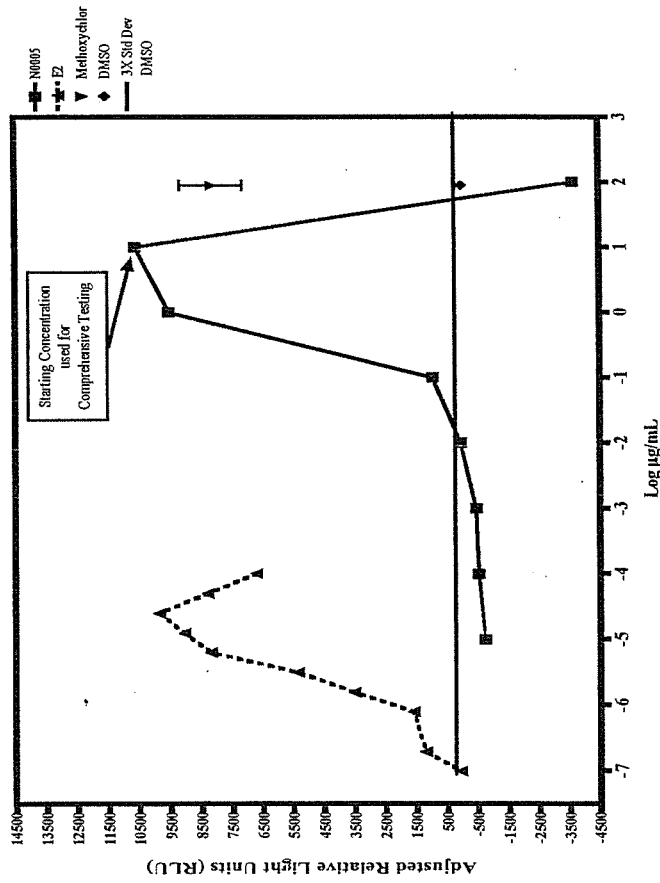
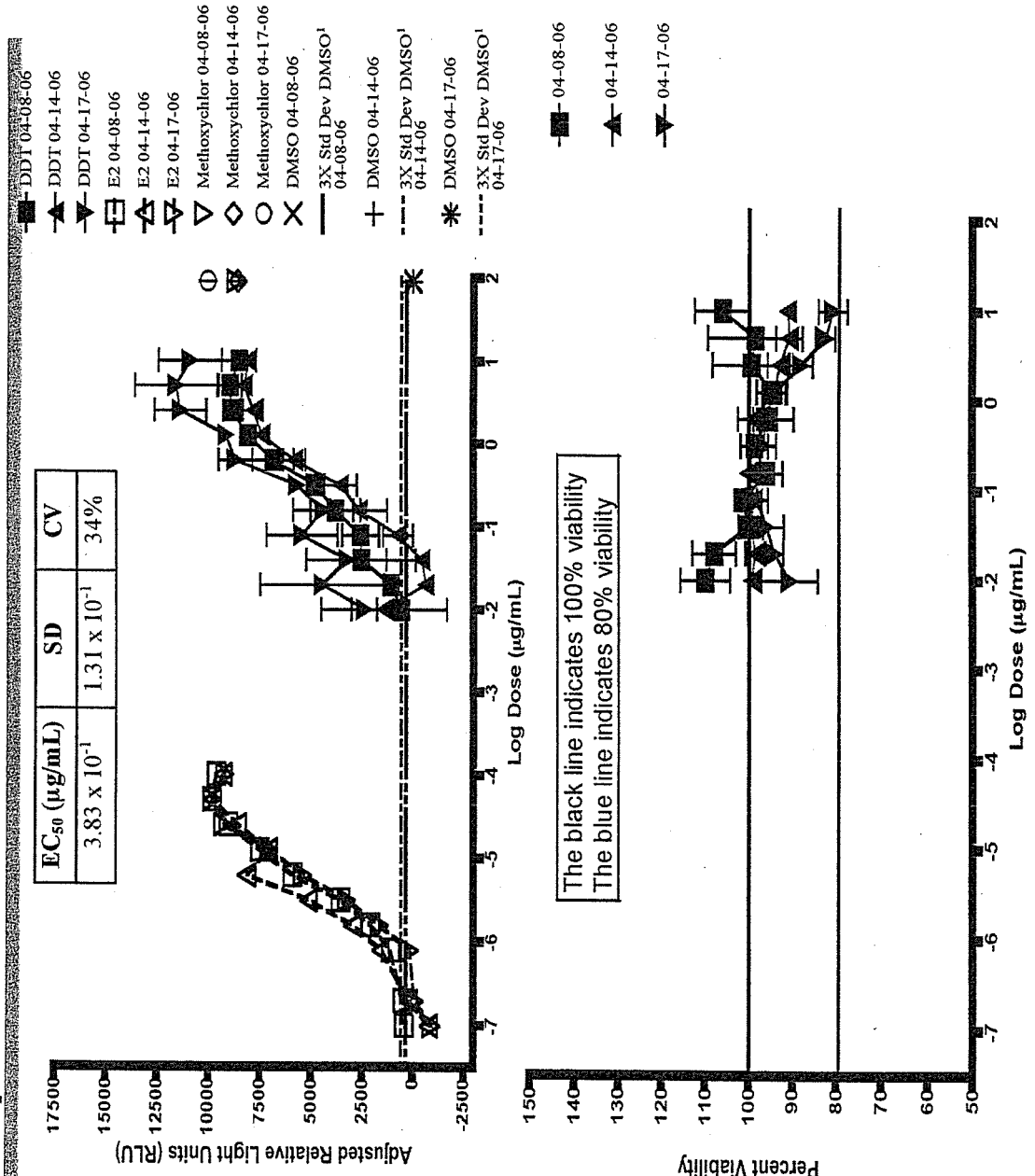


Range Finder Data for N0005-*o,p'*-DDT

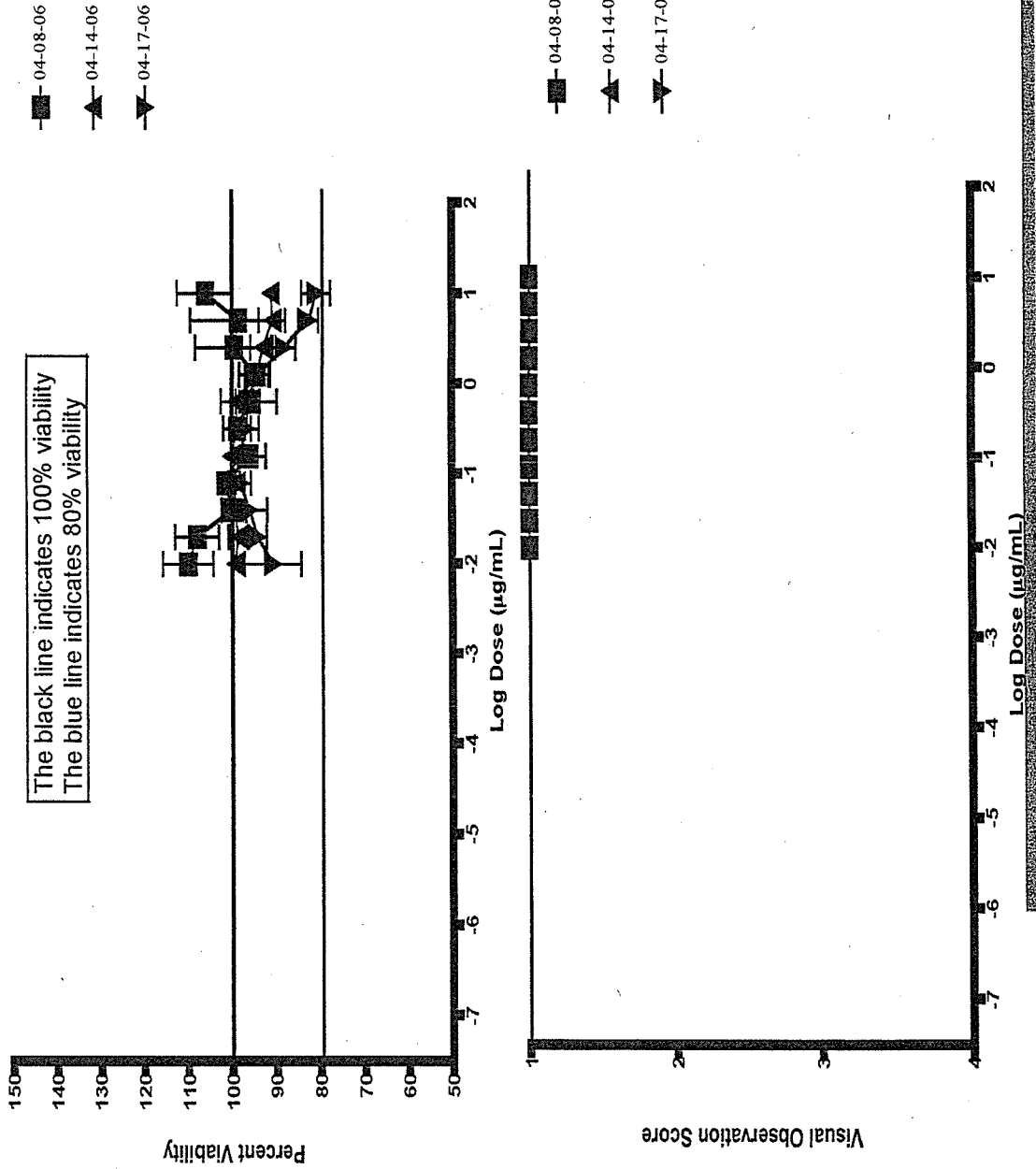


Concentration µg/mL	CellTiterGlo®	Visual Observation Score
$1.00 \times 10^{+2}$	12%	4
$1.00 \times 10^{+1}$	94%	1
$1.00 \times 10^{+0}$	103%	1
1.00×10^{-1}	93%	1
1.00×10^{-2}	97%	1
1.00×10^{-3}	101%	1
1.00×10^{-4}	101%	1
1.00×10^{-5}	108%	1

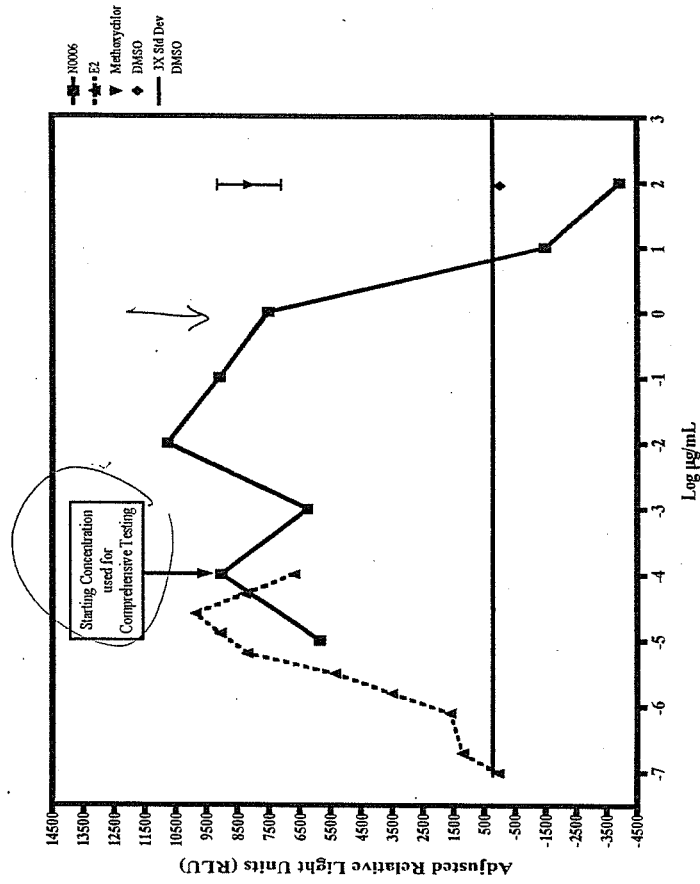
Agonist and Viability Results for N0005- o,p'-DDT



Comparison of CellTiterGlo[®] and Visual Observations for N0005-o,p'-DDT



Range Finder Data for N0006- Diethylstilbestrol

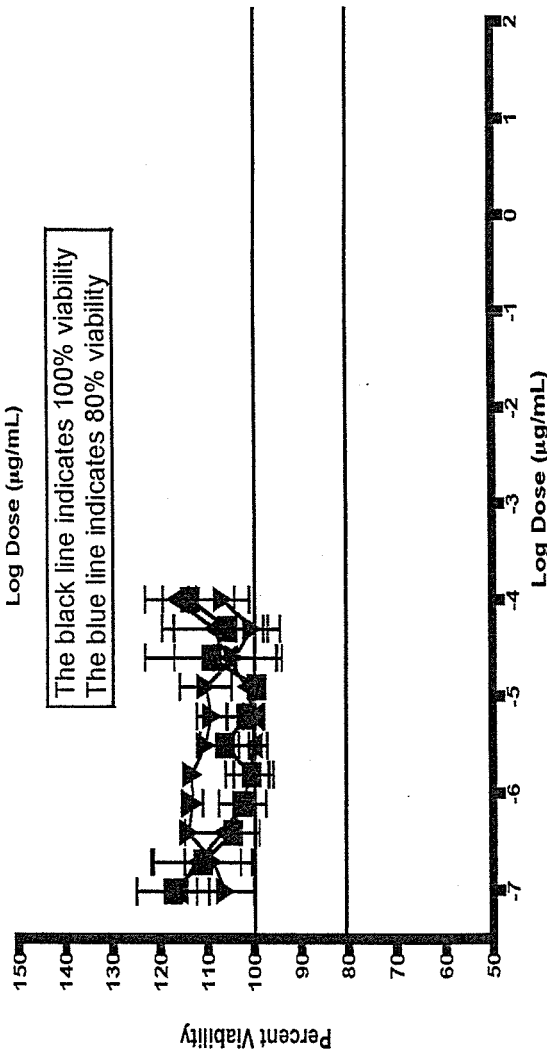
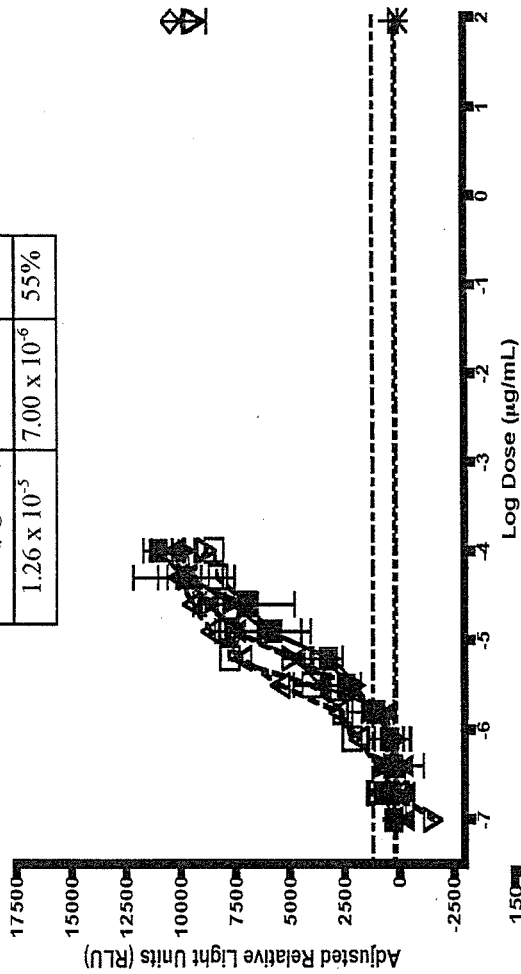


Concentration µg/mL	CellTiterGlo®	Visual Observation Score
1.00 x 10 ⁻²	6%	4
1.00 x 10 ⁺¹	111%	1
1.00 x 10 ⁺⁰	111%	1
1.00 x 10 ⁻¹	107%	1
1.00 x 10 ⁻²	99%	1
1.00 x 10 ⁻³	92%	1
1.00 x 10 ⁻⁴	96%	1
1.00 x 10 ⁻⁵	101%	1

Agonist and Viability Results for N0006- Diethylstilbestrol

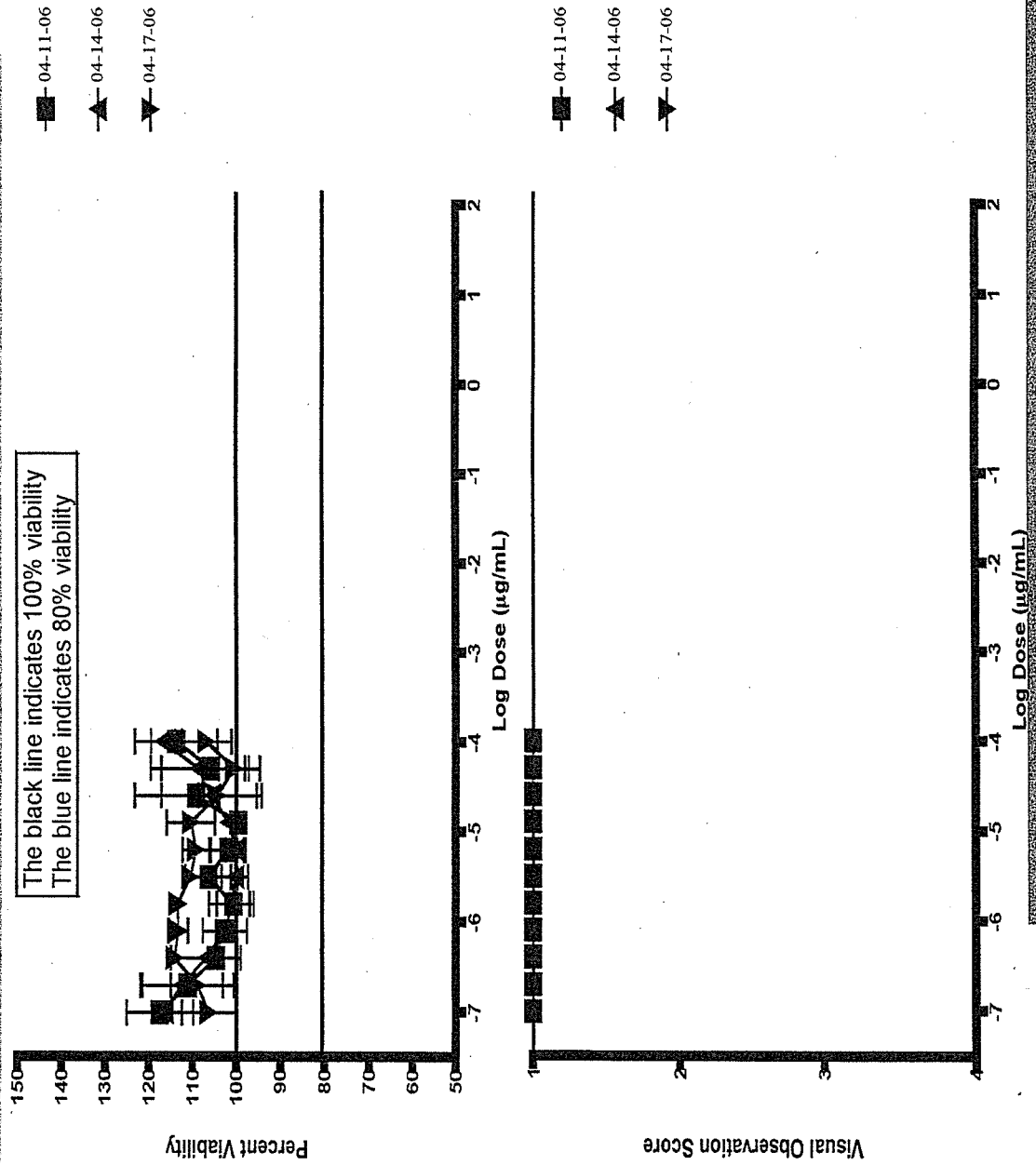
EC ₅₀ (µg/mL)	SD	CV
1.26 x 10 ⁻⁵	7.00 x 10 ⁻⁶	55%

- DES 04-08-06
- ▲ DES 04-14-06
- ▼ DES 04-17-06
- E2 04-08-06
- △ E2 04-14-06
- ▽ E2 04-17-06
- ◇ Methoxychlor 04-08-06
- Methoxychlor 04-14-06
- × Methoxychlor 04-17-06
- DMSO 04-08-06
- 3X Std Dev DMSO¹ 04-08-06
- + DMSO 04-14-06
- 3X Std Dev DMSO¹ 04-14-06
- * DMSO 04-17-06
- 3X Std Dev DMSO¹ 04-17-06

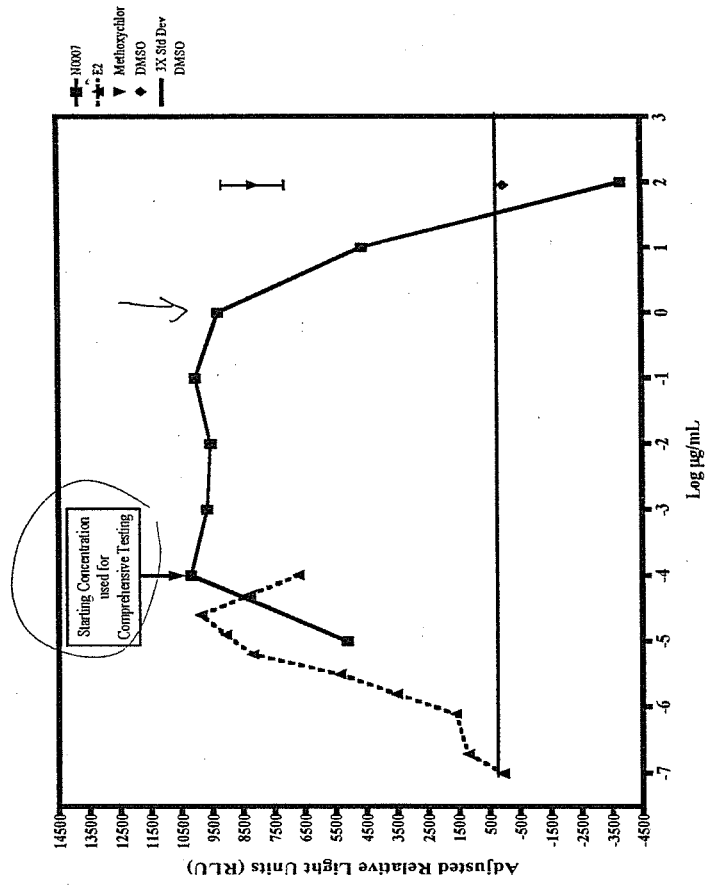


The black line indicates 100% viability
The blue line indicates 80% viability

Comparison of CellTiterGlo[®] and Visual Observations for N0006-DES



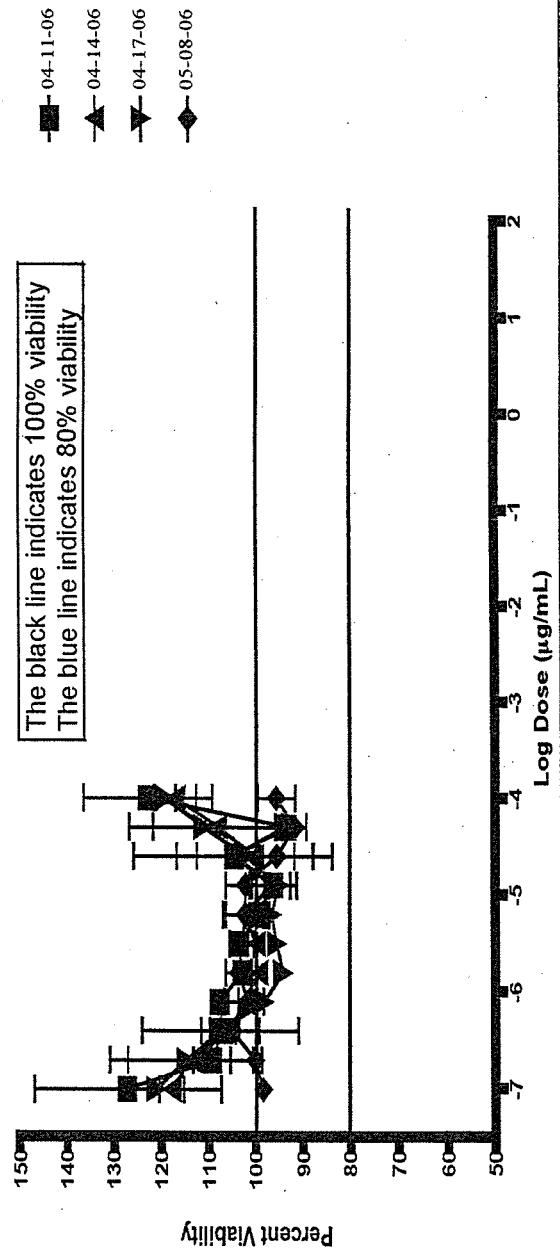
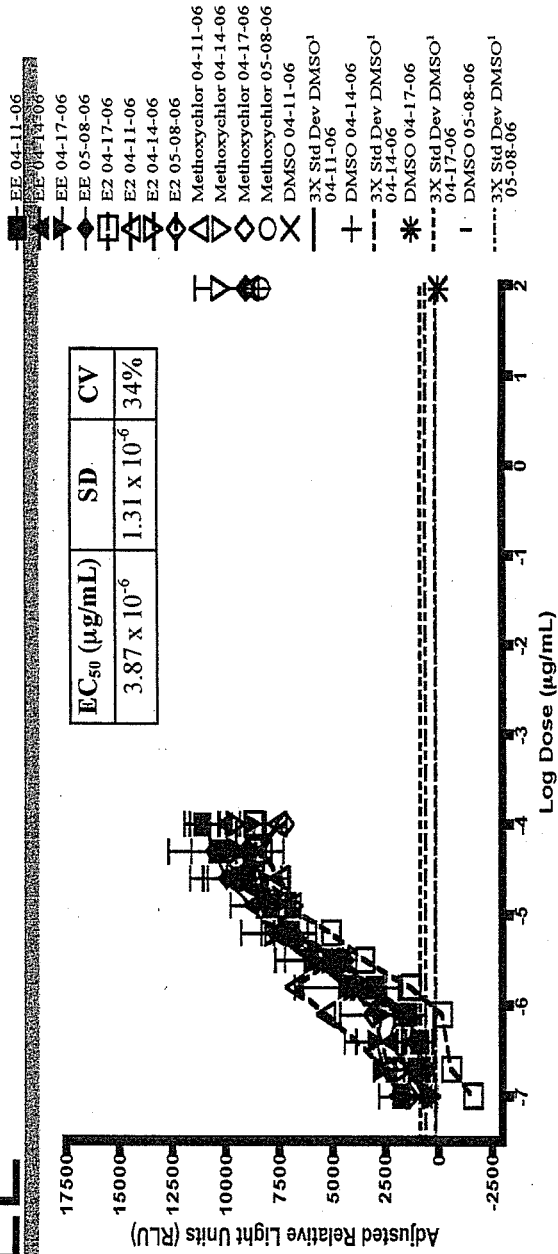
Range Finder Data for N0007- 17 α -ethinyl estradiol (EE)



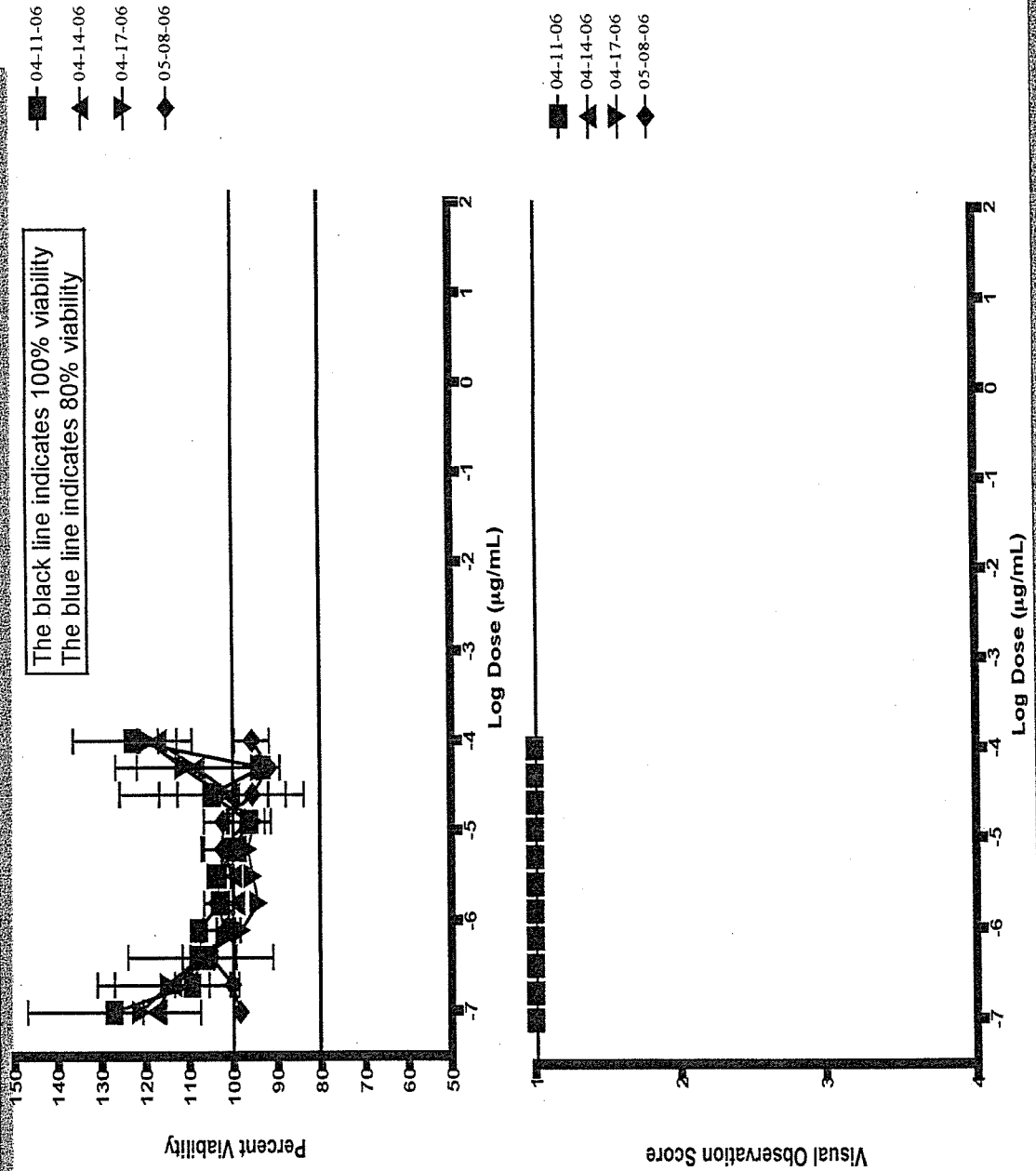
Concentration µg/mL	CellTiterGlo®	Visual Observation Score
1.00 x 10 ⁺²	30%	3
1.00 x 10 ⁺¹	96%	1
1.00 x 10 ⁺⁰	104%	1
1.00 x 10 ⁻¹	107%	1
1.00 x 10 ⁻²	112%	1
1.00 x 10 ⁻³	104%	1
1.00 x 10 ⁻⁴	102%	1
1.00 x 10 ⁻⁵	93%	1

Agonist and Viability Results for N0007-

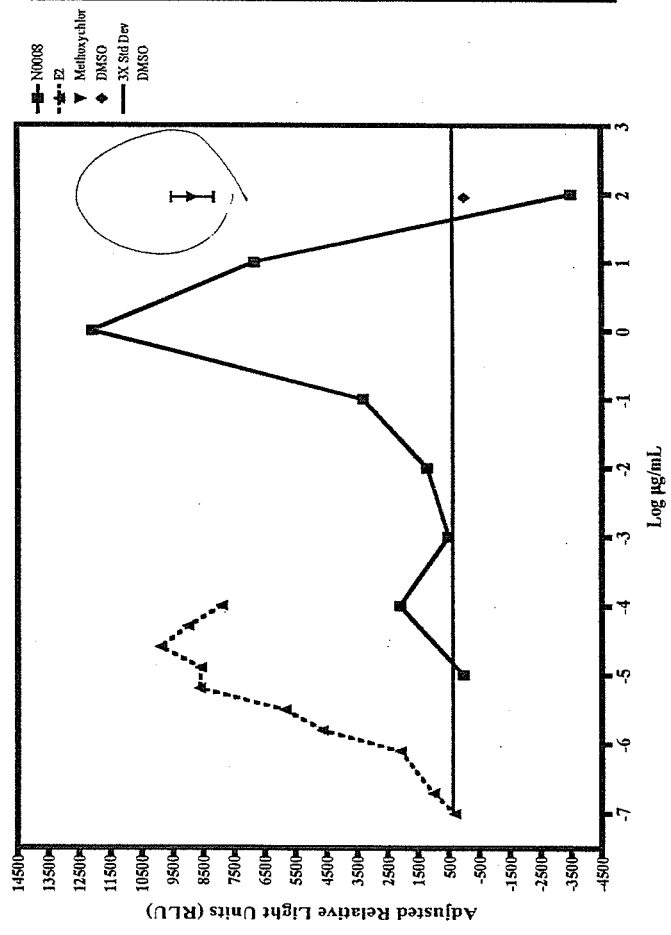
EE



Comparison of CellTiterGlo[®] and Visual Observations for N0007-EE

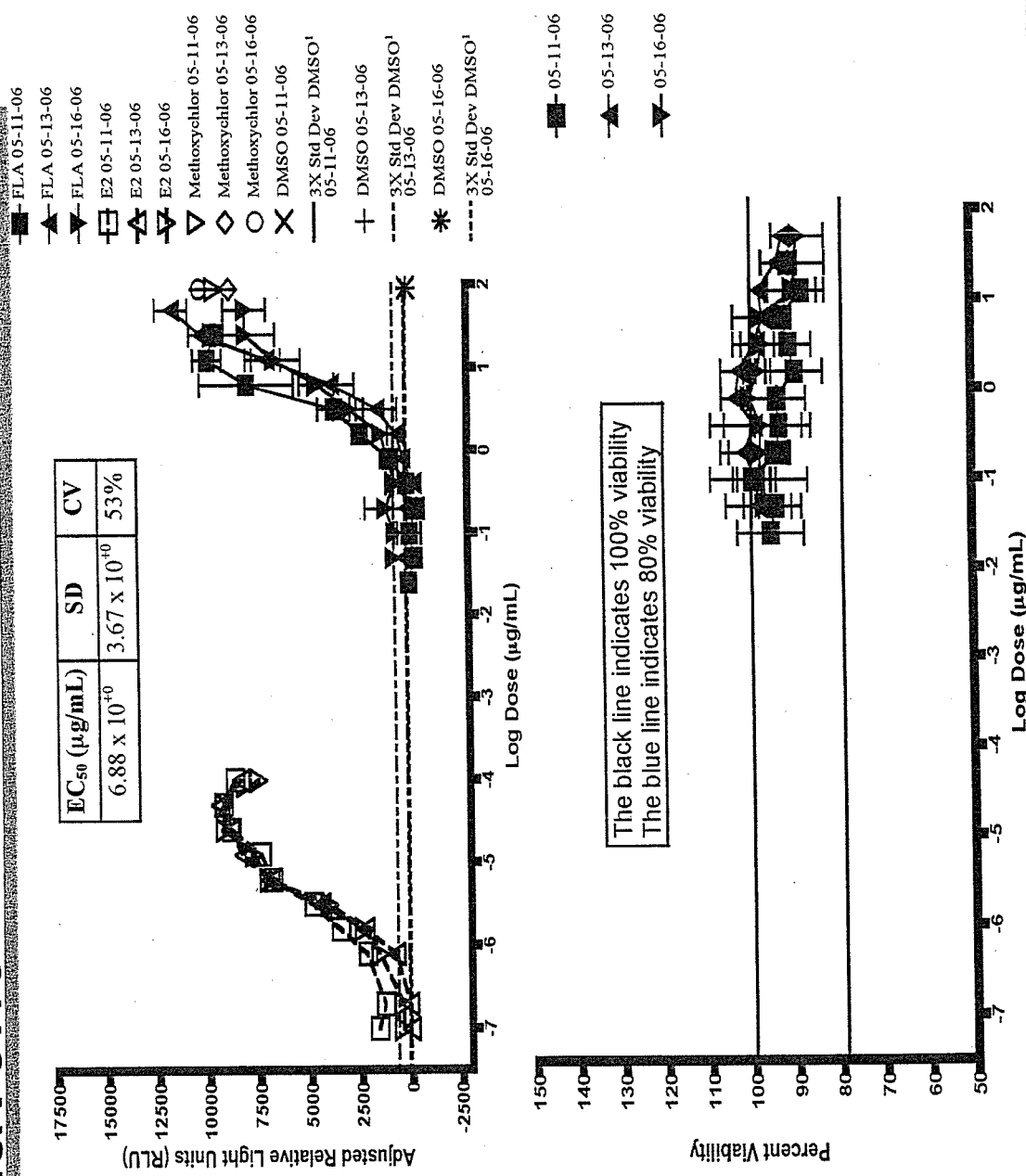


Range Finder Data for N0008-Flavone

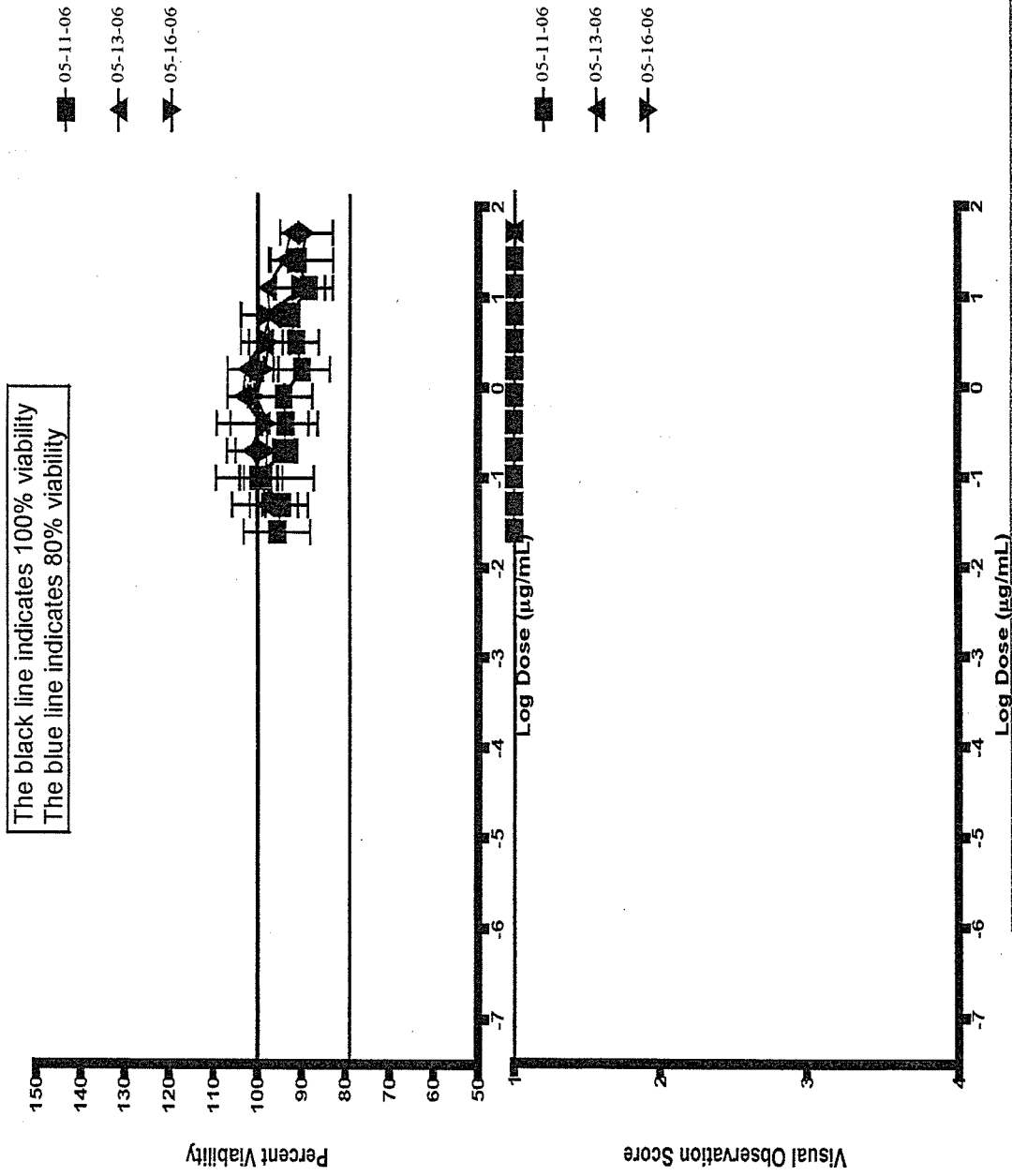


Concentration µg/mL	CellTiterGlo®	Visual Observation Score
1.00 x 10 ⁺²	12%	4
1.00 x 10 ⁺¹	92%	1
1.00 x 10 ⁺⁰	102%	1
1.00 x 10 ⁻¹	103%	1
1.00 x 10 ⁻²	101%	1
1.00 x 10 ⁻³	92%	1
1.00 x 10 ⁻⁴	102%	1
1.00 x 10 ⁻⁵	100%	1

Agonist and Viability Results for N0008-Flavone



Comparison of CellTiterGlo[®] and Visual Observations for N0008-Flavone



Testing of the LUMI-CELL[®] Antagonist Protocol with Coded Substances

General Procedures for Antagonist Testing

- Antagonist range finder and comprehensive testing was conducted on 96 well plates using:
 - Nine concentrations of raloxifene with a fixed amount of E2, in duplicate, as the reference standard.
 - Three replicate wells for the solvent control
 - Three replicate wells for the weak positive control, flavone
 - Three replicate wells for the E2 control
- Luminescence was measured with a luminometer and expressed as RLU
- Data was transferred into Prism[®] and, when possible, the IC₅₀ value was calculated



General Procedures for Antagonist Testing (cont.)

- Antagonist assay acceptance criteria are as follows:
 - Reduction
 - Plate induction, as measured by dividing the highest raloxifene/E2 reference standard RLU value by the lowest raloxifene/E2 reference standard RLU value, must be greater than three fold
 - Reference standard results
 - Calculated raloxifene/E2 reference standard IC_{50} values must be within 2.5 times the standard deviation of the historical database IC_{50} mean values
 - Solvent control values
 - Solvent control RLU values must be within 2.5 times the standard deviation of the historical database solvent 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

Antagonist Testing

- A subset of minimum substances recommended by ICCVAM for validation of *in vitro* ER assays were selected for testing
- They were selected for:
 - ER antagonist activity classification, including those that are negative for antagonism
 - Properties that might make them problematic, including limited solubility or potential cytotoxicity
- None of the substances tested for antagonism could be tested to the intended limit dose of $1.00 \times 10^3 \mu\text{g/mL}$ because of insolubility in cell culture media containing 1% DMSO

Antagonist Testing (cont.)

- Limit dose for antagonist protocol standardization was set one log dose lower than the intended limit dose (1.0×10^{-2} $\mu\text{g/mL}$)
- A protocol deviation in the process of making serial dilutions for range finder testing resulted in 5.00×10^{-1} $\mu\text{g/mL}$ being used as the highest concentration tested
- Concentrations of substance that caused reductions of cell viability below 80% were classified as cytotoxic and were not included in data analyses

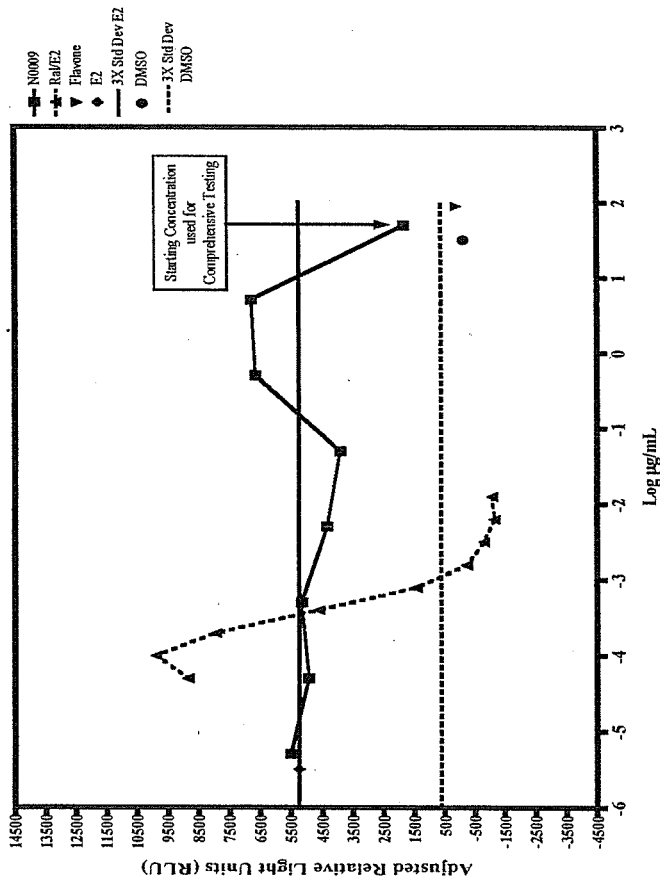
Coded Substances for Antagonist Testing

Code	Substance Name	CASRN	Supplier	Purity	ER TA Antagonist Activity ^{1,2}	Additional Basis for Selection ³
N0009	Butylbenzyl phthalate	85-68-7	Sigma-Aldrich Corp	98%	-	
N0010	Dibenzo [a,h] anthracene	53-70-3	Sigma-Aldrich Corp	99%	##	
N0011	Genistein	446-72-0	Sigma-Aldrich Corp	99%	#	Insoluble
N0012	Flavone	525-82-6	Sigma-Aldrich Corp	99%	###	
N0013	<i>p</i> -n-nonylphenol	104-40-5	Alfa Aesar, Co	100%	#	
N0014	Progesterone	57-83-0	Sigma-Aldrich Corp	100%	-	
N0015	<i>o,p'</i> -DDT	789-2-6	ChemService, Inc	98%	#	Cytotoxic
N0016	Tamoxifen	10540-29-1	Sigma-Aldrich Corp	99%	###	Cytotoxic

Footnotes for Coded Substances for Antagonist Testing

- Abbreviations: CASRN = Chemical Abstracts Service Registry Number; Co = Company; Corp = Corporation, Inc = Incorporated; LLC = Limited Liability Corporation
- ¹Data on agonist and antagonist activities were derived from the March, 2006 Addendum to ICCVAM Evaluation of In Vitro Test Methods For Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays, NIH Publication No. 03-4503. May 2003 (Addendum)
- 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
- ³Information on solubility and cytotoxicity were derived from the addendum and from the scientific literature.

Range Finder Data for N0009- Butylbenzyl phthalate (BBP)



Concentration $\mu\text{g/mL}$	CellTiterGlo®	Visual Observation Score
$5.00 \times 10^{+1}$	111%	1
$5.00 \times 10^{+0}$	102%	1
5.00×10^{-1}	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