

## General Procedures for Agonist Testing

- Agonist range finder and comprehensive testing was conducted on 96 well plates using:
  - o Ten concentrations of E2 in duplicate as the reference standard
  - o Four replicate wells for the solvent control
  - o Three replicate wells for the weak positive control, methoxychlor
- Luminescence was measured with a luminometer and expressed as relative light units (RLU)

## General Procedures for Agonist Testing (cont.)

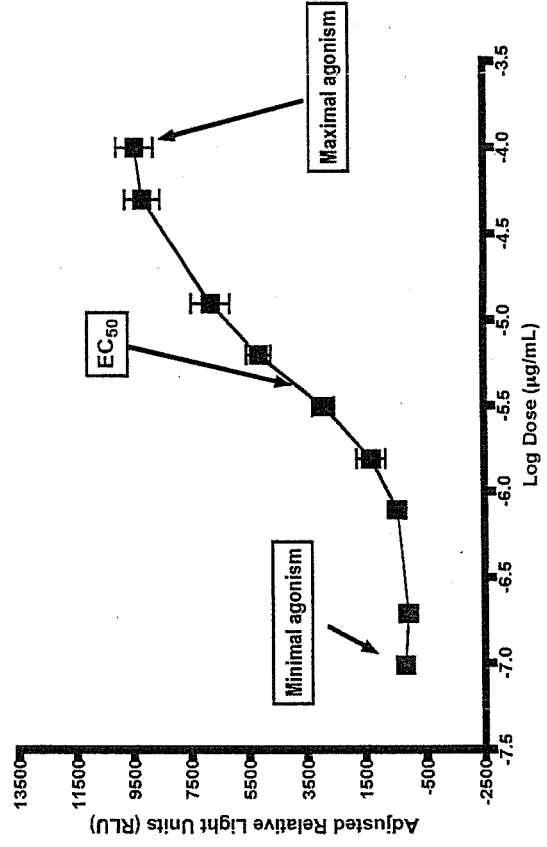
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- Data was transferred into GraphPad PRISM® 4.0 statistical software, graphed, and evaluated for positive or negative response
- EC<sub>50</sub> values were calculated for positive substances using a Hill function analysis
  - four parameter logistic mathematical model relating the substance concentration to RLU values in a sigmoidal shape

# General Procedures for Agonist Testing (cont.)

## ■ Calculation of $EC_{50}$ values (GraphPad Prism<sup>®</sup> Definition):

- The top of the curve is a plateau at a value equal to maximal agonist activity. The bottom of the curve is a plateau equal to minimal agonist activity.
- The concentration of agonist that results in activity halfway between the upper and lower plateaus is called the  $EC_{50}$  (effective concentration 50%).



## General Procedures for Agonist Testing (cont.)

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- Acceptance criteria were based on evaluation of the reference standard and negative and positive control results from each experiment conducted on a 96 well plate
- Results were compared to quality controls from the historical database established during development and standardization of the LUMI-CELL<sup>®</sup> agonist protocol

# General Procedures for Agonist Testing (cont.)

- Agonist assay acceptance criteria are as follows:
  - Induction
    - Plate induction, as measured by dividing the highest E2 reference standard RLU value by the lowest E2 reference standard RLU value, must be greater than three fold
  - 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
  - Solvent control results
    - Solvent 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

# Agonist 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 agonist activity classification, including those that are negative for agonism
  - Properties that might make them problematic, including limited solubility or potential cell viability
- None of the substances tested for agonism could be tested to the intended limit dose of  $1.0 \times 10^{-3}$   $\mu\text{g}/\text{mL}$  because of insolubility in cell culture media containing 1% DMSO
- The limit dose ( $1.00 \times 10^{-2}$   $\mu\text{g}/\text{mL}$ ) for agonist protocol standardization was set one log dose lower than the intended limit dose

# Coded Substances for Agonist Testing

Code	Substance Name	CASRN	Supplier	Purity	ER TA Agonist Activity	Additional Basis for Selection
N0001	Atrazine	1912-24-9	ChemService, Inc	98%	-	Cytotoxic
N0002	Bisphenol A	80-5-7	Sigma-Aldrich Corp	100%	+	
N0003	Bisphenol B	77-40-7	City Chemical, LLC	97%	++	
N0004	Corticosterone	50-22-6	Sigma-Aldrich Corp	99%	-	
N0005	<i>o,p'</i> -DDT	789-2-6	ChemService, Inc	98%	+	Cytotoxic
N0006	Diethylstilbestrol	56-53-1	Sigma-Aldrich Corp	99%	+++	
N0007	17 $\alpha$ -ethinyl estradiol	57-63-6	Sigma-Aldrich Corp	99%	+++	
N0008	Flavone	525-82-6	Sigma-Aldrich Corp	99%	+	

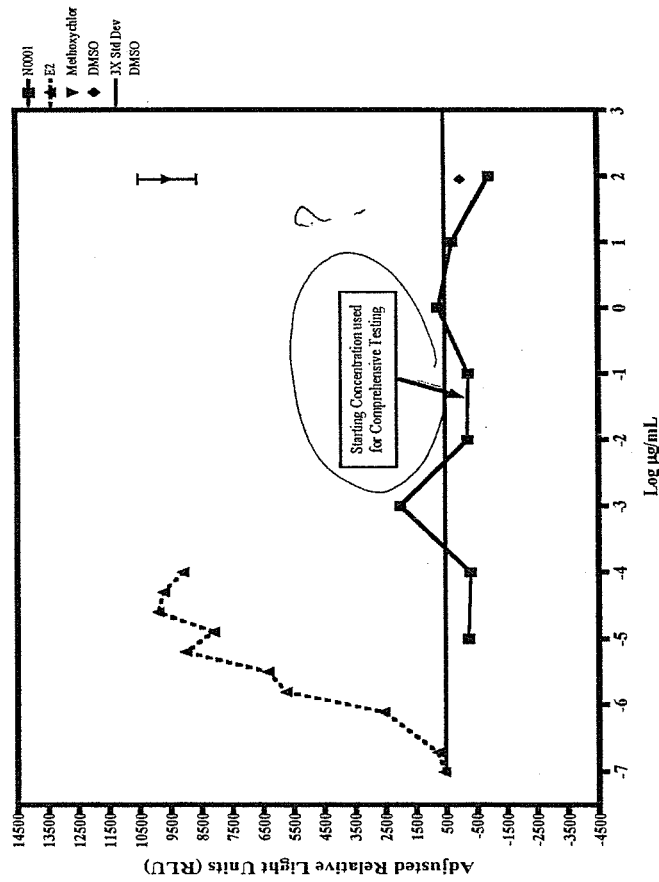
# Footnotes for Coded Substances for Agonist Testing

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- Abbreviations: CASRN = Chemical Abstracts Service Registry Number; Co = Company; Corp = Corporation, Inc = Incorporated; LLC = Limited Liability Corporation
- <sup>1</sup>Data 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)
- <sup>2</sup>+++ Indicates that the substance was strongly active ( $EC_{50}$  value was  $<0.001 \mu\text{M}$ ); ++ indicates that the substance was moderately active ( $EC_{50}$  value was between  $0.001$  and  $0.1 \mu\text{M}$ ); + indicates that the substance was weakly active ( $EC_{50}$  value was  $>0.1 \mu\text{M}$ ), or a positive response was reported without an  $EC_{50}$  value. The  $EC_{50}$  is the effective concentration that causes half-maximal activation of the receptor.
- <sup>3</sup>Information on solubility and cytotoxicity were derived from the addendum and from the scientific literature.

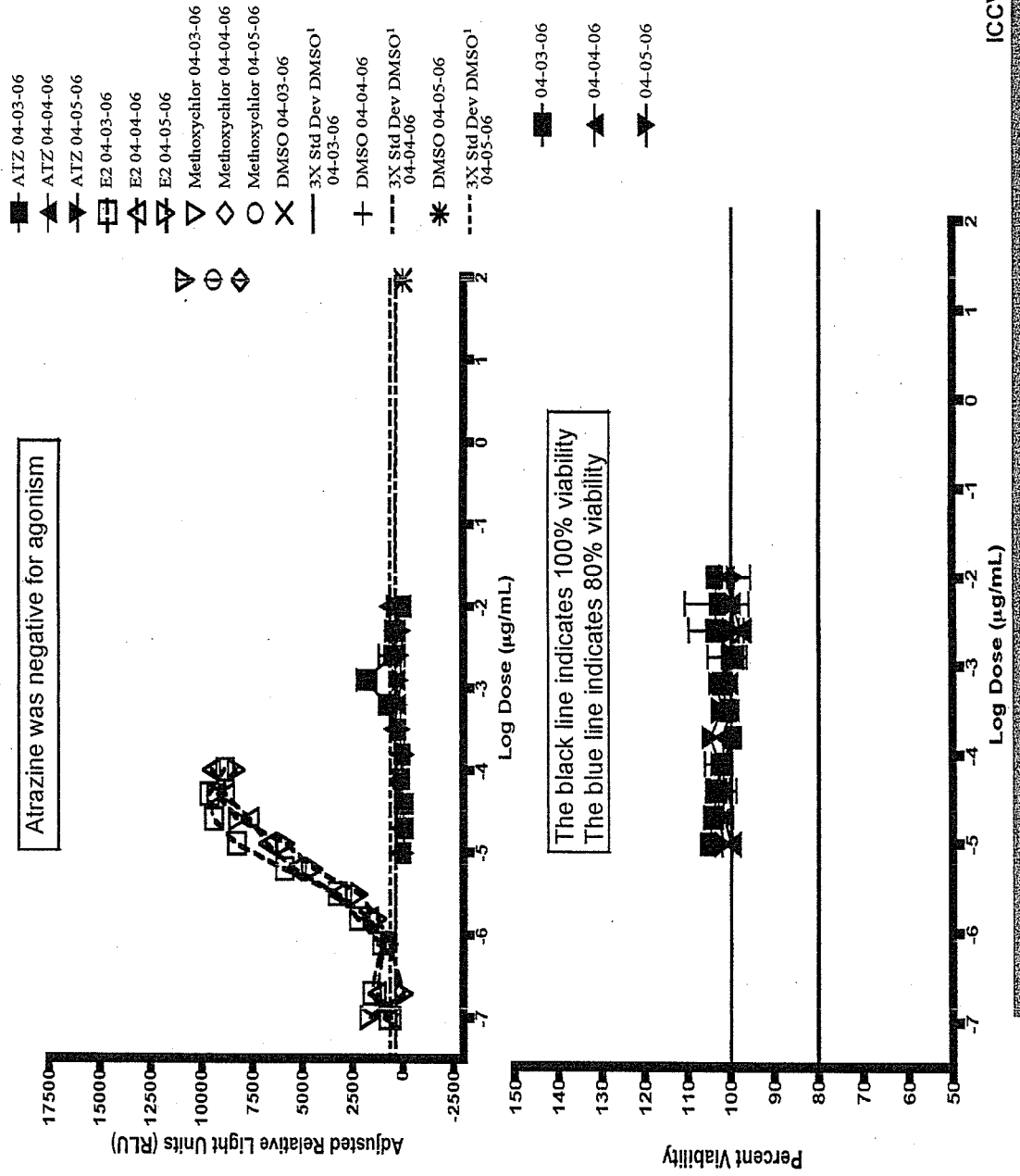


# Range Finder Data for N0001-Atrazine

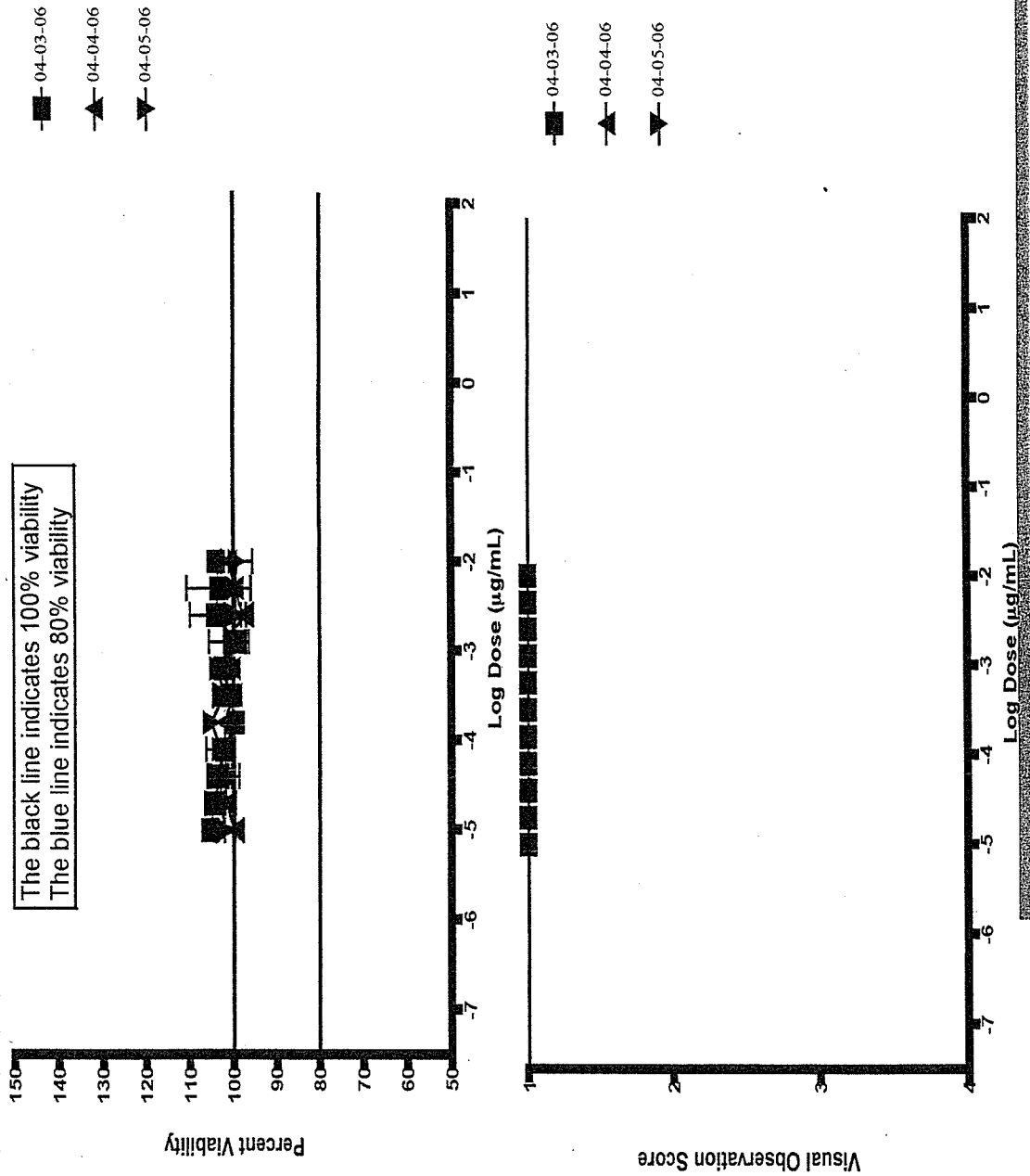


Concentration µg/mL	CellTiterGlo®	Visual Observation Score
$1.00 \times 10^{+2}$	93%	1
$1.00 \times 10^{+1}$	104%	1
$1.00 \times 10^{+0}$	99%	1
$1.00 \times 10^{-1}$	99%	1
$1.00 \times 10^{-2}$	107%	1
$1.00 \times 10^{-3}$	90%	1
$1.00 \times 10^{-4}$	98%	1
$1.00 \times 10^{-5}$	107%	1

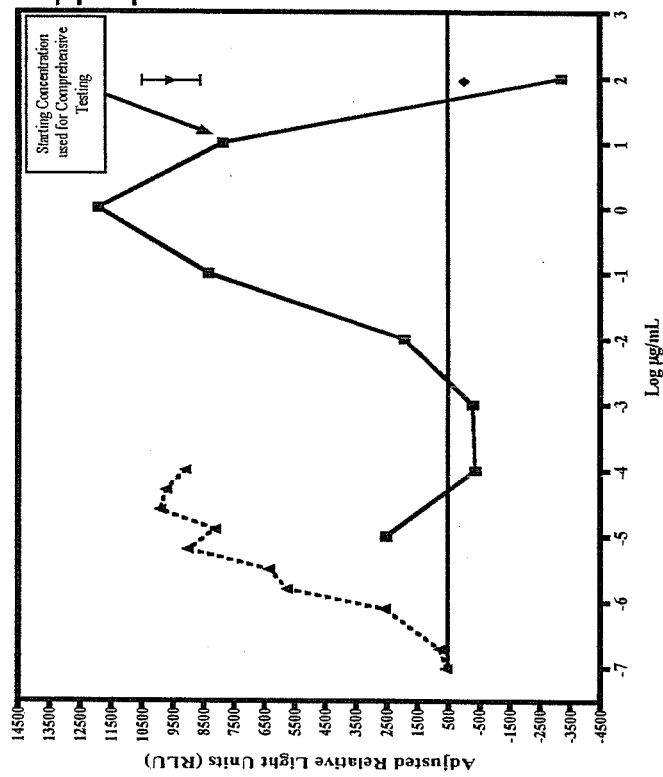
# Agonist and Viability Results for N0001-Atrazine



# Comparison of CellTiterGlo<sup>®</sup> and Visual Observations for N0001-Atrazine



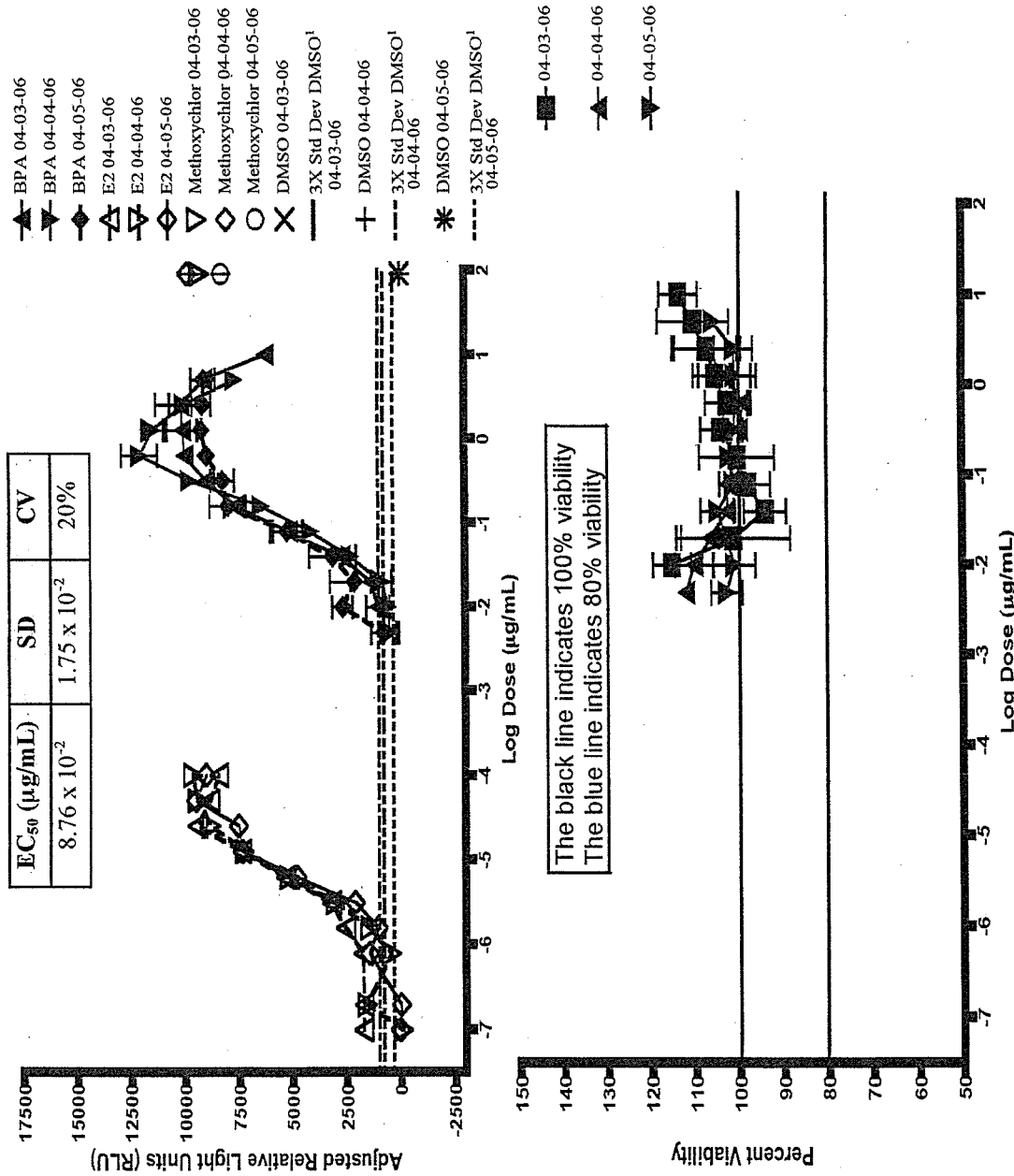
# Range Finder Data for N0002-Bisphenol A



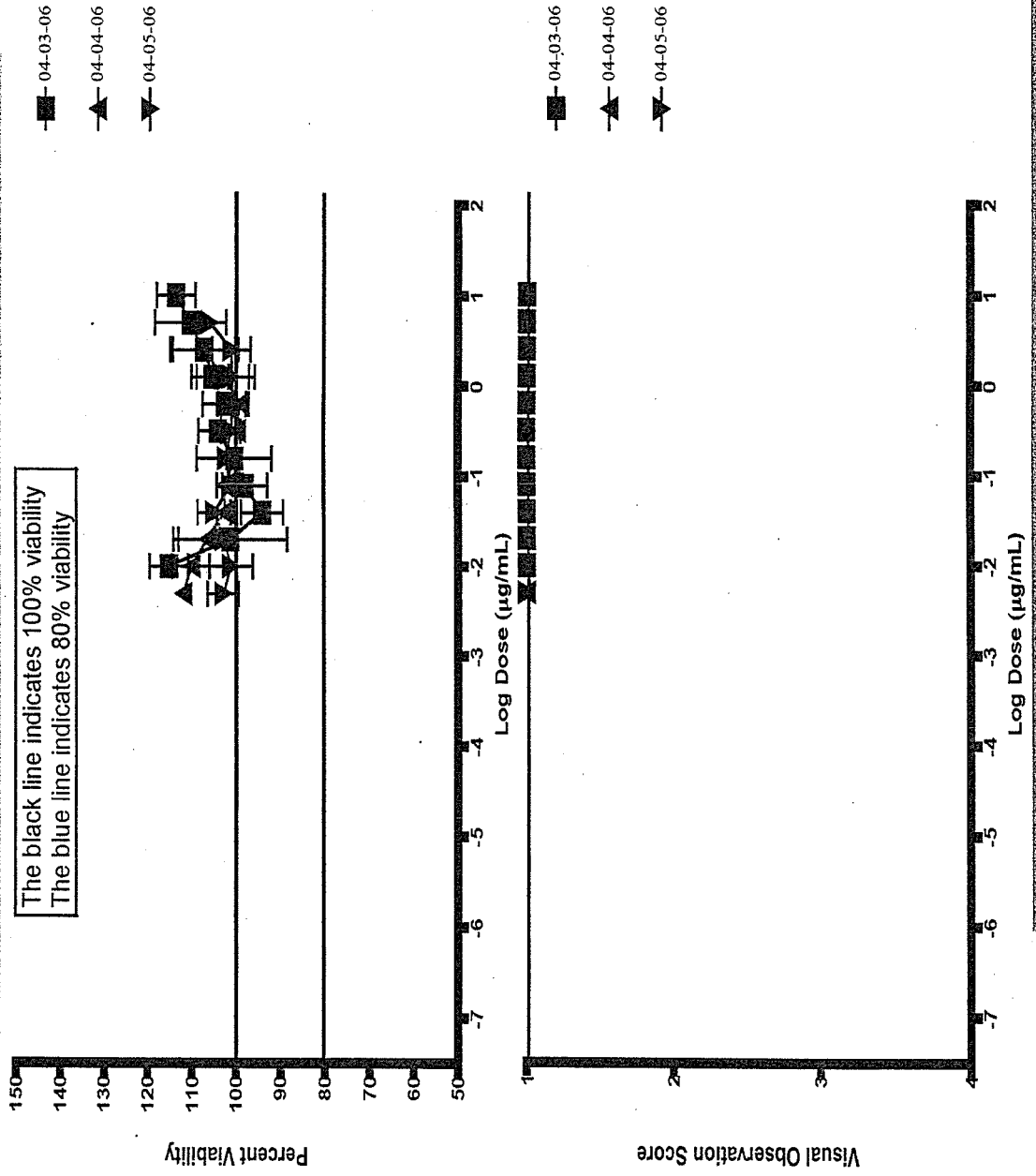
Concentration µg/mL	CellTiterGlo®	Visual Observation Score
1.00 x 10 <sup>+2</sup>	6%	4
1.00 x 10 <sup>+1</sup>	105%	1
1.00 x 10 <sup>+0</sup>	99%	1
1.00 x 10 <sup>-1</sup>	108%	1
1.00 x 10 <sup>-2</sup>	105%	1
1.00 x 10 <sup>-3</sup>	95%	1
1.00 x 10 <sup>-4</sup>	109%	1
1.00 x 10 <sup>-5</sup>	96%	1

■ N0002  
 ▲ EZ  
 ▼ Melanoychlor  
 ◆ DM50  
 — IX 34 Dev.  
 — DM60

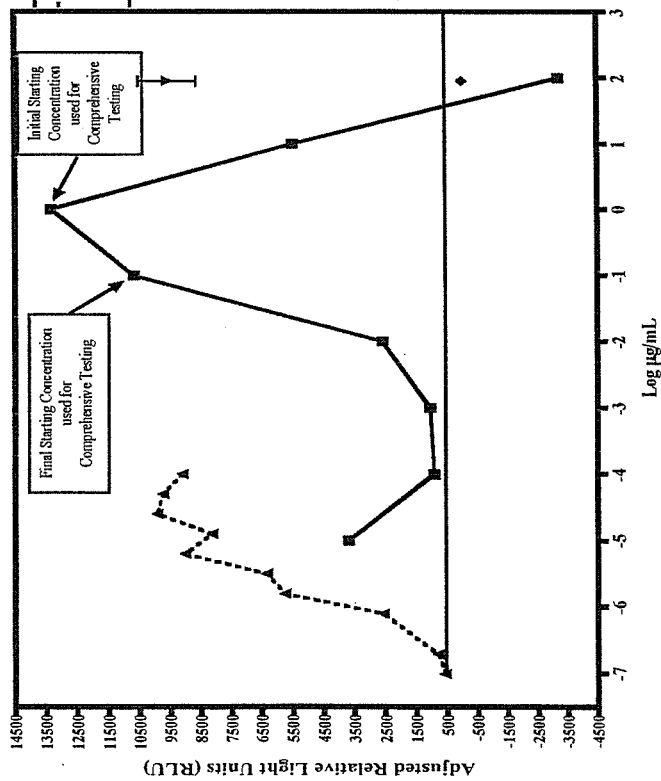
# Agonist and Viability Results for N0002-Bisphenol A



# Comparison of CellTiterGlo<sup>®</sup> and Visual Observations for N0002-Bisphenol A

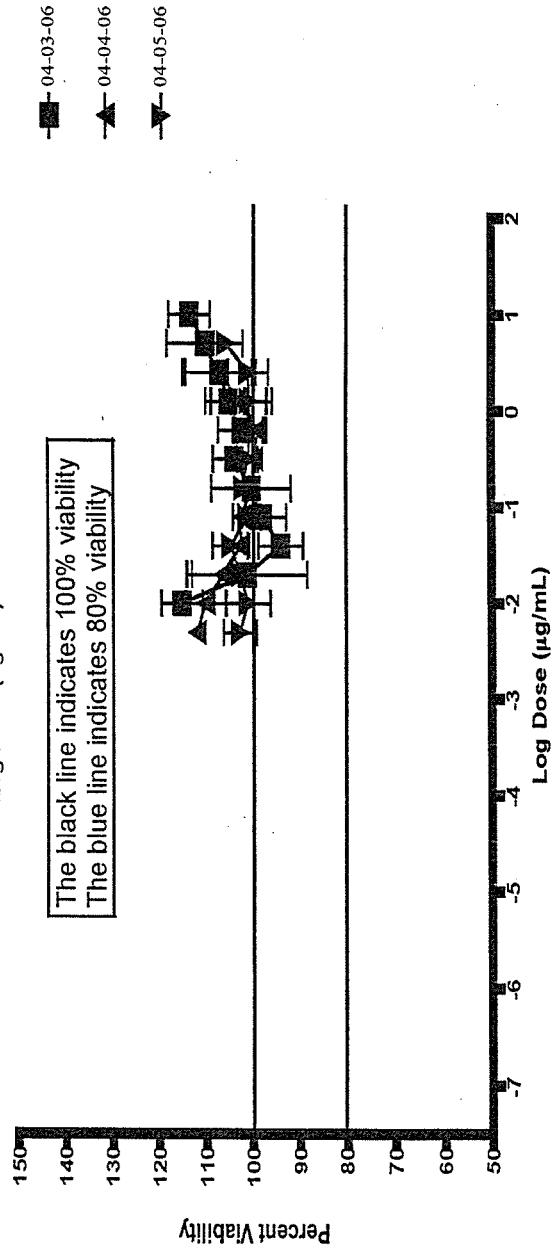
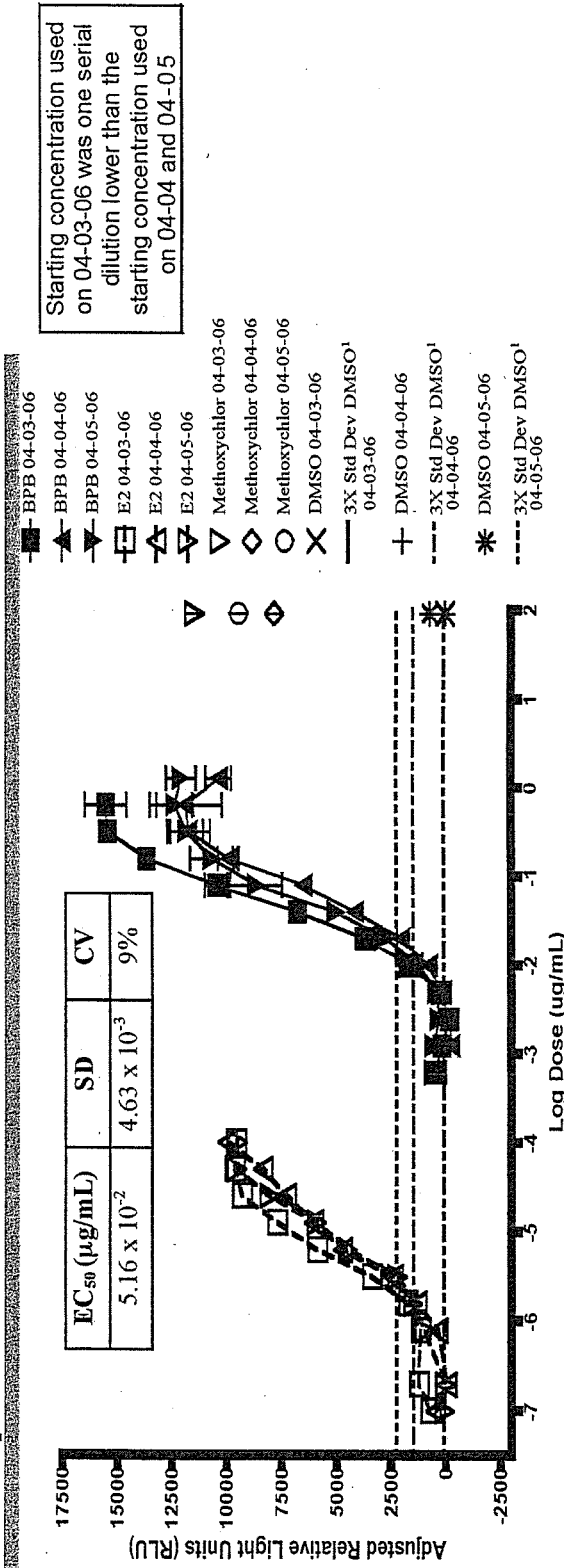


# Range Finder Data for N0003- Bisphenol B



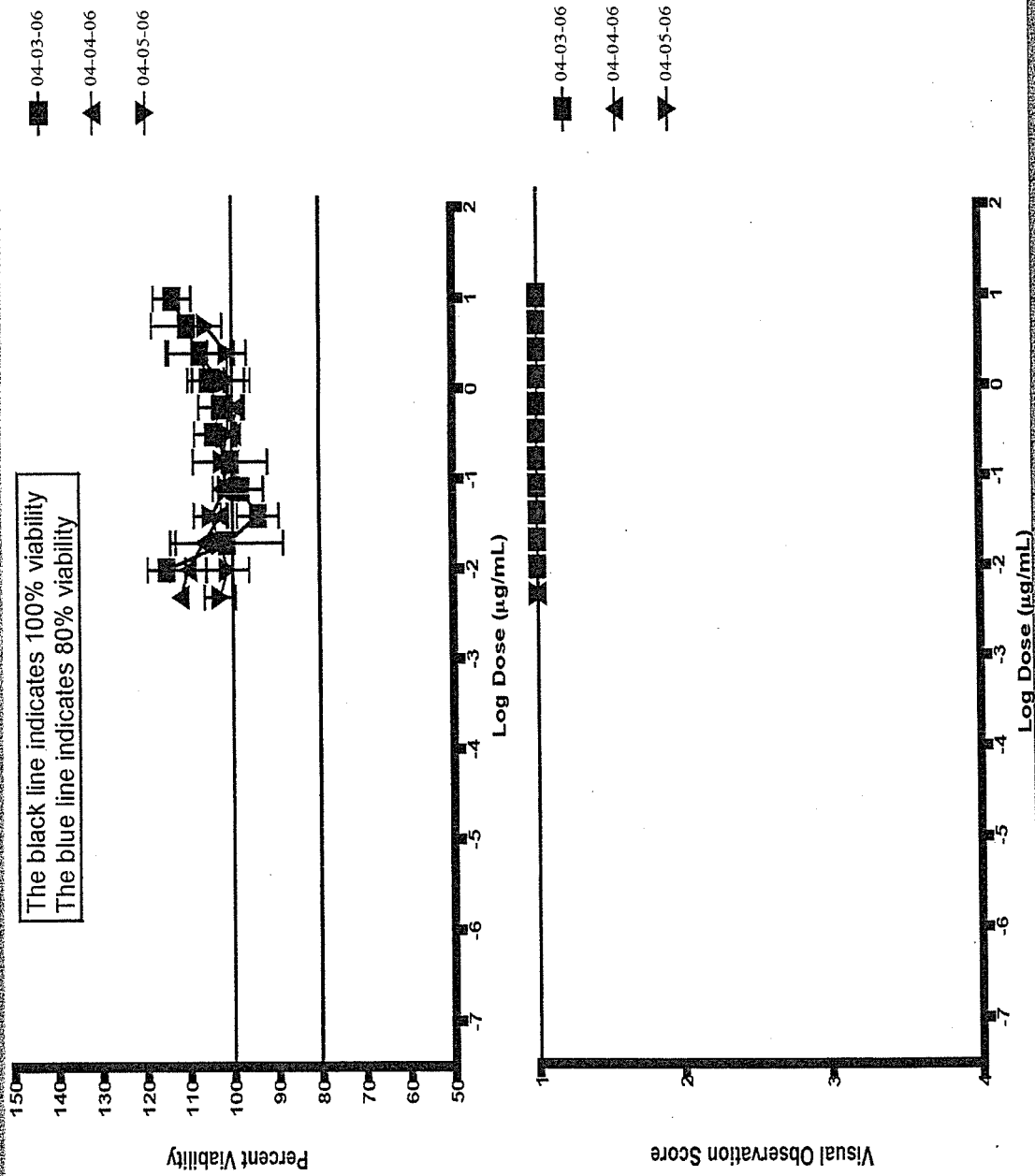
Concentration µg/mL	CellTiterGlo®	Visual Observation Score
1.00 x 10 <sup>+2</sup>	6%	4
1.00 x 10 <sup>+1</sup>	102%	1
1.00 x 10 <sup>+0</sup>	100%	1
1.00 x 10 <sup>-1</sup>	105%	1
1.00 x 10 <sup>-2</sup>	108%	1
1.00 x 10 <sup>-3</sup>	106%	1
1.00 x 10 <sup>-4</sup>	102%	1
1.00 x 10 <sup>-5</sup>	102%	1

# Agonist and Viability Results for N0003-Bisphenol B

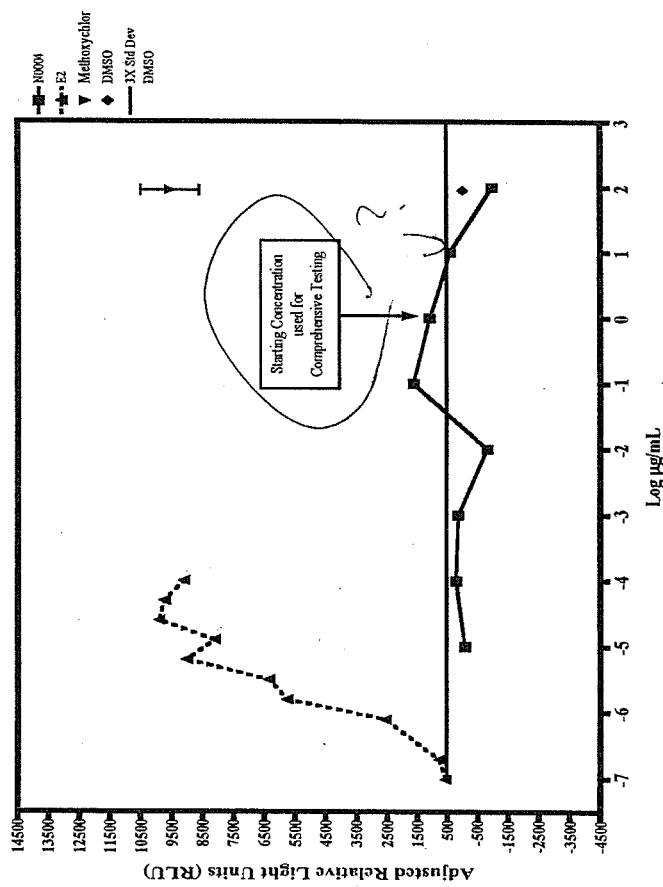




# Comparison of CellTiterGlo<sup>®</sup> and Visual Observations for N0003-Bisphenol B

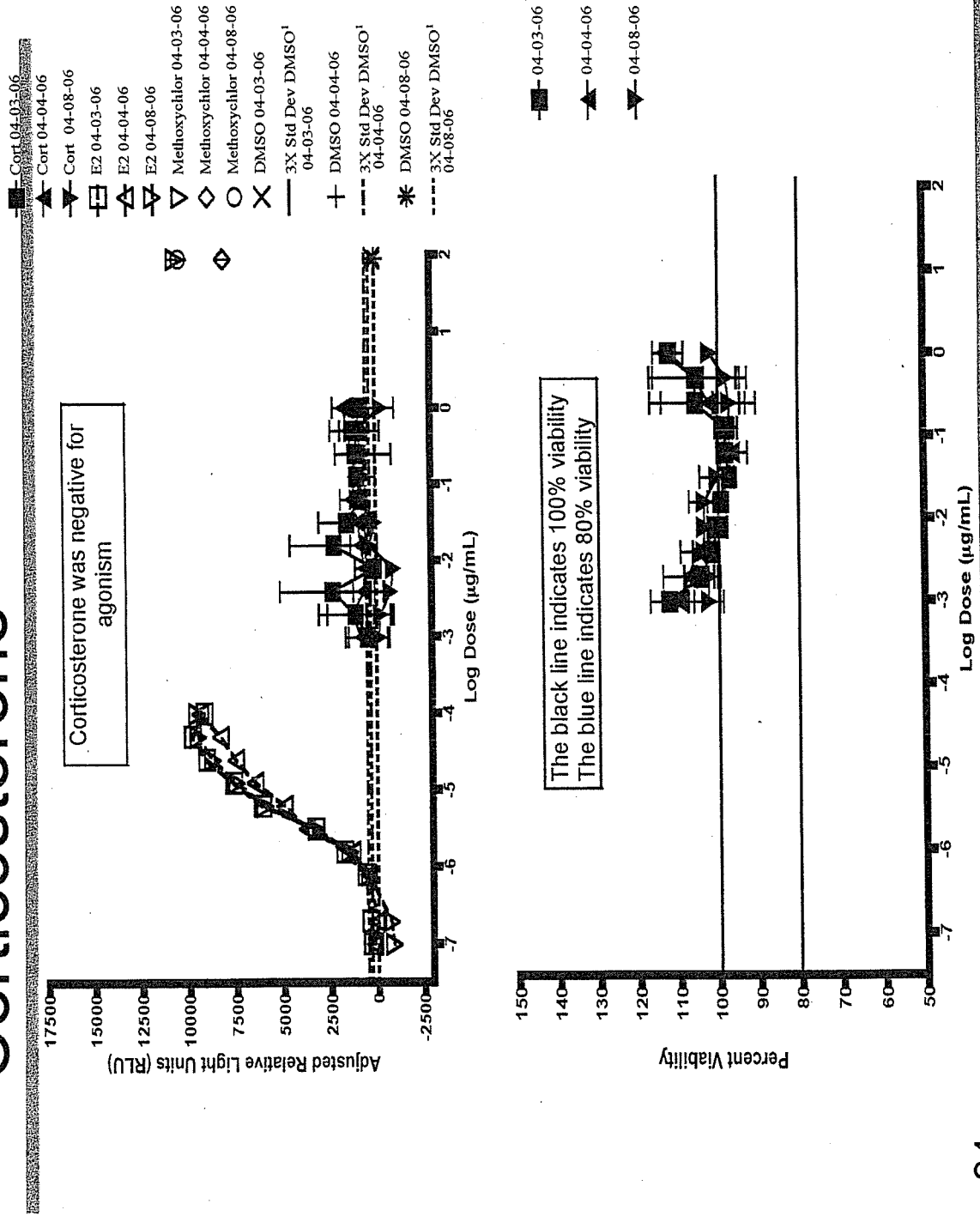


# Range Finder Data for N0004-Corticosterone



Concentration µg/mL	CellTiterGlo®	Visual Observation Score
1.00 x 10 <sup>+2</sup>	80%	2
1.00 x 10 <sup>+1</sup>	94%	1
1.00 x 10 <sup>+0</sup>	97%	1
1.00 x 10 <sup>-1</sup>	102%	1
1.00 x 10 <sup>-2</sup>	104%	1
1.00 x 10 <sup>-3</sup>	103%	1
1.00 x 10 <sup>-4</sup>	107%	1
1.00 x 10 <sup>-5</sup>	103%	1

# Agonist and Viability Results for N0004-Corticosterone



# Comparison of CellTiterGlo<sup>®</sup> and Visual Observations for N0004-Corticosterone

