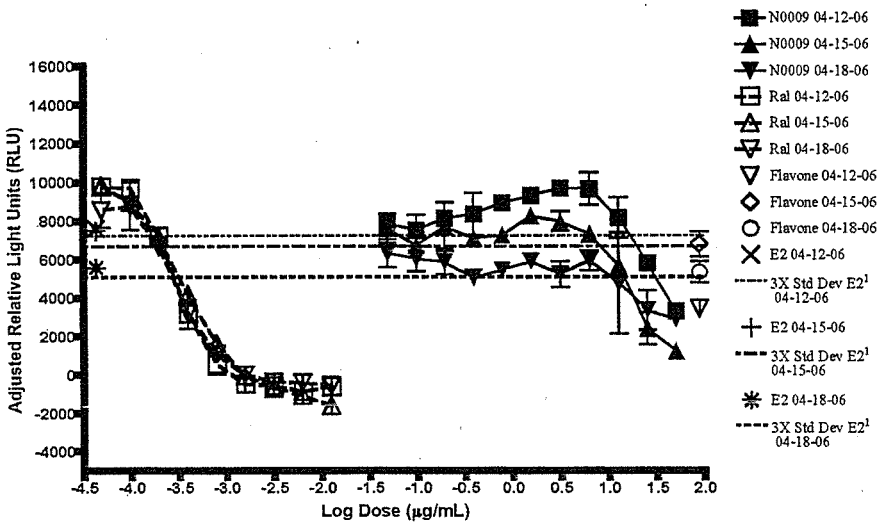
Figure A-4 Antagonist Comprehensive Testing for N0009[1]



[1] Line represents the mean of three raloxifene/E2 replicates minus three times the standard deviation of the raloxifene/E2 mean

Quality Controls:

This section should include graphical representations of quality control data used for acceptance or rejection of experiments conducted during each phase using Excel® as follows:

- Agonist Quality Controls
  - a graph depicting the combined results for the methoxychlor control
  - a graph depicting the combined results for the DMSO control
  - a graph depicting the combined results for the fold induction of the E2 reference standard
  - a graph depicting the combined EC_{50} values of the E2 reference standard
- Antagonist Quality Controls
  - a graph depicting the combined results for the flavone control
  - a graph depicting the combined results for the DMSO control
  - a graph depicting the combined results for the fold reduction of the Ral/E2 reference standard
  - a graph depicting the combined IC_{50} values of the Ral/E2 reference standard

DISCUSSION

Results, including a description of any problems that were encountered and how they were resolved, should be presented and discussed.

SIGNATURE PAGE

Study Director: Name, signature and date

852
853
854
855
856
857
858
859
860
861

862 **APPENDIX B**

863
864

865 **LUMI-CELL® ER AGONIST PROTOCOL**

866

867

868 **APPENDIX C**

869

870

871 **LUMI-CELL® ER ANTAGONIST PROTOCOL**

872

873

874

875

876

877

878

879

880

881

882

883

884 **APPENDIX D**

885

886

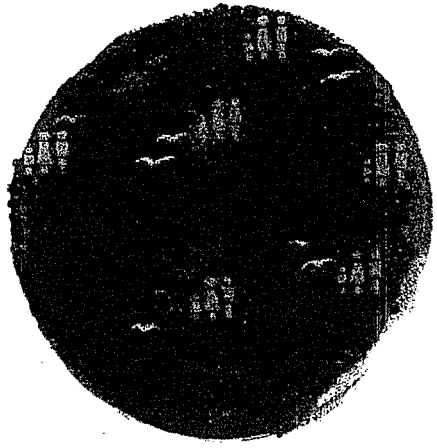
887 **Style Guide for LUMI-CELL® ER Validation Study**

888 **Laboratory Reports and Documents**

NICEATM **ICCVAM**

National Toxicology Program
Interagency Center for the Evaluation of Alternative Toxicological Methods

Interagency Coordinating Committee on the Validation of Alternative Methods



Report on the LUMI-CELL® Protocol Standardization Study

Dr. Ray Tice

Mr. Frank Deal
Ms. Patricia Ceger

International ED SMT Meeting
16 November 2006

National Toxicology Program

NIEHS

Goals of the Protocol Standardization

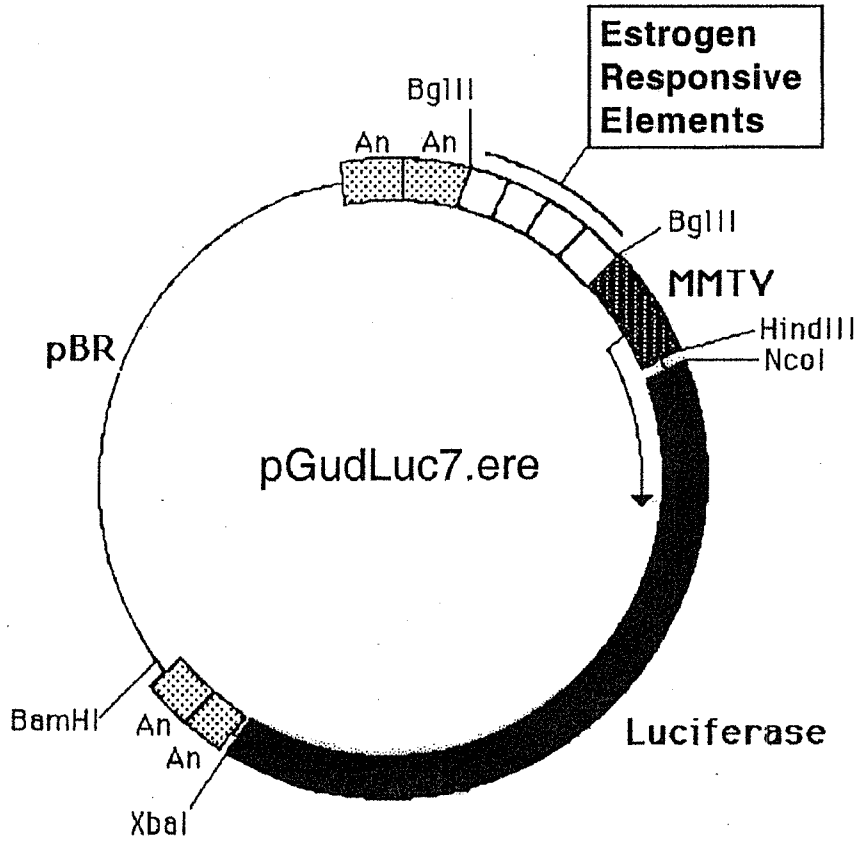
Goals of the LUMI-CELL® Test Method Protocol Standardization Study

- The primary goal of the study was to develop standardized protocols for detecting ER agonists and antagonists that can be transferred to other laboratories for use in validation studies
- Specific goals of the study for both agonist and antagonist studies were to:
  - o Select and standardize:
    - Reference standards and controls
    - Methods for assessing cell viability
    - Establish an historical database for quality control
  - o Test the adequacy of the protocol with a subset of the ICCVAM recommended substances for validation of ER TA binding and TA assays

Overview of the LUMI-CELL® ER TA Test Method

- LUMI-CELL® test method is based on a stable recombinant cell line (BG1Luc4E2)
  - BG1 - human ovarian carcinoma cell that expresses endogenous alpha (95%) and beta (5%) estrogen receptors
  - Plasmid pGudLUC7.ERE used to transfect cell line
    - Contains 4 copies of synthetic oligonucleotide containing estrogen response element (ERE)
    - Mouse mammary tumor promoter
    - Firefly luciferase gene
  - Exposure to estrogenic substances causes activation of ERE, which drives transcription of luciferase
  - Luminometer is used to quantify luciferase expression

LUMI-CELL® ER-TA Plasmid Construct



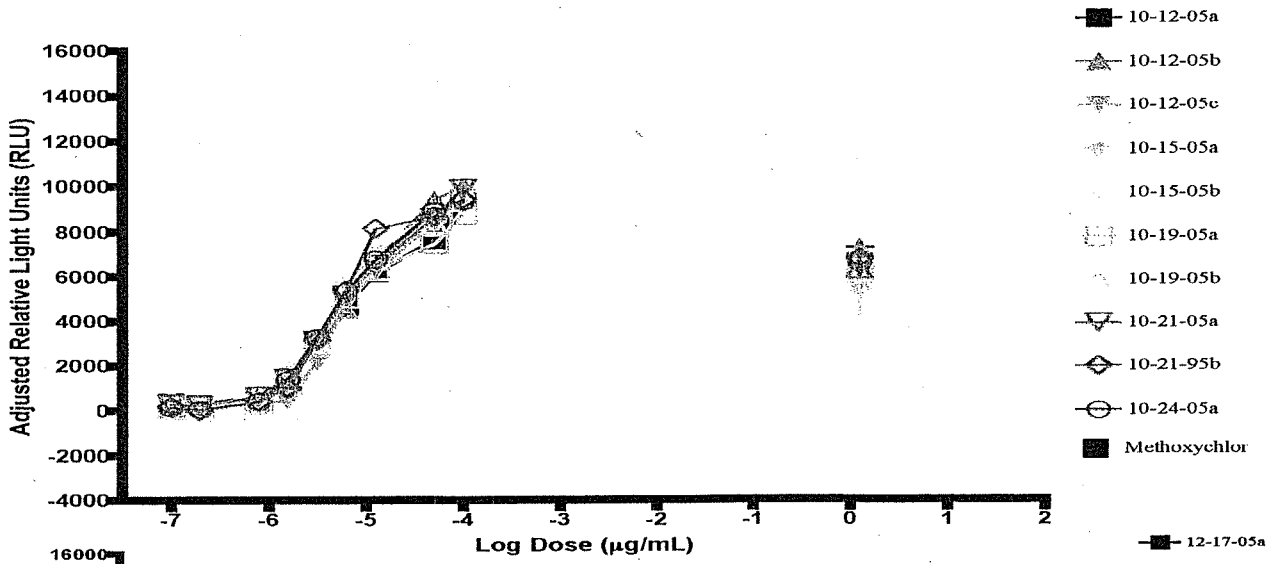
Solvent, Reference Estrogen, Agonist, and Antagonist Controls

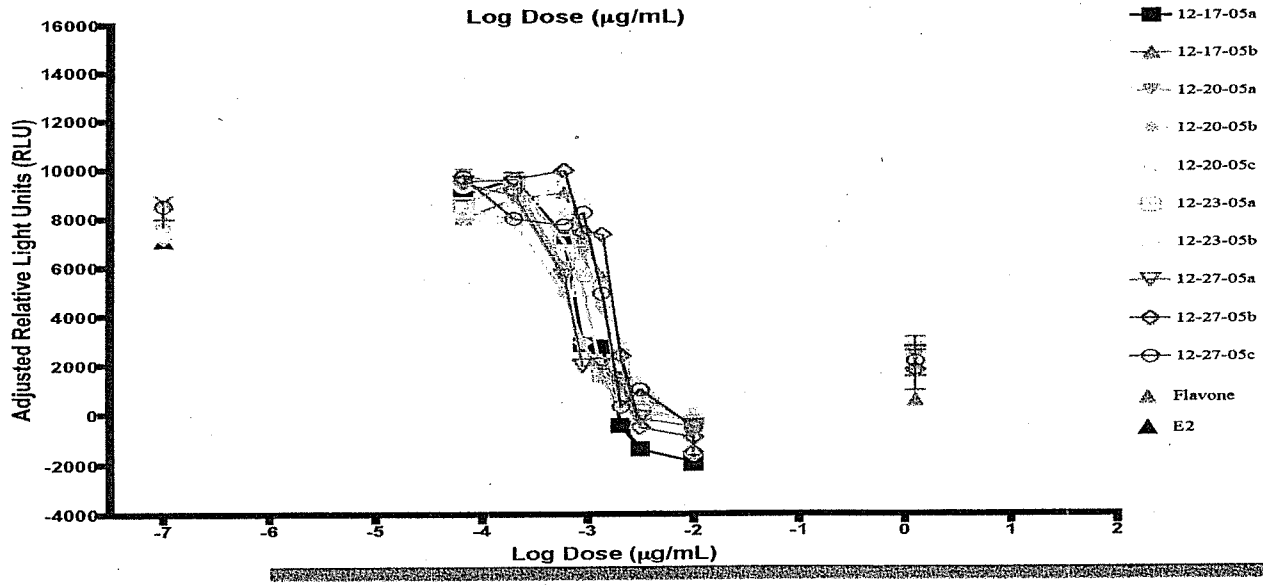
Use	Substance Name	CASRN	Concentration
Solvent	Dimethyl sulfoxide	67-68-5	1%
Agonist Reference Standard	17β-estradiol	50-28-2	10 point serial dilution
Agonist Positive Control	*p,p'*-methoxychlor	72-43-5	3.13 µg/mL
Antagonist Reference Standard	Raloxifene HCl	82640-04-8	9 point serial dilution
Antagonist Positive Control	Flavone	525-82-6	25 µg/mL
Antagonist E2 Control	17β-estradiol	50-28-2	2.5×10^{-5} µg/mL

Creation of the 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 intra-laboratory reproducibility
- The historical databases were established by conducting 10 independent experiments using each protocol

Agonist and Antagonist Historical Databases





Assessment of Cell Viability

Quantitative Evaluation of Cell Viability

- ICCVAM recommended the use of quantitative tests for the measurement of cell viability in ER TA assays
- A commercially available quantitative cytotoxicity assay, CellTiterGlo® was selected.
  - CellTiterGlo® is a luminescence-based assay that measures ATP.
  - CellTiterGlo® requires the use of a separate plate from the one used to evaluate ER TA activity

Quantitative Evaluation of Cell Viability (cont.)

- Cell viability data during evaluation of reference standards indicated that a significant decrease in ER TA response occurred when a reduction of ATP levels (as measured with CellTiterGlo®) exceeded 20%.
- Concentrations of substance that caused reduction in cell viability below 80% were classified as cytotoxic and were not included in data analyses.

Qualitative Evaluation of Cell Viability

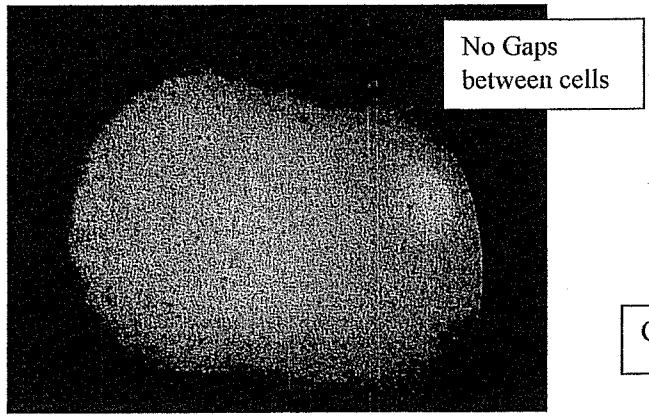
- Assessment of cell viability was also conducted qualitatively using a method developed by XDS based on visual observations of cellular morphology and cell density in the same plates used to evaluate ER TA activity

Visual Observation Rating System

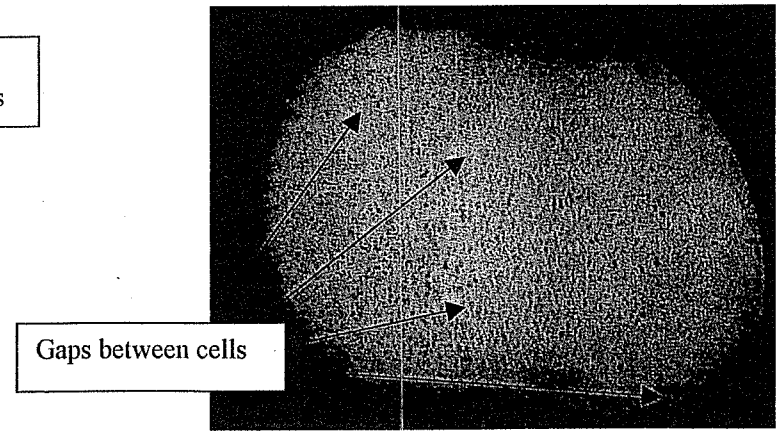
Viability Score	Brief Description
1	Normal Cell Morphology and Cell Density
2	Altered Cell Morphology and/or Small Gaps between Cells
3	Altered Cell Morphology and/or Large Gaps between Cells
4	Few (or no) Visible Cells
1 P	Score of 1 with Precipitate
2 P	Score of 2 with Precipitate
3 P	Score of 3 with Precipitate
4 P	Score of 4 with Precipitate
5 P	Unable to View Cells Due to Precipitate

Visual Observation Rating System Images

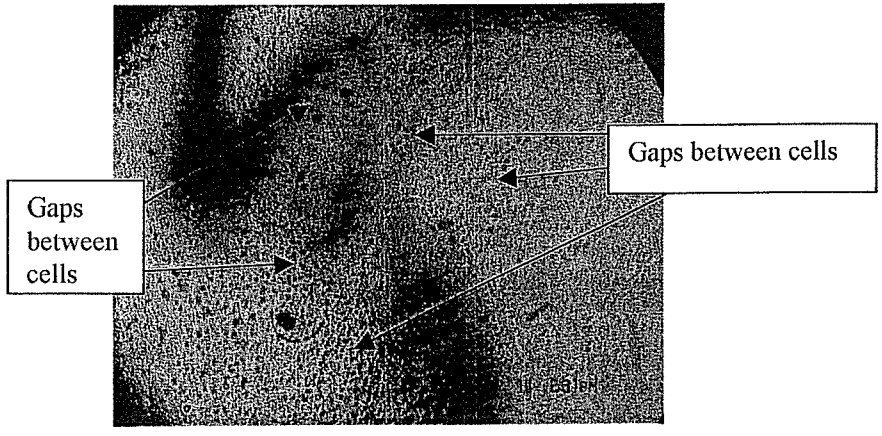
Score of 1



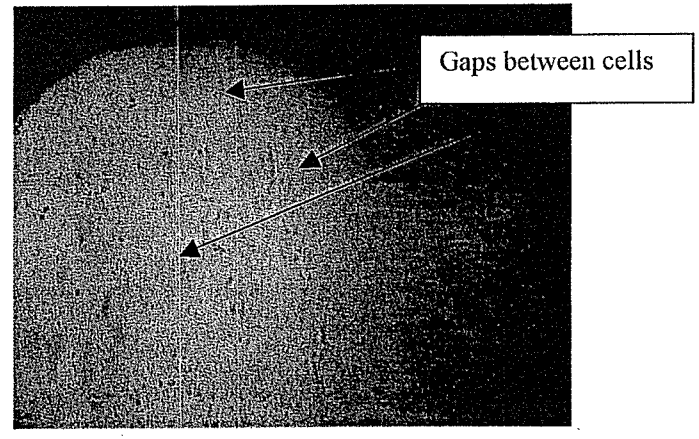
Score of 3



Score of 2



Score of 4



Testing of the LUMI-CELL® Agonist Protocol with Coded Substances