# U. S. ICCVAM EVALUATION OF *IN VITRO* ENDOCRINE DISRUPTOR TESTING METHODS

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**Introduction**

Thank you very much for the kind introduction, Mr. Chairman. I also want to extend my appreciation to the Japanese Ministry of the Environment for inviting me to participate in this 5th International Symposium on Environmental Endocrine Disruptors. International meetings such as this are important to share scientific information and to coordinate effective future international cooperation to address public health and environmental protection issues relating to endocrine disrupting chemicals.

I will discuss the use and current status of *in vitro*, or non-animal test methods, that might be helpful in identifying chemicals and products that may have to potential to disrupt the endocrine system. A comprehensive evaluation of *in vitro* methods for detecting chemicals that can interact with the estrogen and androgen receptors was recently undertaken by the U. S. Interagency Coordinating Committee on the Validation of Alternative Methods, referred to as ICCVAM (pronounced Ick´ vam) in collaboration with the National Toxicology Program Interagency Center for the Evaluation of Alternative Methods, commonly referred to as NICEATM (pronounced ni See´ tum). This paper provides an overview of this interagency and international review of the validation status of these *in vitro* test methods.

***In Vitro* Methods: Background and Proposed Use**

A number of man-made and naturally occurring chemicals have been found to alter endocrine processes in man and animals by binding with estrogen and/or androgen receptors and either initiating or inhibiting sex hormone dependent gene activation. Concern over possible adverse health effects of such chemicals led to legislation requiring the U. S. EPA to develop and implement a screening and testing program to identify endocrine disrupting chemicals in food and water. The laws also require EPA to "appropriately validate" the test methods prior to implementation. Using the advice provided by the Endocrine Disruptor Screening and Testing Advisory Committee, or EDSTAC, the EPA proposed an Endocrine Disruptor Screening and Testing Program in 1998. The Program consists of a Tier 1 screening battery of *in vitro* and *in vivo* test methods that is designed to detect substances capable of interacting with the estrogen, androgen, or thyroid hormone systems. Tier 2 is comprised of multi-generation *in vivo* assays and is designed to establish a quantitative relationship between the dose and any adverse effects. This Tier 2 data will be used to support appropriate risk assessments.

***In Vitro* Methods Proposed for ED Screening**

Figure 1 lists the current *in vitro* methods under consideration for the Tier 1 screening battery. *In vitro* and *in vivo* results will be used as part of a weight-of-evidence decision regarding the need for definitive Tier 2 *in vivo* testing. Either an estrogen receptor (ER) binding or transcriptional activation (TA) assay will be required, as well as an androgen receptor (AR) binding or transcriptional assay. Transcriptional assays can be conducted in two ways: first, as an agonist assay to detect if a substance binds to the receptor and results in gene transcription, and secondly, as an antagonist assay to detect if a substance binds to the receptor and prevents transcription by endogenous hormone. When used as screening assays, a negative TA agonist assay result will need to be followed by a TA antagonist assay if both assays are not run concurrently. Two other *in vitro* assays are also being considered for the Tier 1

screening battery: the steroidogenesis assay and the aromatase assay. The EPA is coordinating evaluation of these assays, and they will not be discussed in this paper.

**Advantages and Limitations of *In Vitro* Assays**

There are many advantages to using *in vitro* methods. They can be rapidly performed, usually in less than 1 to 2 days, and can often be automated for semi- or high–throughput processing of large numbers of samples. Only small amounts of chemical are needed and they can often detect effects from very low concentrations. They can measure highly specific biological effects and indicate whether or not a chemical is capable of acting by certain biological mechanisms. This information can be helpful in reaching a weight-of-evidence decision from Tier 1 screening results. *In vitro* methods can also reduce or avoid the use of animals, although some binding assays currently require animal tissue as a source of receptor protein, such as the rat uterine cytosol ER binding assay.

There are potential limitations to the use of *in vitro* methods. They may not accurately predict effects that could occur *in vivo* due to metabolic activation or inactivation of a chemical, although exogenous metabolic systems can be developed and used with *in vitro* assays. There are also limitations in knowing whether biologically active concentrations *in vitro* can realistically be achieved at target tissues in whole animals due to possible limited absorption and tissue distribution. Finally, there are many possible modes of action by which endocrine disruption can occur, and multiple *in vitro* test systems would be necessary to fully address all of these.

**What is Validation and Why Is It Necessary?**

Prior to incorporating test methods into the EPA screening and testing program, the methods must be adequately validated and found to be acceptable for their proposed use. Validation is necessary to determine the usefulness and limitations of a test method for a specific purpose and is considered a prerequisite for determining whether a test method is acceptable for regulatory purposes (ICCVAM, 1997). Validation is defined as the process by which the reliability and relevance of a test method are established for a specific purpose. Reliability is a measure of the extent to which a test can be performed reproducibly within and among laboratories over time and provides an estimate of the likelihood that different labs will get similar results. Relevance is defined as a measure of the extent to which a test method will correctly predict or measure the biological effect of interest. Also referred to as the accuracy of the test, it indicates the likelihood that it will provide the correct answer.

**ICCVAM Evaluation of ER and AR *In Vitro* Methods**

The U. S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) was established to evaluate the scientific validity of new test methods on behalf of federal agencies (ICCVAM, 1997). In 2000, ICCVAM was asked by the U. S. EPA to conduct an independent scientific peer review of the validation status of existing *in vitro* ER and AR binding and TA assays and to use this information to develop minimum performance criteria that could be used to define acceptable *in vitro* assays. To support the evaluation, the National Toxicology Program Interagency Center for the Evaluation of Alternative Methods (NICEATM) prepared Background Review Documents (BRDs) consisting of comprehensive reviews of existing protocols and data. A public notice called for submission of data and protocols for existing test methods, as well as nomination of expert scientists to review the data. An international expert panel was convened in May 2002 to evaluate the available information and to provide conclusions and recommendations about the current status and future validation of these assays.

Individual BRDs were prepared for each of the four types of receptor assays. Each BRD contained:

- A review of available protocols and data
- A review of the essential protocol components
- Proposed minimum procedural standards for protocols
- Proposed assays for future validation
- Proposed substances for future validation studies

**ER and AR Background Review Documents**

A total of 4037 test results were located for the four different types of assays (Figure 2). The number of different test protocols ranged from 11 to 95 for each type of assay and the number of different chemicals evaluated in each type of assay ranged from 108 to 698. However, very few chemicals that had been evaluated in all of the different protocols for each type assay. Based on a review of the available data, it was concluded that there were no adequately standardized *in vitro* assays with adequate validation data. Specifically, there were no interlaboratory evaluations of a standardized protocol and inadequate data that could serve as the basis for establishing minimum performance standards for a specific assay. Accordingly, EPA and ICCVAM agreed to convene an expert panel that would assess the current validation status of available methods and develop recommendations for future validation efforts.

**Expert Panel Meeting**

NICEATM in collaboration with the ICCVAM convened an Expert panel in May 2002 in Research Triangle Park, North Carolina. A total of 23 scientists from 5 different countries with a broad range of expertise in molecular biology, endocrinology, *in vitro* toxicology, and biostatistics served on the expert panel (Figure 3). All documents and data considered by the expert panel were made available to the public for advance comments, and these comments were provided to the panel for their consideration. The panel met in public session, and the opportunity for public comments was provided during the meeting.

The Panel was charged with reviewing the BRDs and providing conclusions and recommendations on the following:

- Assays that should be considered for further evaluation in validation studies and their relative priority
- Adequacy of the proposed minimum procedural standards for each of the four types of assays
- Adequacy of available test method protocols for assays recommended for validation studies
- Adequacy and appropriateness of the substances recommended for validation studies

**ER and AR Binding Assays**

The panel agreed that no *in vitro* ER and AR binding or TA assay protocols were sufficiently standardized and adequately validated to be considered for regulatory testing. With regard to ER and AR binding assays that should receive priority for future development and validation, the Panel recommended that the highest priority should be development of assays using the following recombinant protein receptors: human AR and human or rat ER alpha and/or beta. Use of receptor protein produced in a recombinant cell system would:

- Eliminate the need for animals as a receptor source
- Result in minimal contamination with other tissue receptors
- Reduce assay variation due to use of a standardized receptor among laboratories
- Be adaptable to high-throughput testing.

The panel also recommended that consideration should be given to further development and validation of non-radioactive methods, such as fluorescent polarization methodologies, in order to reduce the generation of radioactive wastes. The panel acknowledged that development of an exogenous metabolic activation system would be desirable, but recommended that development should be deferred until there is further evaluation of the need for such a system.

The final report of the expert panel's conclusions and recommendations and the background review documents considered by the panel are available at the ICCVAM/NICEATM website: http://iccvam.niehs.nih.gov/methods/endocrine.htm.

**ER and AR TA Assays**

With regard to TA assays, the Panel did not recommend any specific assays of the many that were reviewed. For AR TA assays, they recommended that priority should be given to developing systems using mammalian cell lines with endogenous or stably transfected receptors and a reporter construct with a response element that is relatively specific to the AR. These types of assays would provide for increased efficiency and reduced variability. For ER TA assays, the Panel recommended that a pre-validation study should be conducted to compare stably versus transiently transfected cell lines with a human ER-alpha expression vector, using a reporter construct with multiple vitellogenin estrogen response elements (vit-ERE) and luciferase. For all TA assays, the Panel recommended that development of an exogenous metabolic activation would be desirable, but inclusion should be deferred until there is further evaluation of need.

**Minimum Procedural Standards**

The Panel reviewed and proposed additional minimum procedural standards for each of the four types of assays. They recommended that all standardized test method protocols should incorporate the recommended minimum procedural standards. Selected standard procedures for all assays included:

- Substances should be tested up to a designated limit concentration (1 millimolar), or if not soluble at this level, then tested at the maximum soluble or non-cytotoxic concentration.
- At least 7 concentrations should be tested over a range of at least 7 orders of magnitude, and triplicate measurements should be performed for each concentration.
- Acceptable positive control responses should be defined based on historical control data within each laboratory.
- There should be approval of all studies requiring animals as tissue sources by an Institutional Animal Care and Use Committee (IACUC), or equivalent.
- All assays should be conducted in accordance with Good Laboratory Practice guidelines.

A complete description of recommended minimum procedural standards can be found in the Expert Panel's final report which is available on the ICCVAM/NICEATM website.

Minimum procedural standards for ER and AR binding assays included the following:

- A concurrent positive control with a binding affinity 2-3 orders of magnitude below that of the reference estrogen/androgen should be used and tested at multiple concentrations.
- Specific reference estrogen and androgens should be used as recommended by the Panel.
- Solvent controls should be included in each assay.
- Test substances should be prepared in water, 95-100% ethanol, or DMSO, in that order of preference.

- Sodium molybdate and a cocktail of protease inhibitors should be added to protect the estrogen/androgen receptor from degradation.
- The dissociation constant ($K_d$) of the reference estrogen/androgen should be determined with each assay.

Panel recommendations for minimum procedural standards that should be incorporated in transcriptional activation assays included, but were not limited to, the following:

- Cellular cytotoxicity should be assessed to define the upper limit for test substance concentrations.
- The stability of cell lines with stably transfected reporter constructs should be monitored.
- A constitutive reporter gene assay to assess the efficiency of transfection for transiently transfected assays should be included.
- A suitable nonlinear regression model such as the Hill equation to estimate potency ($EC_{50}$ or $IC_{50}$ values) and slope of the concentration-response curve with a 95% confidence interval should be used.

**Recommended Substances for Validation Studies**

The panel endorsed a list of chemicals proposed for validation studies and made the following recommendations for improving the list:

- To assess the specificity of the assays, at least 25% of the chemicals should be negative for each assay type.
- The same substances should be used for validation of both ER binding and TA assays.
- The same substances should be used for validation of both AR binding and TA assays.
- The number of relevant chemical classes should be increased.
- All substances used for *in vivo* validation studies, including those used for U. S. EPA studies, should be included.
- Highly hazardous substances that require expensive disposal procedures (e.g., PCBs) should be avoided.
- A central repository that can distribute chemicals of known purity should be established.
- Substances representative of the full range of expected activity of test substances from very weak to strong should be used.

**ICCVAM Proposed Substances for Validation Studies**

Following the expert panel meeting, NICEATM and an ICCVAM working group compiled a common list of 78 proposed substances that should be considered for validation studies. The list is intended to ensure that assay reliability and performance are adequately characterized for a broad range of chemical classes and across a wide range of potencies from weak to strong. The list incorporates the chemicals endorsed by the Expert Panel and their recommendations regarding additional chemical selections.

The proposed list includes 78 total substances representing 70 chemical classes and 13 product classes. Activity in all assay types has not been determined for all substances. However, based on existing data, positive results are expected for at least the following number of chemicals:

- ER binding: 41(+); 37(-)
  - ER TA agonists: ≥35
  - ER TA antagonists: ≥11
- AR binding: 34(+); 44(-)
  - AR TA agonists: ≥22
  - AR TA antagonists: ≥21

To fully characterize the usefulness, limitations, and predictive value of a battery of *in vitro* tests methods for predicting *in vivo* responses, all 78 substances are recommended for testing in each of the *in vitro* assays. The generation of both *in vivo* and *in vitro* data on these substances will help facilitate the future development of more predictive *in vitro* endocrine disruptor assays.

A notice of availability of the proposed list of chemicals and selection criteria were published in the U. S. *Federal Register*, with a request for public comments. The final list of recommended substances is provided in the ICCVAM Evaluation Report (ICCVAM, 2003).

**Opportunities for Future Progress**

The ICCVAM/NICEATM detailed evaluation of the validation status of *in vitro* methods for detecting potential endocrine activity produced many substantive recommendations and sound conclusions. Application of this scientific advice will advance the usefulness and application of these *in vitro* methods. Specific activities that will help facilitate validation include:

- More accurate and efficient *in vitro* assays should be developed, especially those that do not require the use of animals for tissue and those that can be accomplished more quickly and with less expense.
- Test method protocols should be standardized using the recommended minimum procedural standards.
- Validation studies should use the recommended standard reference chemicals.
- Valid and acceptable *in vitro* assays should be included in the EDSP.
- *In vitro* test batteries should be evaluated for their ability to predict *in vivo* effects. Useful test batteries should be used to prioritize chemicals for further ED screening and testing.

**Summary**

Implementation of the Expert Panel and ICCVAM recommendations will facilitate validation and adoption of standardized protocols for ER and AR binding and TA assays that can be used in the Tier 1 screening battery to identify potential endocrine disrupting chemicals.

Standardization and validation of *in vitro* ED test methods will:

- Enhance reproducibility and transferability of protocols.
- Facilitate evaluation of comparative performance of various protocols to identify those that are most useful.
- Facilitate establishment of minimum performance standards to evaluate future methods. This will speed the adoption of better methods that also offer other advantages in terms of time and expense.

Generation of *in vitro* and *in vivo* data on the same chemicals will be essential to facilitate development and validation of *in vitro* test batteries that are predictive of potential human effects. Such predictive mechanism-based *in vitro* methods can be expected to support accurate, rapid, and cost-effective chemical screening to identify potential endocrine active chemicals, and that will reduce the use of animals.

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**Figure 1. *In Vitro* Methods Proposed for Endocrine Disruptor Screening**

- **Estrogen receptor (ER) binding assays**
- **ER transcriptional activation (TA) assays**
  - **Agonist assays: detects transcription**
  - **Antagonist assays: detect blocked transcription**
- **Androgen receptor (AR) binding assays**
- **AR TA assays**
  - **Agonist assays**
  - **Antagonist assays**
- **Other *in vitro* test methods**
  - **Steroidogenesis assays**
  - **Aromatase assays**

**Figure 2. NICEATM ER and AR Background Review Documents**

| Assay type | Protocols | Chemicals | Data Entries |
|---|---|---|---|
| ER BA | 14 | 635 | 1567 |
| ER TA | 95 | 698 | 1831 |
| AR BA | 11 | 108 | 276 |
| AR TA | 17 | 145 | 363 |
| Totals | 137 | >698 | 4037 |

**Figure 3. Expert Panel on *In Vitro* Endocrine Disruptor Methods**

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Terry Brown (Section Chair), USA
Grantley Charles, USA
Robert Combes, UK
George Daston (Panel and Section Chair), USA
Kevin Gaido, USA
Thomas Gasiewicz, USA
John Harbell, USA
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