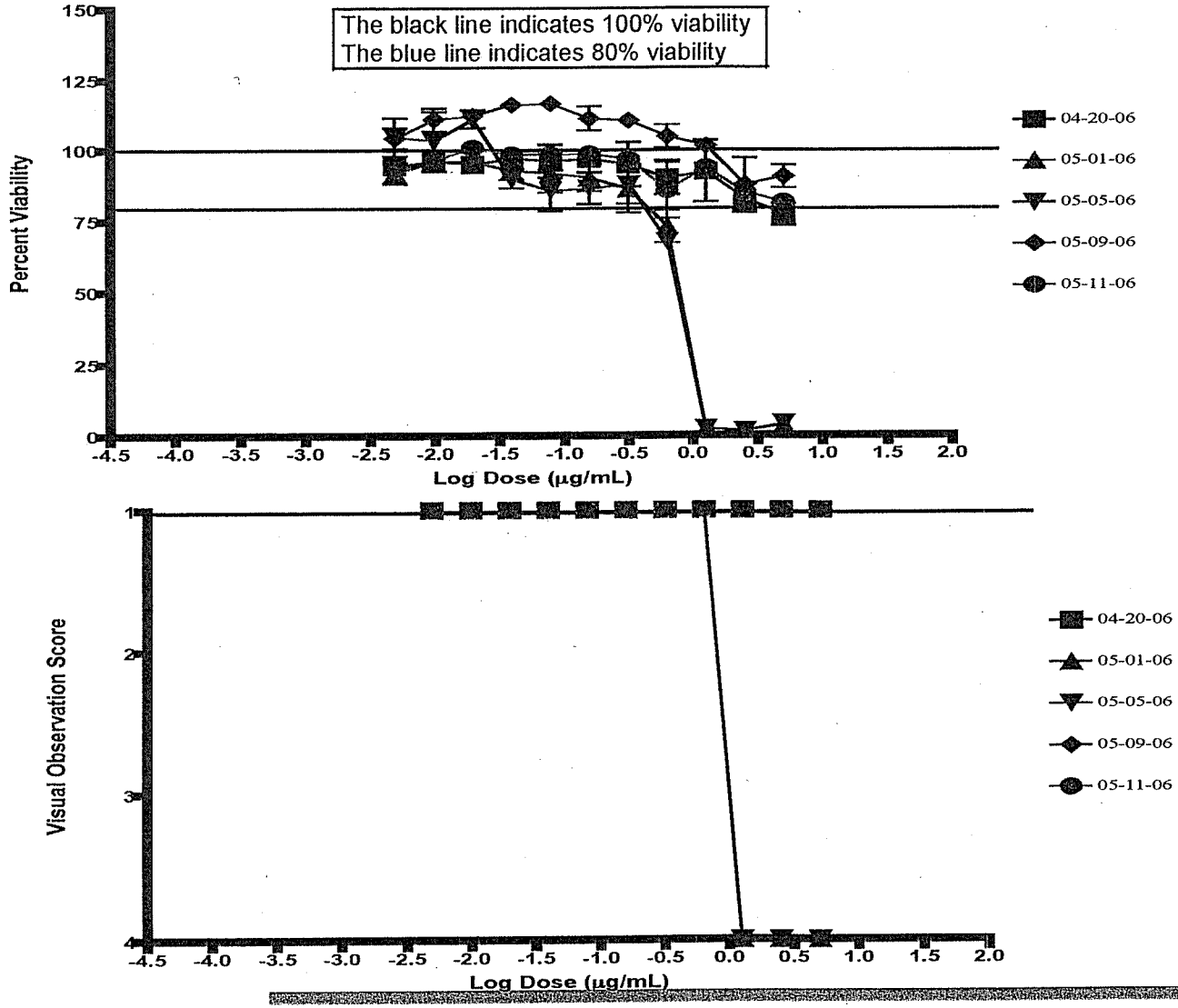
Antagonist and Viability Results for N0016-Tamoxifen

IC_{50} (µg/mL)	SD	CV
1.58×10^{-1}	4.89×10^{-2}	31%





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
  - o 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 EC_{50} 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.
  - 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.)

- 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

426

Summary of EC_{50} and IC_{50} Values

		Antagonist	
Substance Name	Agonist EC_{50} (μg/mL)	Relative[1] IC_{50} (μg/mL) (GraphPad Prism®)	Absolute[2] IC_{50} (μg/mL)
Atrazine	Negative		
Bisphenol A	8.76×10^{-2}		
Bisphenol B	5.16×10^{-2}		
Corticosterone	Negative		
Diethylstilbestrol	1.26×10^{-5}		
EE	3.87×10^{-6}		
Flavone	$6.88 \times 10^{+0}$	Positive	
o,p'-DDT	3.83×10^{-1}	Negative	
BBP		Negative	
DBA		Positive	
Genistein		Positive	
p,n-nonylphenol		Negative	
Progesterone		Negative	
Tamoxifen		1.58×10^{-1}	

[1] Relative IC_{50} - calculated from fitted curve using Hill equation in GraphPad Prism®

[2] Absolute IC_{50} – 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, 17α-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

430

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
  - o 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

431

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

TABLE OF CONTENTS

LIST OF ACRONYMS AND ABBREVIATIONS

13 mm test tube	13 x 100 mm glass test tubes
Absolute EC_{50} value	Concentration of a substance that increases the measured activity in an agonist assay to 50% of the maximum activity induced by the reference substance
DMEM	Dulbecco's Modification of Eagle's Medium
DMSO	Dimethyl Sulfoxide
DMSO control	1% v/v dilution of DMSO in tissue culture media used as a vehicle control
E2	17β-estradiol
E2 reference standard	10 Point Serial Dilution of 17β-estradiol reference standard for the LUMI-CELL® ER agonist assay
ER	Estrogen Receptor
Estrogen-free DMEM	DMEM (phenol red free) supplemented with 1% Penicillin/Streptomycin, 2% L-Glutamine, and 5% Charcoal-dextran treated FBS
FBS	Fetal Bovine Serum
G418	Gentamycin
Methoxychlor	*p,p'*-Methoxychlor
Methoxychlor control	3.13 µg/mL Methoxychlor Positive Control for the LUMI-CELL® ER Agonist Assay
Relative EC_{50} value	Concentration that produces a half-maximal response as calculated using the four parameter Hill function.
RPMI	RPMI 1640 growth medium
TA	Transcriptional Activation
T25	25 cm^2 tissue culture flask