SUPPORT PROTOCOLS

No. 6-1. Chemiluminescence Detection with standard luciferase reagent

Reagents

Cell lysis reagent (4.5x): Dilute 10 mL of 5×Cell Culture Lysis Reagent (CCLR, #E1531) with 45 mL of distilled water.

Luciferase Assay Reagent: Add 1 vial (105 mL) of Luciferase Assay buffer (Promega, #E4550) into a vial containing Luciferase Assay Substrate (Promega, #E4550), and dissolve the substrate thoroughly. Store the substrate below -20□ if necessary.

Chemiluminescence Detection

1. Flick and drain off the contents of the assay plate.
2. Add 100μl of PBS to the well to wash the plate.
3. Flick and drain off the contents of the assay plate.
4. Add 100μl of PBS to the well to wash the plate again.
5. Flick and drain off the contents of the assay plate.
6. Add 15uL of Cell lysis reagent (4.5x) to wells.
7. Incubate for 10 min at room temperature.
8. Add 50uL of Luciferase Assay Reagent to wells.
9. Read plates on a Chemiluminescence plate reader.

SUPPORT PROTOCOLS

No. 6-2. Chemiluminescence Detection with luciferase reagent using Steady-Glo Luciferase Assay System

Reagents

Luciferase Assay Reagent: Add 1 vial (100 mL) of Luciferase Assay buffer into a vial containing Luciferase Assay Substrate (Promega, #E2520), and dissolve the substrate thoroughly. Store the substrate below -20□ if necessary.

Chemiluminescence Detection

1. Remove 50μL of assay medium from all wells of assay plate.
2. Add 100μL of Luciferase Assay Reagent to wells.
3. Allowed to stand for 5 min.
4. Read plates on a Chemiluminescence plate reader

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Current Status of Test Methods for Detecting Endocrine Disruptors: In Vitro Estrogen Receptor Transcriptional Activation Assays, The National Toxicology Program (NTP), Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), National Institute of Environmental Health Sciences (NIEHS), 2002.

ICCVAM Evaluation of In Vitro Test Methods for Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays URL: http://iccvam.niehs.nih.gov/methods/endodocs/edfinrpt/edfinrpt.pdf

OECD conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals URL: http://www.oecd.org/dataoecd/17/33/23652447.doc

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Earl-Gray LJr. (1998) Tiered screening and testing strategy for xenoestrogens and antiandrogens. Toxicology Letters 102-103:677-680

Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) (1998) Final report. URL: http://www.epa.gov/scipoly/oscpendo/history/finalrpt.htm

Organization of Economic Cooperation and Developement. OECD. 2001. 3rd meeting of the validation management group for the screening and testing of endocrine disrupters (mannalian effects). ENV/JM/TG/EDTA (2001). Paris: Joint meeting of the chemicals committee and the working party on chemicals, pesticides and biotechnology .2001.

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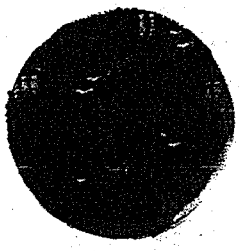
National Toxicology Program
Interagency Center for the Evaluation of Alternative Toxicological Methods

Interagency Coordinating Committee on the Validation of Alternative Methods

ICCVAM/NICEATM ED Activities



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Ms. Patricia Ceger

March 02, 2006

Presentation Overview

I. ICCVAM/OECD Test Method Submission Outline
II. ICCVAM Evaluation of *In Vitro* Test Methods for Detecting Potential Endocrine Disruptors
III. Revised Reference Substance List
IV. Current NICEATM ED Activities
V. Phased Validation Testing
VI. XDS LUMI®-CELL ER Agonist and Antagonist Test Method

I. ICCVAM/OCED Test Method Submission Outline

- ICCVAM Prioritization Criteria
  - o The extent to which the proposed test method is:
    - Applicable to U.S. Federal Regulatory testing needs
    - Applicable to multiple U.S. Federal agencies/programs
    - Warranted, based on the extent of expected use or application and impact on human, animal or ecological health.
  - o The potential for the proposed method, compared to current test methods accepted by U.S. Federal regulatory agencies, to:
    - Refine animal use (decrease or eliminate pain and distress)
    - Reduce animal use
    - Replace animal use

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I. ICCVAM/OCED Test Method Submission Outline (cont.)

- ICCVAM Prioritization Criteria (cont.)
  - o The potential for the proposed test method to provide improved prediction of adverse health or environmental effects, compared to current test methods accepted by U.S. Federal regulatory agencies
  - o The extent to which the test method provides other advantages (e.g., reduced cost and time to perform) compared to current methods.

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I. ICCVAM/OCED Test Method Submission Outline (cont.)

- Guidelines for the Nomination and Submission of Test Methods*
  1. An introduction, including the scientific and regulatory rationale for the proposed test method
  2. Information on the development of the proposed test method protocol and its key components
  3. Characterization of the substances used for validation studies on the proposed test method
  4. The reference data used to assess the accuracy and reliability of the proposed test method
  5. Test method data and results
  6. An assessment of the accuracy of the proposed test method

*ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods (NIH Pub. No. 03-4508).

*Organisation of Economic Co-Operation and Development (OECD) Series on Testing and Assessment Number 34: Guidance Document on the Validation and International Acceptance of New or Updated test Methods for Hazard Assessment

I. ICCVAM/OCED Test Method Submission Outline (cont.)

7. An assessment of the reliability (repeatability/reproducibility) of the proposed test method
8. An assessment of test method data quality
9. Other scientific reports and reviews pertinent to the propose test method
10. An assessment of animal welfare considerations (refinement, reduction, and replacement)
11. Practical considerations (e.g., test method study costs, time needed to perform a study, ease of transferability of the test method among laboratories)
12. A comprehensive and complete list of references
13. Supporting materials (e.g., the proposed test method protocol) in appendices

II. Evaluation of ER TA Test Methods

- Essential test method components are found in *ICCVAM Evaluation of In Vitro Test Methods for Detecting Potential Endocrine Disruptors* (NIH Pub. No. 03-4503)
  - o Test Methods Should Be Able to Detect Both Agonist and Antagonist Activity
    - Substances that test negative for agonism may have antagonist activity and should be tested for antagonism
  - o Essential Test Method Components:
    - Reference estrogen - 17ß-estradiol should be used to generate a full dose response curve for each study
    - Preferred solvent is water, ethanol, or DMSO
    - Solvent should be no more than 1% of reaction mixture
    - In the absence of limited concentrations due to solubility or cytotoxicity, maximum test substance concentration should be 1mM

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II. Evaluation of ER TA Test Methods (cont.)

- o Essential Test Method Components (cont.):
  - Seven test substance concentrations should be used spaced at log intervals up to the limit dose
  - Evaluation of cytotoxicity should be included in each study
  - Concurrent solvent controls should be included in each study
  - A positive control with maximal response two to three orders of magnitude lower than reference estrogen should be included in each study
  - All concentration levels of reference estrogen, controls, and test substance should be tested in triplicate
  - The preferred method for analysis of data is calculation of EC_{50} values for agonists and IC_{50} values for antagonists
  - Studies should be GLP compliant
  - Response for reference estrogen and positive controls should be within appropriate historical acceptance range

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II. Evaluation of ER TA Test Methods (cont.)

- Essential Test Method Components (cont.):
  - Test substance is considered an agonist if response is increased significantly above concurrent solvent control
  - Test substance is considered an antagonist if it causes a significant decrease in the ability of reference estrogen to induce a response
  - Test substance is considered negative if it does not induce a response above solvent control or does not reduce response of reference estrogen after testing to the limit dose or maximum concentration possible based on solubility and cytotoxicity
  - Repeat studies should be used to adequately demonstrate intralaboratory repeatability and reproducibility
  - The study report should include detailed information about the test method (e.g., reference estrogen, solvent, positive control, cell line, study conditions), test substances and include discussion of results and a conclusion

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II. Evaluation of ER TA Test Methods (cont.)

- ICCVAM recommended substances* for validation of *In-Vitro* ER and AR binding and TA test methods
  - Reference substances were selected to:
    - Cover the widest possible range of predicted responses
      - 25% of total substances should be negative
    - Allow determination of test method accuracy
    - Have well defined chemical structures
    - Be readily commercially available
    - Not be associated with excessive hazard or prohibitive disposal costs
  - 53 minimum ER substances
  - 44 minimum AR substances
  - Data on 78 total substances for all methods
- (*note - list of substances with activities and chemical/physical properties is provided in Appendix B of "Addendum to ICCVAM Evaluation of *In Vitro* Test Methods for Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays")

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II. Evaluation of ER TA Test Methods (cont.)

- Criteria for consideration for validation studies:
  - o Reliability (repeatability and intra- and inter-laboratory reproducibility)
  - o The accuracy of the test method for detecting agonist and/or antagonist activity

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III. Revisions to Recommended ED Substance List

- Pricing and commercial availability were made current for 2005.
  - o Four substances were not commercially available and were replaced with available substances that have similar ER and AR activity
  - o Two substances were considered too costly and were replaced with less costly substances that have similar ER and AR activity

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III. Replacement ED Substances from ICCVAM List of 78

Not Commercially Available

Status	*Substance	Action	Min.[a] List for ER	Min.[a] List for AR	EPA List	ER Binding Activity[b]	ER Agonist Activity[c]	ER Antag.[d] Activity[e]	AR Binding Activity[b]	AR Agonist Activity[c]	AR Antag.[d] Activity[e]	Total Cost Per 500 mg
Original	Anastrozole	Aromatase Inhibitor								-		-
Replacement	*4-OH Androstenedione	Aromatase Inhibitor			yes	+	-		+++			$53
Original	CGS 18320B	Aromatase Inhibitor										-
Replacement	Chrysin	Aromatase Inhibitor			yes							$60
Original	Fadrozole	Aromatase Inhibitor	yes	yes								-
Replacement	*Dicofol	Aromatase Inhibitor			yes		+	-				$88
Original	ICI 182,780	ER Antagonist	yes			+++	-	###				Limited to 100 mg/yr
Replacement	*Raloxifene HCl[f]	ER Antagonist			yes	+++	-	###				$235

Excessive Cost

Status	*Substance	Action	Min.[a] List for ER	Min.[a] List for AR	EPA List	ER Binding Activity[b]	ER Agonist Activity[c]	ER Antag.[d] Activity[e]	AR Binding Activity[b]	AR Agonist Activity[c]	AR Antag.[d] Activity[e]	Total Cost Per 500 mg
Original	Methyltrienolone	AR Agonist		yes	yes		-		+++	+++		$15,500
Replacement	*19-Norestosterone	AR Agonist				++	+/-		+++	+++		$90
Original	Zearalenone	ER Agonist	yes			+++	++	#		-		$2,760
Replacement	Resveratrol	ER Agonist				+	++	#			#	$226

Footnotes for this table are available on slide number 14

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III. Footnotes for Slide 13

* on original ICCVAM list of 122.

[a] Min. = Minimum

[b] +++ Indicates that the substance was strongly active as measured by the relative binding affinity (RBA) (RBA value was >1); ++ indicates that the substance was moderately active (RBA value was between 1 and 0.01); + indicates that the substance was weakly active (RBA value was < than 0.01); - indicates that RBA value could not be determined; ± indicates an equivocal response (i.e., in different studies, the substance was reported as positive and negative).

[c] +++ Indicates that the substance was strongly active (half maximal effective concentration [EC_{50}] value was <0.001 mM); ++ indicates that the substance was moderately active (EC_{50} value was between 0.001 and 0.1mM: + indicates that the substance was weakly active (EC_{50} value was >0.1 mM); +/- indicates that the substance was weakly active or negative in different assays.

[d] Antag. is Antagonist

[e] ### 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.

[f] Unlike ICI 182,780, Raloxifene may show agonism in some *in vitro* systems.

- indicates that the substance was uniformly negative in all assays.

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III. Revisions to Recommended ED Substance List (cont.)

- Product class information was changed to reflect information found in the NLM's Hazardous Substances Database (HSDB) and in published literature.
- Chemical class information changed to reflect Medical Subject Headings (MeSH) utilized by the National Library of Medicine (NLM).
  - o Original NICEATM chemical classifications assigned 73 separate chemical classes for 78 substances
  - o Current MeSH chemical class assignments yield 23 classes for 78 substances

III. Evaluation of Minimum Substances Lists

- Examined substances in the same chemical class
  - o Identified those that have similar physical/chemical and activity profiles
  - o Examined those for qualities precluding removal
  - o Considered removing appropriate matches from minimum list, but retain in complete list of 78
- Identified 7 ER & 4 AR substances that met these criteria
- Reducing the minimum lists by this small subset of substances would not significantly reduce resources needed to conduct reliability assessment
- Minimum list remains unchanged

IV. Current NICEATM Test Method Evaluation Activities

- Standardization of XDS LUMI®-CELL ER TA agonist and antagonist test method
  - o Standardization of test method protocols
  - o Testing intra-laboratory repeatability by testing of recommended ER minimum substances (8 for agonism and 8 for antagonism, all coded)
- Evaluation of other ER TA test methods for validation studies or peer review
  - o Certichem ER TA Submission
    - Does submission:
      - Follow ICCVAM guidelines
      - Address ICCVAM prioritization criteria
      - Contain essential test method components
      - Use recommended substances
      - Show adequate performance (reliability and accuracy) to warrant consideration for acceptance or further validation studies

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IV. Current NICEATM Test Method Evaluation Activities (cont.)

- Evaluation of other ER TA test methods for validation studies or peer review (cont.)
  - o CERI ER TA Submission to OECD VMG-NA
    - Tested 46 substances from ICCVAM Reference Substances list to establish concordance with ICCVAM data
      - 34 of those substances were from the ER minimum list
      - 12 substances were from the list of 78
    - Did not address ICCVAM prioritization criteria
    - Partially followed ICCVAM submission guidelines:
      - Did not address whether the test method is warranted, based on the extent of expected use or application and impact on human, animal or ecological health
      - Did not contain an assessment of animal welfare considerations (refinement, reduction, and replacement)
      - Did not address practical considerations (e.g., study costs, time needed to perform a study)
      - Did not contain a comprehensive list of references and supporting materials

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IV. Current NICEATM Test Method Evaluation Activities (cont.)

- Partially contained essential test method components:
  - Did not contain antagonist data
  - Did not include an E2 reference standard curve (concentrations from 1 pM to 1 μM) in all studies.
  - Did not test substance concentrations to 1 mM limit dose (tested to 10 μM)
  - Did not run positive controls in addition to E2
  - Ran 10 substances for the assessment of interlaboratory reproducibility
  - Reported some data as positive or negative for agonism, or as PC_{10} or PC_{50} values, rather than EC_{50} values or fold induction.
- Showed adequate performance (reliability and accuracy) to warrant consideration for further development toward validation studies

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V. Phased Validation Testing Scheme

- Phase I - Laboratory qualification/Protocol refinement
  - Test reference standards, positive controls and solvent controls in 3 laboratories
- Phase II - Limited testing of protocol and protocol refinement
  - Phase IIa (4 substances from ER or AR minimum list) - Initial evaluation of intra- and inter-laboratory reproducibility and accuracy in 3 laboratories
  - Phase IIb (8 substances from ER or AR minimum list) - Further evaluation of intra- and inter-laboratory reproducibility and accuracy in 3 laboratories

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V. Phased Validation Testing Scheme (cont.)

- Phase III - Expanded testing with final standard protocol
  - o Test remainder of substances on ER (41) or AR (32) Minimum List once in each of 3 laboratories
- Phase IV - Testing the remainder of the 78 substances on the ICCVAM ED List tested once in 1 laboratory

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VI. Submission and Evaluation of LUMI-CELL® Test Method

- NICEATM authored Federal Register notice (April 2004) inviting the nomination of *in vitro* test methods for the detection of potential endocrine disruptors
- Received submission from XDS nominating LUMI-CELL® ER TA test method
- NICEATM evaluated submission to determine if nomination addressed ICCVAM:
  - o Submission guidelines
  - o Prioritization criteria
  - o Recommendations for standardization and validation of *in vitro* ED test methods

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VI. LUMI-CELL® ER TA Test Method

- LUMI-CELL® test method is based on a stable recombinant cell line (BG1Luc4E2)
  - o BG1 - human ovarian carcinoma cell that expresses endogenous alpha (95%) and beta (5%) estrogen receptors
  - o 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
  - o Exposure to estrogenic substances causes activation of ERE, which drives transcription of luciferase
  - o Luminometer is used to quantify luciferase expression

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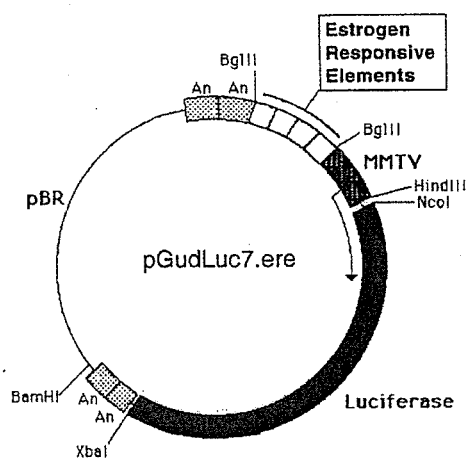
VI. LUMI-CELL® ER-TA Plasmid Construct



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VI. LUMI-CELL® Submission Data

Compound	CAS Number	EC 50 Value	Relative Induction to β-Estradiol	Relative Induction to highest Pesticide
ß-Estradiol *	50-28-2	1.92E-11 ± 1.37E-12	1.00E-00	-
α-Chlordane	12789-03-6	8.92E-07 ± 2.20E-07	2.15E-05	1.0000
DDD	72-54-8	1.92E-06 ± 3.63E-07	9.99E-06	0.4643
Kepone *	143-50-0	2.00E-06 ± 3.97E-07	9.61E-06	0.4465
pp' DDT *	789-02-6	2.95E-06 ± 3.49E-07	6.50E-06	0.3021
Metoxychlor *	72-43-5	3.53E-06 ± 6.10E-07	5.43E-06	0.2524
γ-Chlordane	57-74-9	4.86E-06 ± 1.67E-06	3.95E-06	0.1835
pp' DDE *	72-55-9	5.69E-06 ± 1.89E-06	3.37E-06	0.1567
Fenarimol *	60168-88-9	8.15E-06 ± 1.26E-06	2.36E-06	0.1095
Dieldrin	60-57-1	8.18E-06 ± 9.65E-07	2.34E-06	0.1089
Linuron *	330-55-2	1.26E-05 ± 5.28E-06	1.53E-06	0.0711
2,4,5-Trichlorophenoxyacetic Acid *	93-76-5	1.30E-05 ± 2.60E-06	1.48E-06	0.0687
Mirex	2385-85-5	Non-active	N/A	N/A
Vinclozolin *	50471-44-8	Non-active	N/A	N/A
Progesterone *	57-83-0	Non-active	N/A	N/A

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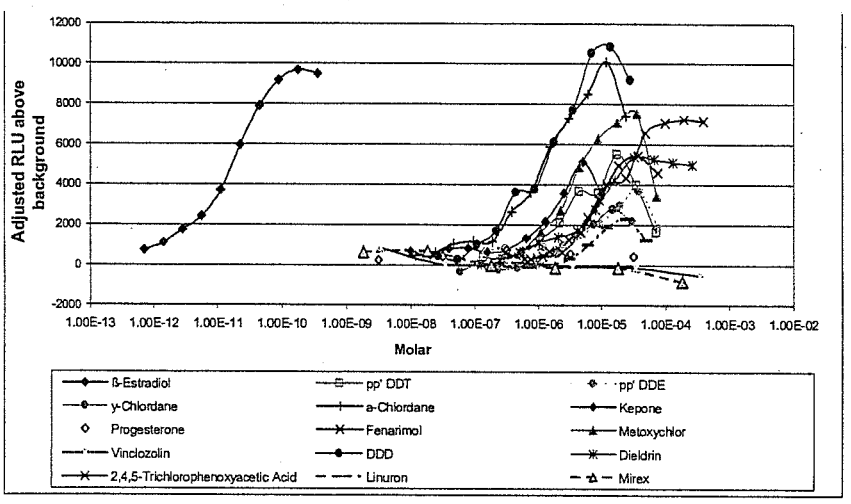
VI. LUMI-CELL® Submission Data



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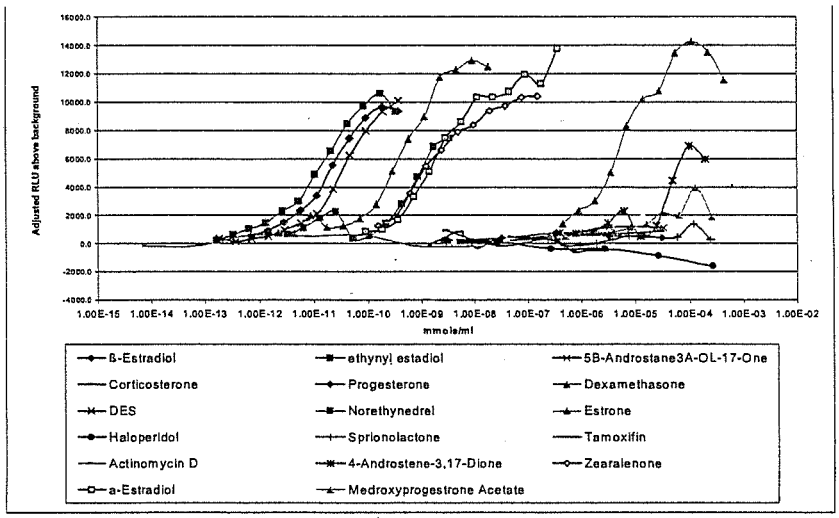
VI. LUMI-CELL® Submission Data



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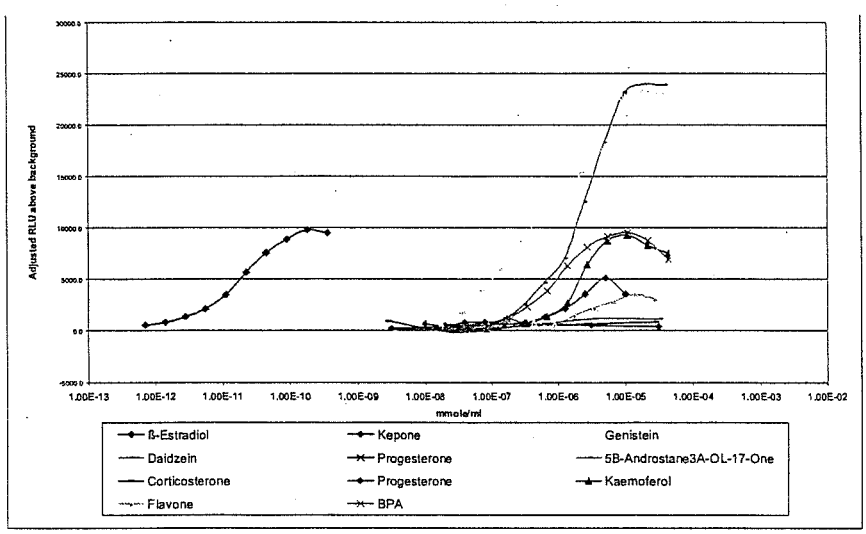
VI. LUMI-CELL® Submission Data



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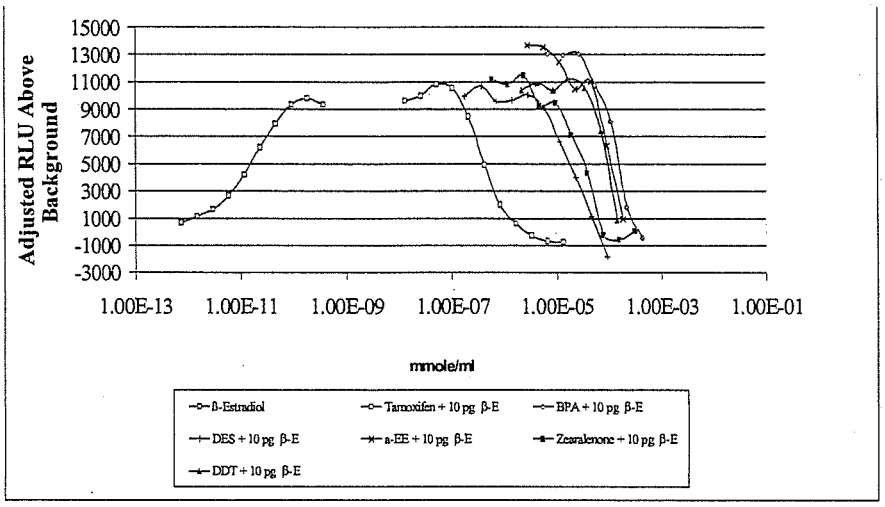
VI. LUMI-CELL® Submission Data



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VI. Evaluation of LUMI-CELL® Test Method

- Satisfied submission guidelines
- Satisfied prioritization criteria
- Satisfied most recommendations for:
  - o Essential test method components
    - Used DMSO versus water or ethanol
    - Used limit dose of 0.1mM instead of 1mM, however, there were no unexpected negatives at this lower concentration
  - o Validation substances
    - Tested 56 of 78 ICCVAM recommended substances
  - o Reliability
    - Well to well variability within 96 well plates was low
    - Plate to plate (intralaboratory) variability was low
- Showed adequate performance (reliability and accuracy) to warrant consideration for further development toward validation studies

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VI. NICEATM/ICCVAM Recommendations for LUMI-CELL® Nomination

- LUMI-CELL® to be considered a high priority for validation studies as a test method for detecting substances for ER agonist and antagonist activity
- XDS to conduct additional pre-validation antagonist studies
- NICEATM to sponsor and manage XDS protocol standardization study (October '05 to July '06)
- ICCVAM International Validation Studies (to begin July '06).
  - Study Management Team
    - NICEATM, JaCVAM, ECVAM
    - NICEATM: Responsible for validation study coordination and substance distribution

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VI. XDS Standardization Study Timeline

STANDARDIZATION TIMELINE	WEEK
Award of Contract	0
Program plate reader and Excel spreadsheet.	1
Begin plate design and historical dat abase	2
Complete plate design ph ase.	7
Compare cell viability assays and establish historical datab ase	8
Complete cell viability and historical datab ase dat a collection	17
Complete GLP-compliant protocols for testing receptor agonists and ant agonists	17
Begin Range Finding for 8 agonist and 8 ant agonist chemicals	18
Complete Range Finding for 8 agonists and 8 ant agonists	20
Begin testing of 8 agonists and 8 ant agonists for protocol standardization	21
Complete testing of 8 agonists and 8 antagonists for protocol standardization	38
Proposed Fin al GLP -compliant protocols for testing receptor agonists and ant agonists	39

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VI. XDS Staff

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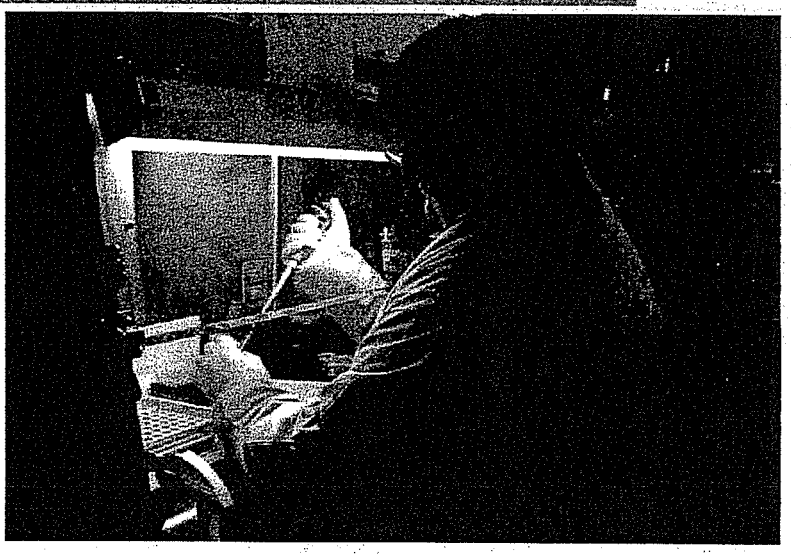
VI. Dr. Gordon in XDS Tissue culture lab



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