International Validation Study of an *In Vitro* Cell Proliferation Test Method for Screening Potential Estrogenic Agonists and Antagonists in MCF-7 cells

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Abstract

The MCF-7 Cell Proliferation test method developed by CertiChem Inc. (CCi) uses a human cell line that endogenously expresses estrogen receptor (hER-alpha and hER-beta) to screen for substances that may induce or inhibit cell proliferation via ER-mediated pathways. NICEATM, in collaboration with JaCVAM and KoCVAM, has coordinated an international validation study to evaluate test method accuracy and reliability. Three laboratories (one each in the U.S., Japan, and Korea) are testing ICCVAM recommended reference substances with well-characterized *in vitro* ER data. Initial testing of a subset of these substances was conducted at CCi to refine protocols and demonstrate intralaboratory reproducibility and accuracy of the test method. Multi-phased interlaboratory studies were subsequently initiated at laboratories in Japan and Korea to demonstrate transferability and further evaluate test method accuracy and reliability. Phases 1 and 2 of the interlaboratory study will be used to demonstrate proficiency, establish historical databases, and to evaluate intra- and interlaboratory reproducibility by testing 12 substances in three independent experiments at both laboratories. Phase 3 testing of 14 additional ICCVAM reference substances (tested once at each laboratory) will provide data for a more comprehensive evaluation of interlaboratory reproducibility and accuracy. A final intralaboratory phase is being conducted only at CCi, to complete testing for the entire list of 78 ICCVAM reference substances. Results from this validation study will be used as the basis for ICCVAM recommendations on usefulness and limitations of *in vitro* endocrine disruptor test methods, and to develop performance standards for the expedited validation of functionally and mechanistically similar test methods.

Introduction

- Endocrine disruptors are natural and man-made substances in the environment that interfere with the normal function of hormones in the endocrine system.
- Studies indicating that animal populations exposed to high levels of these substances have an increased incidence of reproductive and developmental abnormalities have raised public health concerns.
- In 2005, CertiChem Inc. (CCi) nominated their *in vitro* MCF-7 cell proliferation test method proposed for screening potential estrogen agonists and antagonists (CCi MCF-7 Assay) to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM).
- The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) coordinated an international validation study with its counterparts in Japan and Korea. Participating laboratories were sponsored by validation organizations in each country: NICEATM, the Japanese Center for the Validation of Alternative Methods (JaCVAM) and the Korean Center for the Validation of Alternative Methods (KoCVAM).
- The participating laboratories include:
  - CCi, Austin, Texas, USA
  - Hiyoshi Corporation, Omihachiman, Japan
  - Korean Food and Drug Administration (KFDA), Cheongwon-gun, Republic of Korea

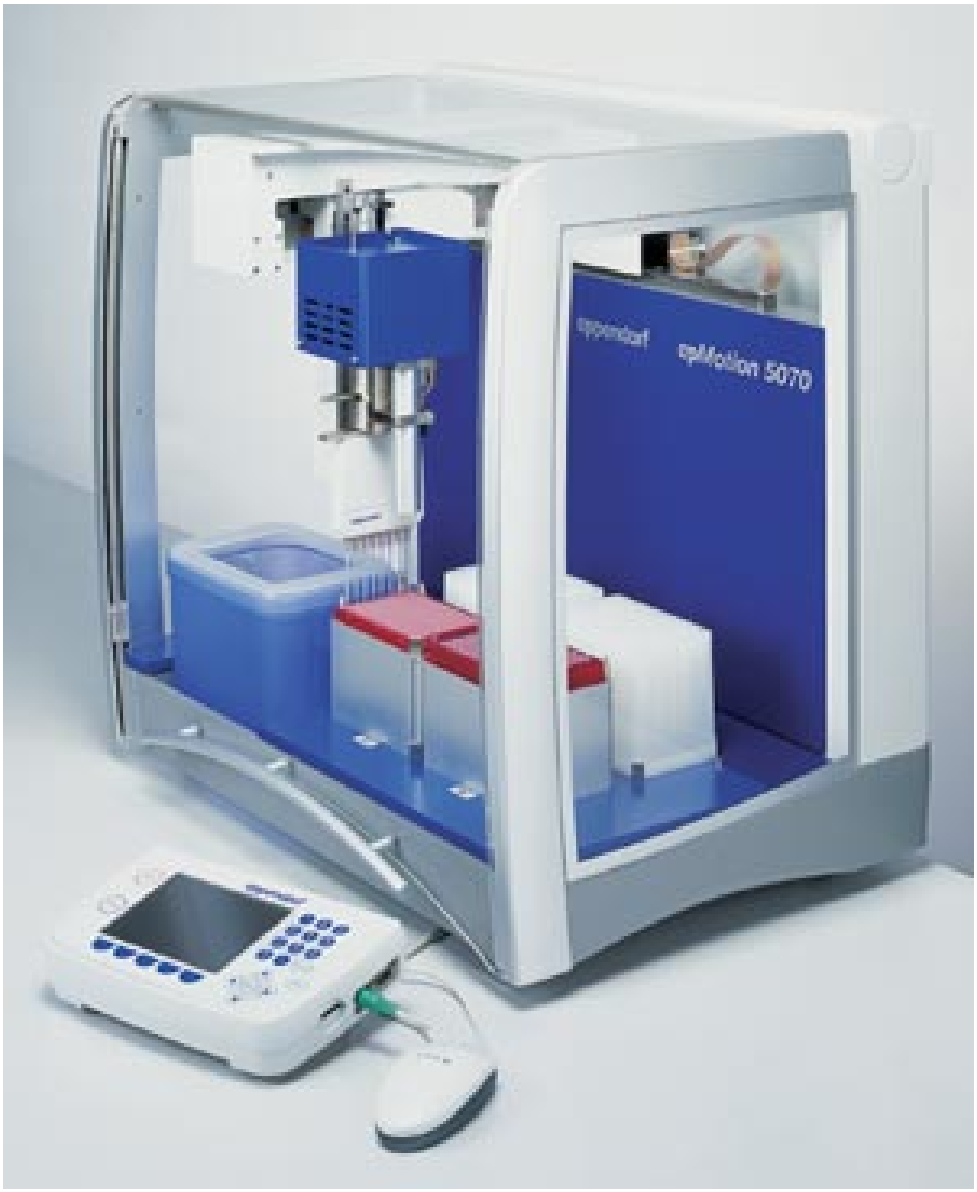
Overview of CCi MCF-7 Cell Proliferation Assay

Basis of Assay

- MCF-7 is a human breast adenocarcinoma cell line that endogenously expresses estrogen receptors (ERα and ERβ).
- The MCF-7 cell proliferation assay measures whether and to what extent a substance induces (ER agonist) or reduces (ER antagonist) cell proliferation via ER dependent pathways.

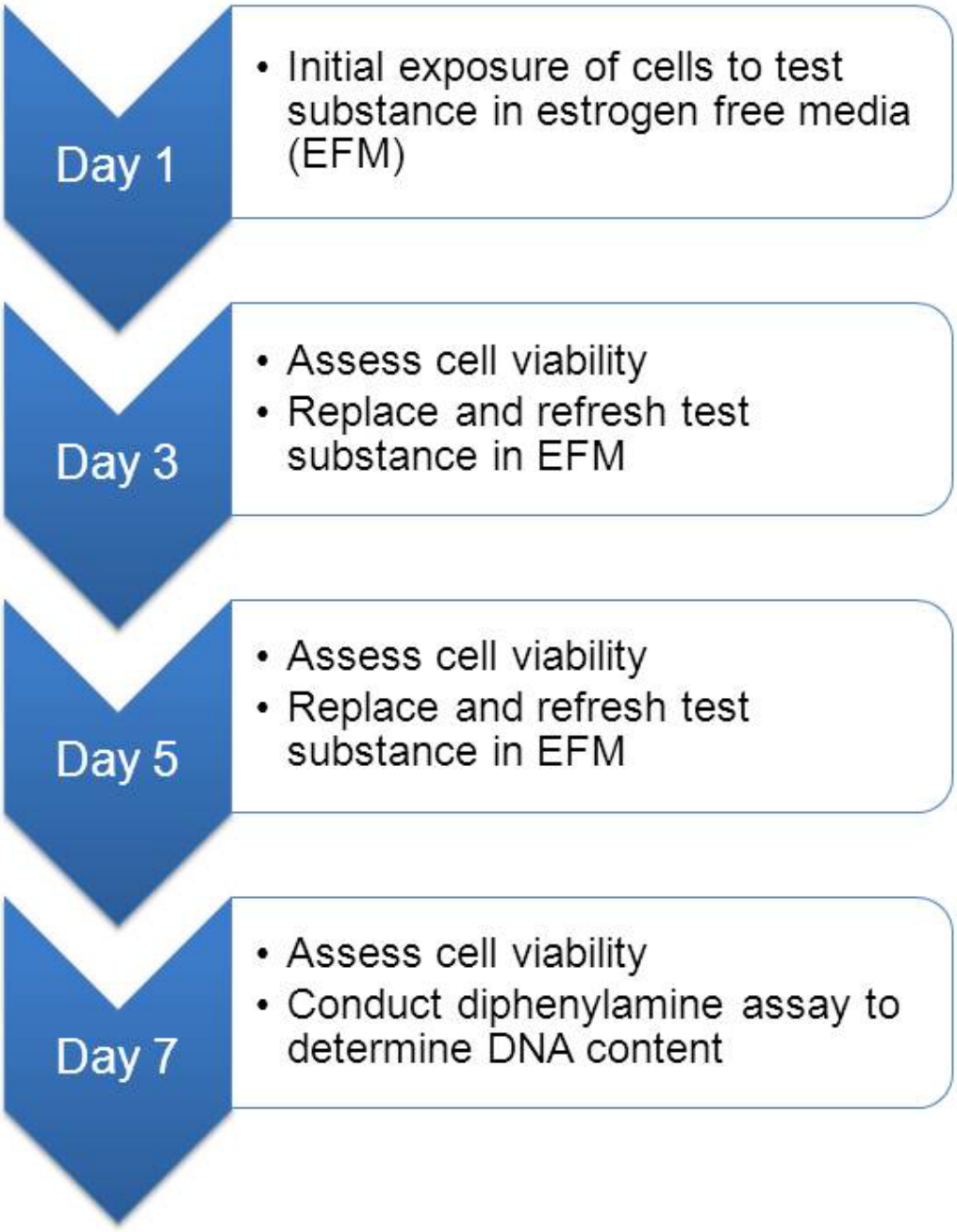
Key Aspects:

- Uses an automated liquid handling system (EpMotion 5070, Eppendorf)
- Cells treated for 6 days in estrogen free media (EFM)
- Cell viability assessed by visual inspection
- On day 7, DNA content measured using a diphenylamine (DPA) assay
- Solubility and volatility of test substances evaluated in 1% ethanol in estrogen free media (EFM) (1 mM limit concentration)
- Range finder testing (8 concentrations at log serial dilutions)
- Comprehensive testing (12 concentrations at 2.5:1 or 5:1 serial dilutions)
- If positive in comprehensive test, additional testing conducted to confirm effect on cell proliferation is ER mediated



EpMotion 5070 Automated Liquid Handling System

CCi MCF-7 Cell Proliferation Testing Scheme



Agonist and Antagonist Testing

Agonist Testing

- If a substance is positive in agonist testing, additional testing is conducted to confirm that the effect on cell proliferation is ER mediated.
- ER-mediated induction is confirmed by adding ICI 182,780 and observing a reduction in test-substance stimulated cell proliferation.

Antagonist Testing

- If a substance is positive in antagonist testing, additional testing is conducted to confirm that the effect on cell proliferation is ER mediated.
  - Confirmation testing is performed by treating cells with test substance plus 2.0 x 10^{-12} M E2 and comparing to cells treated with test substance plus 2.0 x 10^{-9} M E2.
  - If cell proliferation is significantly higher in cells treated with test substance plus 2.0 x 10^{-9} M E2, ER-mediated reduction is confirmed.
  - If cell proliferation is not significantly higher in cells treated with test substance plus 2.0 x 10^{-9} M E2, ER-mediated reduction is not confirmed.

Intralaboratory Study (CCi)

Protocol Optimization Phase

(Repeat Testing of Coded Reference Substances)

- 12 reference substances tested independently three times for agonist and antagonist activity
- Intralaboratory reproducibility and initial assessment of accuracy
- Protocol modification (if necessary to reduce variability and/or to improve accuracy)

Intralaboratory Validation Testing Phase

(Additional Substance Testing/Evaluation of Protocol Modifications)

- 41 reference substances tested once each for agonist and antagonist activity
- Comprehensive accuracy evaluation

Expansion of Validation Database Using Additional Reference Substances

- 26 reference substances tested once for agonist and antagonist activity to complete the list of 78 substances (ICCVAM 2006)
- Further characterize test method accuracy

Interlaboratory Study (Hiyoshi and KFDA)

Phase 1: Initial Laboratory Qualification

(Development of Historical Database for Each Laboratory)

- Initial qualification of laboratories - test agonist and antagonist reference standards and controls
- Establish individual laboratory historical database for standards and controls (five independent experiments each for the agonist and antagonist protocols)
- Establish test acceptance criteria for each laboratory based on historical database

Phase 2: Laboratory Qualification/Repeat Testing Phase

- Each laboratory - 12 reference substances tested independently three times for agonist and antagonist activity
- Intra- and interlaboratory reproducibility
- Protocol modification (if necessary to reduce variability and/or clarify)

Phase 3: Interlaboratory Validation Testing Phase

- Each laboratory – 14 reference substances tested once for agonist and antagonist activity using the final optimized protocols
- Data used to evaluate overall test method performance

Reference Substances Used in the Validation Study

Substance[a]	CASRN	MESH Chemical Class[b]
17ß-Estradiol	50-28-2	Steroid
17ß-Trenbolone	10161-33-8	Steroid
17α-Estradiol	57-91-0	Steroid
17α-Ethinyl estradiol	57-63-6	Steroid
19-Nortestosterone	434-22-0	Steroid
4-Cumylphenol	599-64-4	Phenol
4-tert-Octylphenol	140-66-9	Phenol
5α-Dihydrotestosterone	521-18-6	Steroid
Apigenin	520-36-5	Heterocyclic Compound
Bisphenol A	80-05-7	Phenol
Bisphenol B	77-40-7	Phenol
Butylbenzyl phthalate	85-68-7	Carboxylic Acid, Phthalic Acid
Chrysin	480-40-0	Flavonoid, Heterocyclic Compound
Clomiphene citrate	50-41-9	Amine, Carboxylic Acid, Heterocyclic Compound
Coumestrol	479-13-0	Heterocyclic Compound
Daidzein	486-66-8	Flavonoid, Heterocyclic Compound
Di-*n*-butyl phthalate	84-74-2	Ester, Phthalic Acid
Dicofol	115-32-2	Hydrocarbon (Cyclic), Hydrocarbon (Halogenated)
Diethylstilbestrol	56-53-1	Hydrocarbon (Cyclic)
Estrone	53-16-7	Steroid
Ethyl paraben	120-47-8	Carboxylic Acid, Phenol
Fenarimol	60168-88-9	Heterocyclic Compound, Pyrimidine
Genistein	446-72-0	Flavonoid, Heterocyclic Compound
Kaempferol	520-18-3	Flavonoid, Heterocyclic Compound

Substance[a]	CASRN	MESH Chemical Class[b]
Kepone	143-50-0	Hydrocarbon (Halogenated)
L-Thyroxine	51-48-9	Amino Acid
Medroxyprogesterone acetate	71-58-9	Steroid
Methyl testosterone	58-18-4	Steroid
Mifepristone	84371-65-3	Steroid
Morin	480-16-0	Flavonoid, Heterocyclic Compound
Norethynodrel	68-23-5	Steroid
o,*p*'-DDT	789-02-6	Hydrocarbon (Halogenated)
p-*n*-Nonylphenol	104-40-5	Phenol
p,*p*'- Methoxychlor	72-43-5	Hydrocarbon (Halogenated)
p,*p*'-DDE	72-55-9	Hydrocarbon (Halogenated)
Resveratrol	501-36-0	Hydrocarbon (Cyclic)
Tamoxifen	10540-29-1	Hydrocarbon (Cyclic)
2-sec-Butylphenol	89-72-5	Phenol
Atrazine	1912-24-9	Heterocyclic Compound
Bicalutamide	90357-06-5	Amide
Flutamide	13311-84-7	Amide
Haloperidol	52-86-8	Ketone
Hydroxy flutamide	52806-53-8	Amide
Ketoconazole	65277-42-1	Heterocyclic Compound
Linuron	330-55-2	Urea
Phenobarbital	50-06-6	Heterocyclic Compound, Pyrimidine
Phenolpthalin	81-90-3	Carboxylic Acid, Phenol
Procymidone	32809-16-8	Polycyclic Compound
Spironolactone	52-01-7	Lactone, Steroid
Finasteride	98319-26-7	Steroid

Substance[a]	CASRN	MESH Chemical Class[b]
12-*O*-Tetradecanoylphorbol-13-acetate	16561-29-8	Hydrocarbon (Cyclic)
2,4,5-Trichloro-phenoxyacetic acid	93-76-5	Carboxylic Acid
4-Androstenedione	63-05-8	Steroid
4-Hydroxyandrostenedione	566-48-3	Steroid
4-Hydroxytamoxifen	68047-06-3	Hydrocarbon (Cyclic)
Actinomycin D	50-76-0	Heterocyclic Compound, Polycyclic Compound
Ammonium perchlorate	7790-98-9	Amine, Onium Compound
Apomorphine	58-00-4	Heterocyclic Compound
Corticosterone	50-22-6	Steroid
Cycloheximide	66-81-9	Heterocyclic Compound
Cyproterone acetate	427-51-0	Steroid
Dexamethasone	50-02-2	Steroid
Dibenzo[*a*,*h*]anthracene	53-70-3	Polycyclic Compound
Diethylhexyl phthalate	117-81-7	Phthalic Acid
Flavone	525-82-6	Flavonoid, Heterocyclic Compound
Fluoranthene	206-44-0	Polycyclic Compound
Fluoxymestrone	76-43-7	Steroid
meso-Hexestrol	84-16-2	Steroid
Nilutamide	63612-50-0	Heterocyclic Compound, Imidazole
Oxazepam	604-75-1	Heterocyclic Compound
Pimozide	2062-78-4	Heterocyclic Compound
Progesterone	57-83-0	Steroid
Propylthiouracil	51-52-5	Heterocyclic Compound, Pyrimidine
Raloxifene HCl	82640-04-8	Hydrocarbon (Cyclic)

Substance[a]	CASRN	MESH Chemical Class[b]
Reserpine	50-55-5	Heterocyclic Compound, Indole
Sodium azide	26628-22-8	Azide, Salt (Inorganic)
Testosterone	58-22-0	Steroid
Vinclozolin	50471-44-8	Heterocyclic Compound

[a] The 78 reference substances recommended by ICCVAM for the validation of *in vitro* ER and androgen receptor binding and TA test methods (ICCVAM 2006) are being tested in the validation study.

[b] Substances were assigned into one or more chemical classes using the U.S. National Library of Medicine's Medical Subject Headings (MeSH), an internationally recognized standardized classification scheme (available at: http://www.nlm.nih.gov/mesh).

Post-Validation Evaluation

- Results and analyses will be compiled in a draft background review document (BRD).
- Draft ICCVAM recommendations based on the information in the BRD will be developed for:
  - Usefulness and limitations of the test method
  - Test method protocols
  - Test method performance standards
  - Future studies that may be useful
- The draft BRD and draft ICCVAM recommendations will be made available to the public and to an independent international scientific peer review panel.
- The peer review panel will meet in public session and their report will be made available to the public and to 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 that will contain final ICCVAM test method recommendations.
- The test method evaluation report 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.

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Current validation study information is available on the NICEATM-ICCVAM website at http://iccvam.niehs.nih.gov/methods/endocrine.htm.