NICEATM/ECVAM/JaCVAM Multi-phased International Validation Study of an *In Vitro* Estrogen Receptor Transcriptional Activation Assay to Detect Agonist and Antagonist Activity

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Introduction

The proposed U.S. EPA Tier 1 endocrine disruptor screening program (EDSP) (EPA 1998) includes validated *in vitro* test methods to determine if chemicals interact with the estrogen receptor (ER). The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) developed recommendations that include essential test method components (referred to as "minimum procedural standards" in the report) and a list of 78 reference substances that should be used to standardize and validate *in vitro* ER binding and transcriptional activation (TA) test methods (ICCVAM, 2003). A *U.S. Federal Register Notice* (*FR* notice) by ICCVAM requested nomination of *in vitro* ER binding and TA test methods for validation studies (*FR* Vol. 68, No. 106, pp. 33171-33172, 3 June 2003). In response, a stably transfected ER TA assay (LUMI-CELL® ER) developed by Xenobiotic Detection Systems, Inc. (XDS) to detect *in vitro* ER agonist and antagonist activity was nominated. An ICCVAM pre-screen evaluation of the XDS background review document supporting the nomination resulted in a ICCVAM recommendation that it should be a high priority for validation studies.

In preparation for the validation study, NICEATM conducted a protocol standardization study for the detection of ER agonists and antagonists using the LUMI-CELL® ER assay. ICCVAM-recommended essential test method components (ICCVAM, 2003) were incorporated into the protocols and the intralaboratory reproducibility and accuracy of the standardized protocols were evaluated using a representative subset of the recommended reference substances (ICCVAM, 2003, 2006).

Based on the results obtained in the protocol standardization study, NICEATM, ECVAM, and JaCVAM designed and initiated a collaborative international validation study using three laboratories (one each in Japan, the United States, and Europe) to evaluate the reproducibility and accuracy of the LUMI-CELL® ER assay for detecting ER agonists and antagonists. The validation study will evaluate the 78 reference substances recommended by ICCVAM for validation of *in vitro* ER test methods (see **Table 1**) (ICCVAM, 2006). The study will proceed in four phases (see **Flowchart**) and is being conducted according to Good Laboratory Practices (EPA 2001, 2002; FDA 2002; OECD 1998). Phase I will focus on the transferability of the protocols developed during the standardization study by establishing and comparing a historical control database in each laboratory. Positive and vehicle controls for the ER agonist and antagonist protocols will be evaluated and test acceptance criteria established for each laboratory. Phase II will evaluate 12 coded reference substances selected from the ICCVAM recommended minimum list of 53 reference substances, with each substance tested three times, in each laboratory in two stages. Intra- and inter- laboratory reproducibility and accuracy for agonist and antagonist detection will be assessed during and after each of the first two phases. Excessive variation and discordance will be investigated and protocols modified accordingly. Optimized final test method agonist and antagonist protocols will be used for Phases III and IV. Phase III will evaluate the performance (accuracy and reliability) of the optimized test method protocols using the remaining coded 41 minimum validation substances (each compound tested once for agonist or antagonist activity in each laboratory). The final phase (Phase IV) will test the remaining 25 substances on the ICCVAM list of 78 reference substances, each substance tested once for ER agonist or antagonist activity in a single laboratory.

Study Phases and Activities

Phase I: Initial Laboratory Qualification

(Development of Historical Database for Each Laboratory)

- Initial qualification of laboratories by testing agonist and antagonist reference standards and controls
- Establish individual laboratory historical database for standards and controls by conducting independent experiments (10 each for the agonist and antagonist protocols)
- Establish initial test acceptance criteria for each lab based on historical database
- Evaluate test method repeatability and reproducibility
- If necessary, modify test method protocols to reduce intra- and/or inter-laboratory variability
- Repeat testing if major protocol changes are required

Phase IIa: Laboratory Qualification/Protocol Optimization Phase

(Limited Substance Testing, Possible Protocol Modification)

- At each laboratory, four substances from the ER minimum list tested independently three times for agonist and antagonist activity
- Evaluate accuracy and reliability
- If necessary, modify test method protocols to reduce variability and/or to improve accuracy
- Repeat testing if major protocol changes are required

Phase IIb: Laboratory Qualification/Protocol Optimization Phase

(Additional Substance Testing/Evaluation of Protocol Modifications)

- At each laboratory, eight substances from ER minimum list tested independently three times for agonist and antagonist activity
- Evaluate accuracy and reliability
- If necessary, modify test method protocols to reduce variability and/or to improve accuracy
- Repeat testing if major protocol changes are required
- Finalize optimized test method protocols for use in Phases III and IV

Phase III: Laboratory Validation Testing Phase

- At each laboratory, 41 coded substances are tested once for agonist and antagonist activity using the final optimized protocols
- Data used to evaluate overall test method performance

Phase IV: Expansion of Validation Database Using Additional Reference Substances

- In a single qualified laboratory, the 25 remaining coded substances are tested once for agonist and antagonist activity using the final optimized protocols
- Data used to further characterize test method accuracy

NIEHS National Institute of Environmental Health Sciences

ICCVAM The Interagency Coordinating Committee on the Validation of Alternative Methods

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

More information on ICCVAM and NICEATM can be accessed at http://iccvam.niehs.nih.gov/

Table 1 ICCVAM Recommended Substances for Interlaboratory Validation of ER Test Methods

Substance	CASRN[a]	Agonist Activity[b]	Antagonist Activity[c]	MeSH[d] Chemical Class
Subset of Substances Listed on the Minimum Lists for ER Binding and TA Test Methods				
Actinomycin D	50-76-0	-[e]	-[e]	Heterocyclic Compound, Polycyclic Compound
4-Androstenedione	63-05-8	+[f]	-[e]	Steroid
Apigenin	520-36-5	+++	+/-	Heterocyclic Compound
Atrazine	1912-24-9	-	-[e]	Heterocyclic Compound
Bisphenol A	80-05-7	+	+/-	Phenol
Bisphenol B	77-40-7	++	-[e]	Phenol
2-*sec*-Butylphenol	89-72-5	-[e]	-[e]	Phenol
Clomiphene citrate	50-41-9	+[f]	+[f]	Amine, Carboxylic Acid, Heterocyclic Compound
Corticosterone	50-22-6	-	+	Steroid
Coumestrol	479-13-0	++	-	Heterocyclic Compound
4-Cumylphenol	599-64-4	+	-[e]	Phenol
Daidzein	486-66-8	+	-	Flavonoid, Heterocyclic Compound
p,p'-DDE[g]	72-55-9	+	-	Hydrocarbon (Halogenated)
o,p'-DDT[h]	789-02-6	+	-[e]	Hydrocarbon (Halogenated)
Dexamethasone	50-02-2	+/-	-[e]	Steroid
Dibenzo[*a,h*]-anthracene	53-70-3	-	+	Polycyclic Compound
Dicofol	115-32-2	+	-	Hydrocarbon (Cyclic), Hydrocarbon (Halogenated)
Diethylstilbestrol	56-53-1	+++	-	Hydrocarbon (Cyclic)
5α-Dihydro testosterone	521-18-6	++	-[e]	Steroid
17α-Estradiol	57-91-0	++	-[e]	Steroid
17α-Ethinyl estradiol	57-63-6	+++	-	Steroid
17ß-Estradiol	50-28-2	+++	-[e]	Steroid
Estrone	53-16-7	+++	-	Steroid
Flavone	525-82-6	+/-	+	Flavonoid, Heterocyclic Compound
Fluoranthene	206-44-0	-	-	Polycyclic Compound
Genistein	446-72-0	+	+	Flavonoid, Heterocyclic Compound
meso-Hexestrol	84-16-2	+++	-[e]	Steroid
Hydroxyflutamide	52806-53-8	-[e]	-[e]	Amide
4-Hydroxytamoxifen	68047-06-3	+/-	+++	Hydrocarbon (Cyclic)
Kaempferol	520-18-3	+	+	Flavonoid, Heterocyclic Compound
Kepone	143-50-0	+	-[e]	Hydrocarbon (Halogenated)
p,p'- Methoxychlor	72-43-5	+	-	Hydrocarbon (Halogenated)
Morin	480-16-0	+	-[e]	Flavonoid Heterocyclic Compound
p-n-Nonylphenol	104-40-5	++	+/-	Phenol
Norethynodrel	68-23-5	+++	-[e]	Steroid
4-*tert*-Octylphenol	140-66-9	++	-[e]	Phenol
Ethyl paraben	120-47-8	+	-[e]	Carboxylic Acid, Phenol
Phenobarbital	50-06-6	-	-[e]	Heterocyclic Compound, Pyrimidine
Phenolphtalin	81-90-3	-[e]	-[e]	Carboxylic Acid, Phenol
Butylbenzyl phthalate	85-68-7	++	-	Carboxylic Acid, Phthalic Acid
Diethylhexyl phthalate	117-81-7	+/-	-[e]	Phthalic Acid
Di-*n*-butyl phthalate	84-74-2	+	-	Ester, Phthalic Acid
Progesterone	57-83-0	-	+	Steroid
Propylthiouracil	51-52-5	-[e]	-[e]	Heterocyclic Compound, Pyrimidine
Raloxifene HCl	82640-04-8	+	+++	Hydrocarbon (Cyclic)
Resveratrol	501-36-0	++	+	Hydrocarbon (Cyclic)
Sodium azide	26628-22-8	-[e]	-[e]	Azide, Salt (Inorganic)
Tamoxifen	10540-29-1	+/-	+++	Hydrocarbon (Cyclic)
Testosterone	58-22-0	+	-[e]	Steroid
Methyl testosterone	58-18-4	+	-[e]	Steroid
12-*O*-Tetradecaonyl phobol-13-acetate	16561-29-8	-[e]	-[e]	Hydrocarbon (Cyclic)
2,4,5-Trichloro-phenoxyacetic acid	93-76-5	+	+	Carboxylic Acid
Vinclozolin	50471-44-8	+	+	Heterocyclic Compound
25 Additional ICCVAM Recommended Substances for Validation of *In Vitro* ER Binding and TA Test Methods				
Ammonium perchlorate	7790-98-9	-[e]	-[e]	Amine, Onium Compound
4-OH Androstenedione	566-48-3	+/-	-[e]	Steroid
Apomorphine	58-00-4	-[e]	-[e]	Heterocyclic Compound
Bicalutamide	90357-06-5	-[e]	-[e]	Amide
Chrysin	480-40-0	+	+	Flavonoid, Heterocyclic Compound
Cyclohexamide	66-81-9	-[e]	-[e]	Heterocyclic Compound
Cyproterone acetate	427-51-0	+	-[e]	Steroid
Fenarimol	60168-88-9	+	+	Heterocyclic Compound, Pyrimidine
Finasteride	98319-26-7	-[e]	-[e]	Steroid
Fluoxymestrone	76-43-7	-	-	Steroid
Flutamide	13311-84-7	-	-[e]	Amide
Haloperidol	52-86-8	-[e]	-[e]	Ketone
Ketoconazole	65277-42-1	-[e]	-[e]	Heterocyclic Compound
Linuron	330-55-2	-	-[e]	Urea
Medroxyprogesterone acetate	71-58-9	+	-[e]	Steroid
Mifepristone	84371-65-3	+	-[e]	Steroid
Nilutamide	63612-50-0	-[e]	-[e]	Heterocyclic Compound, Imidazole
19-Nortestosterone	434-22-0	+/-	-[e]	Steroid
Oxazepam	604-75-1	-[e]	-[e]	Heterocyclic Compound
Pimozide	2062-78-4	-[e]	-[e]	Heterocyclic Compound
Procymidone	32809-16-8	-[e]	-[e]	Polycyclic Compound
Reserpine	50-55-5	-[e]	-[e]	Heterocyclic Compound, Indole
Spironolactone	52-01-7	-[e]	-[e]	Lactone, Steroid
L-Thyroxine	51-48-9	+	-[e]	Amino Acid
17ß-Trenbolone	10161-33-8	+	-[e]	Steroid

[a]CASRN = Chemical Abstracts Service Registry Number

[b]+++ = substance was strongly active (half maximal effective concentration [EC_{50}] value was <0.001 µM); ++ = substance was moderately active (EC_{50} value was between 0.001 and 0.1 µM); + = substance was weakly active (EC_{50} value was >0.1 µM); +/- = substance was weakly active or negative in different assays; - = substance was negative.

[c]+++ = substance was strongly active (concentration inhibiting reference estrogen or androgen response by 50% [IC_{50}] value was <0.001 µM); + = substance was weakly active (IC_{50} value was >0.1 µM); +/- = substance was weakly active or negative in different assays; - = substance was negative.

[d]MeSH = Medical Subject Headings, information on chemical class criteria can be obtained at www.nlm.nih.gov/MeSH

[e]Represents substances that have no relevant quantitative receptor binding or TA data available for the respective test method but which are presumed negative based on their known mechanism of action, or their responses in other endocrine disruptor screening test methods.

[f]Represents substances that have no relevant quantitative receptor binding or TA data available for the respective test method but which are presumed positive based on their known mechanism of action or their responses in other endocrine disruptor screening test methods.

[g]*p,p'*-DDE = 1,1-dichloro-bis[4-chlorophenly]ethylene

[h]*o,p'*-DDT = 1,1,1-Trichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl)ethane

Overview of the LUMI-CELL® ER Assay

1. Cells are cultured in estrogen-free tissue culture medium for 24 hours prior to seeding onto 96-well plates to form a sub-confluent monolayer
2. Culture medium is removed and cells are exposed to test substance for 24 hours
3. Treatment medium is removed; cells are washed with phosphate buffered saline
4. Cells are evaluated microscopically for morphological alterations and cell density
5. Cells are incubated for one minute in lysis reagent while being shaken on an orbital shaker.
6. Plates are placed in a luminometer that injects luciferase substrate into each well immediately prior to measuring luminescence at 300 to 650 nm
7. Reference standard and control data are evaluated to determine whether the experiment has met acceptance criteria. If an experiment does not meet acceptance criteria, data from the experiment is not used to assess estrogenic activity and the experiment is repeated
8. Data is transferred into GraphPad PRISM® 4.0 statistical software and evaluated for positive or negative response

Figure 1 Proposed Agonist Test Plate Layout

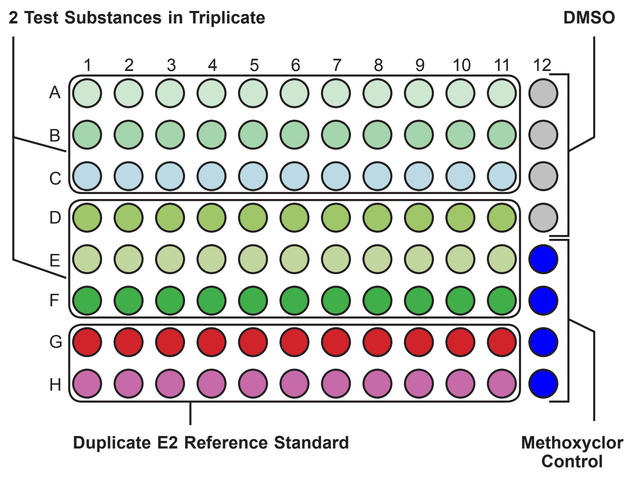
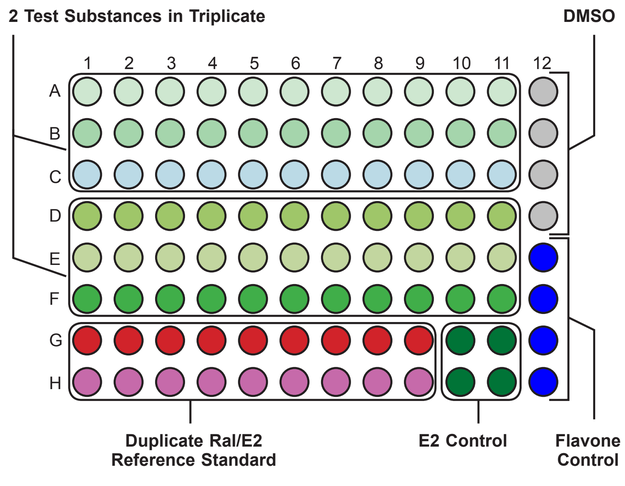


Figure 2 Proposed Antagonist Test Plate Layout



Post-Validation Evaluation and Peer Review Process

After completion of the validation study, results and analyses will be compiled in a draft Background Review Document (BRD). ICCVAM will use this BRD as a reference when developing draft recommendations on proposed test method usefulness and limitations, test method protocols, performance standards, and other future studies that might be determined to be useful. Performance standards will serve as the basis for determining if similar ER TA methods have equal or better performance. The draft BRD and draft ICCVAM recommendations will be made available to the public and to an independent scientific peer review panel. The peer review panel will meet in public session to review the validation status of the test method, and to comment on the extent that ICCVAM draft recommendations are supported by the validation database. The independent peer review panel report will be made available to the public and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) for comments. ICCVAM will consider the peer review panel report along with public and SACATM comments when preparing a Test Method Evaluation Report (TMER) that will contain final ICCVAM test method recommendations. The TMER and the supporting BRD will be forwarded to U.S. Federal agencies for acceptance decisions in accordance with provisions of the ICCVAM Authorization Act of 2000.

This multi-phased approach is expected to identify and resolve sources of variation early in the validation process and to generate a highly reproducible test method protocol for international regulatory use.

Acknowledgements

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