NICEATM/ECVAM/JaCVAM Multi-phased International Validation Study of a Stably-Transfected Estrogen Receptor (ER) Transcriptional Activation (TA) Test Method

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Abstract

The U.S. EPA proposed Tier 1 endocrine disruptor screening program (EDSP) (EPA 1998) includes validated *in vitro* test methods to determine if chemicals interact with the ER. A stably transfected ER TA method (LUMI-CELL® ER, Xenobiotic Detection Systems, Inc.) to detect ER agonists and antagonists was subsequently nominated to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and recommended with high priority for validation. NICEATM, ECVAM, and JaCVAM designed and initiated a four phase international validation study to evaluate the reproducibility and accuracy of the LUMI-CELL® ER bioassay. The study will use three laboratories, one each in Japan, the United States, and Europe. A standardized test method protocol incorporating ICCVAM recommended ER TA essential test method components is being used to evaluate 78 coded reference substances recommended by ICCVAM for validation of *in vitro* ER test methods (ICCVAM 2006). The first phase will evaluate positive and vehicle controls and establish test acceptance standards for each laboratory. The second phase will evaluate 12 coded reference substances in each laboratory in two stages. Intra- and inter laboratory reproducibility and accuracy will be assessed during and after each of the first two phases. Excessive variation and discordance will be investigated and protocols modified accordingly. The third phase will evaluate the performance of the optimized test method protocol using the remaining coded 41 minimum validation substances. The final phase will test the remaining 25 substances on the ICCVAM reference substances list in one laboratory. Performance standards will be developed to serve as the basis for determining if similar ER TA methods have comparable or better performance. 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. Supported by NIEHS Contract N01-ES-85424.

Introduction

The proposed U.S. EPA Tier 1 endocrine disruptor screening program (EDSP) (EPA, 1998) will include a validated *in vitro* test method to determine if chemicals interact with the ER. The proposed EDSP states that this *in vitro* test method may be either a receptor binding or TA assay. A stably transfected ER TA assay (LUMI-CELL® ER, Xenobiotic Detection Systems, Inc.) to detect *in vitro* ER agonist and antagonist activity was subsequently nominated to ICCVAM and recommended by ICCVAM for validation studies with a high priority.

NICEATM, ECVAM, and JaCVAM subsequently designed and initiated a collaborative international validation study to evaluate the reproducibility and accuracy of the LUMI-CELL® ER assay using three laboratories, one each in Japan, the United States, and Europe. In preparation for the validation study, NICEATM conducted a protocol standardization study for the ER agonist and antagonist protocols for the Xenobiotic Detection Systems, Inc., LUMI-CELL® ER assay (see 2007 SOT poster #1165-439). The standardized protocols incorporate ICCVAM recommended ER TA essential test method components (ICCVAM, 2003). Based on the results of the standardization study, the revised protocols were determined to have sufficient performance to use as initial protocols for the international validation study.

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 Below) and will be conducted according to Good Laboratory Practices. 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 will be evaluated and test acceptance standards established for each laboratory. Phase II will evaluate 12 coded reference substances in each laboratory in two stages. Intra- and inter- laboratory reproducibility and accuracy will be assessed during and after each of the first two phases. Excessive variation and discordance will be investigated and protocols modified accordingly during Phases I and II. An optimized final test method protocol will be used for Phases III and IV. Phase III will evaluate the performance (accuracy and reliability) of the optimized test method protocol using the remaining coded 41 minimum validation substances. Phases II and III will be restricted to the testing of substances from a subset of 53 substances from the ICCVAM list that have been identified as the minimum that should be used in any validation of an *in vitro* ER TA assay (ER minimum list). The final phase (Phase IV) will test the remaining 25 substances on the ICCVAM list in a single laboratory.

Phases of the International Validation Study

Phase I

Phase I is for initial laboratory qualification and protocol modification. This phase is limited to repeat testing of agonist and antagonist reference standards and controls in order to demonstrate individual laboratory proficiency with the test method. An initial historical control database is generated for each laboratory, and initial test acceptance standards are established using this data. Test results are compared to the acceptance criteria to determine if the individual test meets these criteria and therefore can be considered an acceptable test result. A comparison of quantitative and qualitative methods of assessing cell viability will also be conducted during this phase. The Study Management Team (SMT) will evaluate the repeatability and reproducibility of the reference standard and control data to determine if there is excessive variation within or among laboratories. If so, the SMT will work with respective laboratories to take appropriate steps to reduce variation to acceptable limits, such as modification of protocols and SOPs, further training, and repeat testing as necessary.

Phase IIa

Phase IIa is the second phase of laboratory qualification/ protocol modification using the test method protocol with modifications made in Phase I. It is also the initial phase for testing substances with well-characterized ER activities (one strong positive, one moderate positive, one weak positive and one negative), and with no problematic issues relating to solubility or cytotoxicity. Four coded substances from the ER minimum list will be tested in the LUMI-CELL® ER agonist and antagonist assays. Each coded substance will be tested independently three times at each laboratory. A comparison of quantitative and qualitative methods of assessing cell viability will also be continued during this phase. The SMT will evaluate the repeatability, reproducibility, and accuracy of the four test substances and the reference standard and control data to determine if there is excessive variation or discordance in results within or among laboratories. If so, the SMT will work with respective laboratories to take appropriate steps to reduce variation and to obtain concordant results (e.g., modifying/refining protocols and SOPs, further training and repeating testing as necessary).

Phase IIb

After review of the Phase IIa data and any necessary modifications to protocols and/or re-testing as needed, eight coded substances from the ER minimum list with well-characterized activity (a mix of strong positive, moderately positive, weakly positive, and negative) and with a range of solubility and cytotoxicity (insoluble, cytotoxic) characteristics will be tested in the LUMI-CELL® ER agonist and antagonist assays in Phase IIb. Each coded substance will be tested independently three times at each laboratory. The SMT will again evaluate the repeatability and reproducibility of the eight test substances and the reference standard and control data to determine if there is excessive variation or discordant results within or among laboratories. If so, the SMT will work with respective laboratories to take appropriate steps to reduce variation and to obtain concordant results (e.g., modifying/refining protocols and SOPs, further training and repeating testing as necessary). After review of the Phase IIb data and modifications to protocols and/or re-testing as needed, an optimized test method protocol will be finalized for use in Phases III and IV.

Phase III

The remaining 41 substances from the ER minimum list (blinded/coded) will be tested in the LUMI-CELL® ER agonist and antagonist assays in Phase III using the final optimized test method protocols. Each coded substance will be tested once in each of the three laboratories. The SMT will evaluate the reproducibility of the 41 test substances and the reference standard and control data to determine if additional testing is needed to reconcile discordant results.

Phase IV

The final 25 substances from the ICCVAM list will be tested in both the LUMI-CELL® ER agonist and antagonist assays. Substances will be tested once in one qualified laboratory using the final optimized test method protocols. This will generate high quality data that can be used to further evaluate the accuracy of the test method, and data that can be used to evaluate the performance for detecting endocrine activity of various combinations of *in vitro* and *in vivo* test methods proposed for the EPA EDSP Tier 1 testing battery. This phase will also generate *in vitro* reference data for many substances for which no ER RBA and/or TA data is available.

Study Phases and Activities

Phase I: Initial Laboratory Qualification

(Development of Historical Database for Each Laboratory)

- Initial qualification of laboratories by testing 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
- Modify test method protocol as necessary to reduce intra- and inter-laboratory variation
- Repeat testing if major changes

Phase IIa: Laboratory Qualification/ Protocol Optimization Phase

(Limited Substance Testing, Possible Protocol Modification)

- Four substances from ER minimum list tested independently three times for agonism and antagonism at each laboratory
- Evaluate repeatability, reproducibility, and concordance
- Modify test method protocol as necessary to reduce variation and to achieve concordant results
- Repeat testing if major changes

Phase IIb: Laboratory Qualification/ Protocol Optimization Phase

(Additional Substance Testing/ Evaluation of Protocol Modifications)

- Eight substances from ER minimum list tested independently three times for agonism and antagonism at each laboratory
- Evaluate repeatability, reproducibility, and concordance
- Modify test method protocol as necessary to reduce variation and to achieve concordant results
- Repeat testing if major changes
- Finalize optimized test method protocol for use in Phases III and IV

Phase III: Laboratory Validation Testing Phase

- Each of the three laboratories tests 41 coded test substances for agonism and antagonism once using the final optimized protocols
- Data used to assess test method inter-laboratory reproducibility and concordance/accuracy

Phase IV: Expansion of Validation Database Using Additional Reference Substances

- Test 25 remaining coded test substances for agonism and antagonism once in a single qualified laboratory using the final optimized protocols
- Data used to further characterize test method accuracy

Table 1 ICCVAM Recommended Substances for the Validation of ER Test Methods (ICCVAM, 2006)

Substance	CASRN[a]	Agonist Activity[b]	Antagonist Activity[c]	MeSH 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	-	-[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	72-55-9	+	-	Hydrocarbon (Halogenated)
o,p'-DDT	789-02-6	+	-[e]	Hydrocarbon (Halogenated)
Dexamethasone	50-02-2	+/-	-[e]	Steroid
Dibenzo[*a,h*]-anthracene	53-70-3	-	-[e]	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]	-[e]	Flavonoid Heterocyclic Compound
p-n-Nonylphenol	104-40-5	++	+[f]	Phenol
Norethynodrel	68-23-5	+[f]	-[e]	Steroid
4-*tert*-Octylphenol	140-66-9	++	-[e]	Phenol
Ethyl paraben	120-47-8	+[f]	-[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*-Tetradecaoyl phobol-13-acetate	16561-29-8	-[e]	-[e]	Hydrocarbon (Cyclic)
2,4,5-Trichloro-phenoxyacetic acid	93-76-5	+	-[e]	Carboxylic Acid
Vinclozolin	50471-44-8	-	-[e]	Heterocyclic Compound
25 Additional ICCVAM Recommended Substances for Evaluation in *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]	-[e]	Steroid
Apomorphine	58-00-4	-[e]	-[e]	Heterocyclic Compound
Bicalutamide	90357-06-5	-[e]	-[e]	Amide
Chrysin	480-40-0	-[e]	-[e]	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]	-[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]	-[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 moderately active (IC_{50} value was between 0.001 and 0.1 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 (e.g., crysin, a known aromatase inhibitor, is presumed negative in ER binding and TA assays).

[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.

Major Steps in Performance 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) Test substance data from a successful experiment is analyzed and presented graphically

Figure 1 Proposed Agonist Test Plate Layout

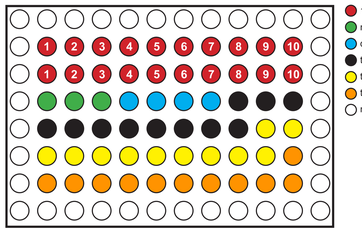
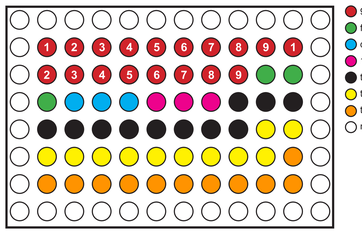


Figure 2 Proposed Antagonist Test Plate Layout



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. ICCVAM will review the BRD and develop draft recommendations on proposed test method usefulness and limitations, test method protocol, 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 comparable 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 and public and SACATM comments, and prepare a Test Method Evaluation Report that will contain its final recommendations. This Report and the supporting BRD will be forwarded to 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.

Acknowledgments

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Current validation study information available at: http://iccvam.niehs.nih.gov/methods/endocrine.htm

NIEHS National Institute of Environmental Health Sciences

U.S. Department of Health and Human Services National Institutes of Health

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/