ICCVAM Evaluation of *In Vitro* Test Methods for Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)

National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)
The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) was established in 1997 by the Director of the National Institute of Environmental Health Sciences (NIEHS) to implement NIEHS directives in Public Law 103-43. P.L. 103-43 directed NIEHS to develop and validate new test methods, and to establish criteria and processes for the validation and regulatory acceptance of toxicological testing methods. P.L. 106-545, the ICCVAM Authorization Act of 2000, establishes ICCVAM as a permanent committee. The Committee is composed of representatives from 15 Federal regulatory and research agencies that generate, use, or provide information from toxicity test methods for risk assessment purposes. P.L. 106-545 directs ICCVAM to coordinate technical reviews and evaluations of new, revised, and alternative test methods of interagency interest. ICCVAM also coordinates cross-agency issues relating to development, validation, acceptance, and national/international harmonization of toxicological test methods.

The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) was established in 1998 to provide operational support for the ICCVAM and to collaborate with the ICCVAM to carry out committee-related activities such as test method peer reviews and workshops. NICEATM and ICCVAM coordinate the scientific review of the validation status of proposed methods and provide recommendations regarding their usefulness to appropriate agencies. NICEATM and ICCVAM seek to promote the validation and regulatory acceptance of toxicological test methods that will enhance agencies' abilities to assess risks and make decisions, and that will refine, reduce, and replace animal use. The ultimate goal is to improve public health by gaining the regulatory acceptance of new scientifically valid test methods that are more predictive of human and ecological effects than currently available methods.

Additional Information
Additional information can be found at the ICCVAM/NICEATM Website: http://iccvam.niehs.nih.gov and in the publication: Validation and Regulatory Acceptance of Toxicological Test Methods, a Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods (NIH Publication No. 97-3981), or you may contact the Center at telephone 919-541-3398, or by e-mail at iccvam@niehs.nih.gov. Specific questions about ICCVAM and the Center can be directed to the Director of NICEATM:

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Agency for Toxic Substances and Disease Registry  Food and Drug Administration
Consumer Product Safety Commission  National Cancer Institute
Department of Agriculture  National Institute of Environmental Health Sciences
Department of Defense  National Institutes of Health, Office of the Director
Department of Energy  National Institute of Occupational Safety and Health
Department of Interior  National Library of Medicine
Department of Transportation  Occupational Safety and Health Administration
Environmental Protection Agency

On the Cover
The ICCVAM/NICEATM graphic symbolizes the important role of new and alternative toxicological methods in protecting and advancing the health of people, animals, and our environment.
ICCVAM Evaluation of *In Vitro* Test Methods for Detecting Potential Endocrine Disruptors:

Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)

National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

National Toxicology Program
P.O. Box 12233
Research Triangle Park, NC 27709

May 2003
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National Institute of Environmental Health Sciences
National Institutes of Health
U.S. Public Health Service
Department of Health and Human Services
# Table of Contents

List of Tables ................................................................................................................................v 
List of Abbreviations and Acronyms ............................................................................................vii 
ICCVAM Representatives .............................................................................................................xi 
Acknowledgments ........................................................................................................................xii 
Preface ........................................................................................................................................xvii 
Executive Summary ......................................................................................................................xxi 

1.0 ICCVAM Evaluation and Recommendations on Estrogen and Androgen Receptor Binding and Transcriptional Activation Assays ..........................................................1 
  1.1 Introduction .........................................................................................................................1 
  1.2 ICCVAM Proposed Substances for Validation of In Vitro Endocrine Disruptor Assays ..........................................................................................................................3 
  1.3 Public Comments ..............................................................................................................3 
  1.4 ICCVAM Recommendations ..............................................................................................6 
  1.5 Other Considerations .......................................................................................................10 

2.0 Proposed Substances and Selection Criteria for Validation of In Vitro Endocrine Disruptor Screening Assays .............................................................................................11 
  2.1 Introduction .......................................................................................................................11 
  2.2 Draft Background Review Document Recommendations ..............................................11 
  2.3 Expert Panel Recommendations on Proposed Substances for Validation Studies ..........11 
  2.4 ICCVAM, EDWG and NICEATM Proposed List of Substances for Validation ...........12 

3.0 In Vitro Estrogen Receptor Binding Assays .........................................................................27 
  3.1 Minimum Procedural Standards .......................................................................................27 
  3.2 Recommended Substances for Validation of In Vitro Estrogen Receptor Binding Assays .........................................................................................................................34 

4.0 In Vitro Estrogen Receptor Transcriptional Activation Assays ...........................................43 
  4.1 Minimum Procedural Standards .......................................................................................43 
  4.2 Recommended Substances for Validation of In Vitro Estrogen Receptor Transcriptional Activation Assays ..........................................................................................50 

5.0 In Vitro Androgen Receptor Binding Assays .....................................................................69 
  5.1 Minimum Procedural Standards .......................................................................................69 
  5.2 Recommended Substances for Validation of In Vitro Androgen Receptor Binding Assays .........................................................................................................................76 

6.0 In Vitro Androgen Receptor Transcriptional Activation Assays .........................................87 
  6.1 Minimum Procedural Standards .......................................................................................87 
  6.2 Recommended Substances for Validation of In Vitro Androgen Receptor Transcriptional Activation Assays ..........................................................................................94 

7.0 References .............................................................................................................................113
Table of Contents

(continued)

8.0 Appendices


   B-1 Evaluation Guidance for Estrogen Receptor and Androgen Receptor Binding Assays .......................................................... B-3
   B-2 Evaluation Guidance for Estrogen Receptor and Androgen Receptor Transcriptional Activation Assays .......................................................... B-7

C. Expert Panel Evaluation Meeting Agenda ........................................... C-1

D. Expert Panel Meeting Minutes ............................................................... D-1

E. ICCVAM Expert Panel Evaluation *Federal Register* Notices ...................... E-1
       Request for Data and Nominations of Expert Scientists
   E-2 Vol. 67, No. 66, pp. 16415-16416, April 5, 2002 ........................................ E-5
       Notice of an Expert Panel Meeting and Request for Comments
   E-3 Vol. 67, No. 204, pp. 64902-64903, October 22, 2002 ................................ E-7
       Announcing Availability of Expert Panel Report, Proposed List of Substances and Final Background Review Documents

F. Public Comments in Response to the *Federal Register* Request for Comments .... F-1
# List of Tables

## 2.0 Proposed Substances and Selection Criteria for Validation of Endocrine Disruptor Screening Assays

<table>
<thead>
<tr>
<th>Table 2-1</th>
<th>Numbers of Substances Recommended in the BRDs for the Validation of \textit{In Vitro} ER and AR Binding and TA Assays</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2-2A</td>
<td>Distribution of Anticipated Responses of the 78 Recommended Test Substances in \textit{In Vitro} ER and AR Binding and TA Assays—\textit{In Vitro} ER-Based Assays</td>
<td>17</td>
</tr>
<tr>
<td>Table 2-2B</td>
<td>Distribution of Anticipated Responses of the 78 Recommended Test Substances in \textit{In Vitro} ER and AR Binding and TA Assays—\textit{In Vitro} AR-Based Assays</td>
<td>18</td>
</tr>
<tr>
<td>Table 2-3</td>
<td>Distribution of the 78 Recommended Substances Among Chemical Classes</td>
<td>19</td>
</tr>
<tr>
<td>Table 2-4</td>
<td>Distribution of the 78 Recommended Substances Among Product Classes</td>
<td>21</td>
</tr>
<tr>
<td>Table 2-5</td>
<td>Chemical and Product Classes of the 78 Recommended Substances</td>
<td>22</td>
</tr>
</tbody>
</table>

## 3.0 \textit{In Vitro} Estrogen Receptor Binding Assays

| Table 3-1 | ICCVAM Recommended Substances for Validation of \textit{In Vitro} ER Binding Assays | 36 |

## 4.0 \textit{In Vitro} Estrogen Receptor Transcriptional Activation Assays

| Table 4-1 | ICCVAM Recommended Substances for Validation of \textit{In Vitro} ER TA Agonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems | 52 |
| Table 4-2 | ICCVAM Recommended Substances for Validation of \textit{In Vitro} ER TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems | 60 |

## 5.0 \textit{In Vitro} Androgen Receptor Binding Assays

| Table 5-1 | ICCVAM Recommended Substances for Validation of \textit{In Vitro} AR Binding Assays | 78 |

## 6.0 \textit{In Vitro} Androgen Receptor Transcriptional Activation Assays

| Table 6-1 | ICCVAM Recommended Substances for Validation of \textit{In Vitro} AR TA Agonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems | 96 |
| Table 6-2 | ICCVAM Recommended Substances for Validation of \textit{In Vitro} AR TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems | 104 |
[This Page Intentionally Left Blank]
### List of Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1G</td>
<td>One-generation assay</td>
</tr>
<tr>
<td>2G</td>
<td>Two-generation assay</td>
</tr>
<tr>
<td>407</td>
<td>OECD Test Guideline 407</td>
</tr>
<tr>
<td>A</td>
<td>Avian</td>
</tr>
<tr>
<td>ACC</td>
<td>American Chemistry Council</td>
</tr>
<tr>
<td>ACLAM</td>
<td>American College of Laboratory Animal Medicine</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>β-gal</td>
<td>Gene for β-galactosidase</td>
</tr>
<tr>
<td>B\textsubscript{max}</td>
<td>Number of binding sites in a cytosolic preparation</td>
</tr>
<tr>
<td>BRD</td>
<td>Background Review Document</td>
</tr>
<tr>
<td>CALUX\textsuperscript{®}</td>
<td>Chemical-activated luciferase expression assay</td>
</tr>
<tr>
<td>CASRN</td>
<td>Chemical Abstracts Service Registry Number</td>
</tr>
<tr>
<td>CAT</td>
<td>Chloramphenicol acetyl transferase (protein)</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CFR</td>
<td>Code of Federal Regulations</td>
</tr>
<tr>
<td>CHO</td>
<td>Cell line derived from Chinese hamster ovary</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>comp.</td>
<td>Computed</td>
</tr>
<tr>
<td>COS</td>
<td>Cell line derived from monkey kidney</td>
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<tr>
<td>%CV</td>
<td>Percent coefficient of variation</td>
</tr>
<tr>
<td>CV\textsubscript{1}</td>
<td>Cell line derived from monkey kidney</td>
</tr>
<tr>
<td>DABT</td>
<td>Diplomate, American Board of Toxicology</td>
</tr>
<tr>
<td>DACVP</td>
<td>Diplomate, American College of Veterinary Pathologists</td>
</tr>
<tr>
<td>DCC</td>
<td>Dextran-coated charcoal</td>
</tr>
<tr>
<td>DDE</td>
<td>1,1-Dichloro-bis[4-chlorophenyl]ethylene</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichlorodiphenyltrichloroethane</td>
</tr>
<tr>
<td>DEAE</td>
<td>2-(Diethylamino)ethyl</td>
</tr>
<tr>
<td>def</td>
<td>Three domains of the estrogen receptor comprising its ligand binding domain</td>
</tr>
<tr>
<td>Dept.</td>
<td>Department</td>
</tr>
<tr>
<td>DHT</td>
<td>5α-Dihydrotestosterone</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPM</td>
<td>Disintegrations per minute</td>
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<tr>
<td>EC\textsubscript{50}</td>
<td>Half-maximal effective concentration</td>
</tr>
<tr>
<td>EDMVS</td>
<td>Endocrine Disruptor Methods Validation Subcommittee</td>
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<td>EDSP</td>
<td>Endocrine Disruptor Screening Program</td>
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<td>EDSTAC</td>
<td>Endocrine Disruptor Screening and Testing Advisory Committee</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EDWG</td>
<td>Endocrine Disruptor Working Group</td>
</tr>
<tr>
<td>EPA</td>
<td>U.S. Environmental Protection Agency</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
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List of Abbreviations and Acronyms

(continued)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>ERα</td>
<td>Estrogen receptor alpha</td>
</tr>
<tr>
<td>ERβ</td>
<td>Estrogen receptor beta</td>
</tr>
<tr>
<td>ERE</td>
<td>Estrogen response element</td>
</tr>
<tr>
<td>F</td>
<td>Fish</td>
</tr>
<tr>
<td>FACB</td>
<td>Fellow, National Academy of Clinical Biochemistry</td>
</tr>
<tr>
<td>FATS</td>
<td>Fellow, Academy of Toxicological Sciences</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S. Food and Drug Administration</td>
</tr>
<tr>
<td>FFDCA</td>
<td>Federal Food, Drug, and Cosmetic Act</td>
</tr>
<tr>
<td>FIFRA</td>
<td>Federal Insecticide, Fungicide and Rodenticide Act</td>
</tr>
<tr>
<td>fmol</td>
<td>Femtomole</td>
</tr>
<tr>
<td>FP</td>
<td>Fluorescence polarization</td>
</tr>
<tr>
<td>F-PA</td>
<td>Female pubertal assay</td>
</tr>
<tr>
<td>FQPA</td>
<td>Food Quality Protection Act</td>
</tr>
<tr>
<td>FR</td>
<td>U.S. Federal Register</td>
</tr>
<tr>
<td>FRAME</td>
<td>Fund for the Replacement of Animals in Medical Experiments</td>
</tr>
<tr>
<td>FRS</td>
<td>Fish reproductive screen</td>
</tr>
<tr>
<td>GAL4</td>
<td>A yeast protein that acts as a transcriptional activator</td>
</tr>
<tr>
<td>GLP</td>
<td>Good Laboratory Practices</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>H</td>
<td>Hershberger assay</td>
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<tr>
<td>HAP</td>
<td>Hydroxylapate</td>
</tr>
<tr>
<td>hAR</td>
<td>Human androgen receptor</td>
</tr>
<tr>
<td>HDT</td>
<td>Highest dose tested</td>
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<tr>
<td>HeLa</td>
<td>Cell line derived from a human cervical carcinoma</td>
</tr>
<tr>
<td>HepG2</td>
<td>Cell line derived from a human hepatoblastoma</td>
</tr>
<tr>
<td>hER</td>
<td>Human estrogen receptor</td>
</tr>
<tr>
<td>hERα</td>
<td>Human estrogen receptor alpha</td>
</tr>
<tr>
<td>hERα-FP</td>
<td>Human estrogen receptor alpha-fluorescence polarization assay</td>
</tr>
<tr>
<td>hERβ</td>
<td>Human estrogen receptor beta</td>
</tr>
<tr>
<td>HGF</td>
<td>Human genital fibroblasts</td>
</tr>
<tr>
<td>HPTE</td>
<td>2,2-Bis-(p-hydroxyphenyl)-1,1,1-trichloroethane</td>
</tr>
<tr>
<td>HRE</td>
<td>Hormone responsive elements</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
</tr>
<tr>
<td>IC</td>
<td>Inhibitory concentration</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>Concentration of a test substance inhibiting the reference estrogen or androgen response by 50%</td>
</tr>
<tr>
<td>ICCVAM</td>
<td>Interagency Coordinating Committee on the Validation of Alternative Methods</td>
</tr>
<tr>
<td>IM</td>
<td>Intact male assay</td>
</tr>
<tr>
<td>IUL</td>
<td>In utero through lactation assay</td>
</tr>
<tr>
<td>IUPAC</td>
<td>International Union of Pure and Applied Chemistry</td>
</tr>
<tr>
<td>JME</td>
<td>Japanese Ministry of the Environment</td>
</tr>
<tr>
<td>Kd</td>
<td>Dissociation constant</td>
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### List of Abbreviations and Acronyms (continued)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>$K_i$</td>
<td>Equilibrium dissociation constant of a receptor-ligand complex</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand binding domain</td>
</tr>
<tr>
<td>LNCaP</td>
<td>Cell line derived from lymph node of a patient with metastatic prostatic adenocarcinoma</td>
</tr>
<tr>
<td>LOEC</td>
<td>Lowest observed effective concentration</td>
</tr>
<tr>
<td>Luc</td>
<td>Luciferase reporter gene</td>
</tr>
<tr>
<td>M2</td>
<td>A metabolite of vinclozolin</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Cell line derived from a human mammary adenocarcinoma</td>
</tr>
<tr>
<td>MCRG</td>
<td>Mammalian cell reporter gene</td>
</tr>
<tr>
<td>MDA-kb2</td>
<td>Cell line derived from MDA-MB-453 cells that are stably transfected with a luciferase reporter gene</td>
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<tr>
<td>MDA-MB-453</td>
<td>Cell line derived from a human mammary carcinoma</td>
</tr>
<tr>
<td>meas.</td>
<td>Measured</td>
</tr>
<tr>
<td>mER</td>
<td>Mouse estrogen receptor</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse mammary tumor virus</td>
</tr>
<tr>
<td>M-PA</td>
<td>Male pubertal assay</td>
</tr>
<tr>
<td>MPA</td>
<td>Medroxyprogesterone acetate</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MTD</td>
<td>Maximum tolerated dose</td>
</tr>
<tr>
<td>MVLN</td>
<td>Cell line derived from MCF-7 cells that are stably transfected with a vitellogenin-luciferase reporter gene</td>
</tr>
<tr>
<td>NAS</td>
<td>National Academy of Sciences</td>
</tr>
<tr>
<td>N/C</td>
<td>Amino(N)- and carboxyl(C)-terminal regions of a protein</td>
</tr>
<tr>
<td>Neg.</td>
<td>Negative</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
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<tr>
<td>NICEATM</td>
<td>National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods</td>
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<tr>
<td>NIEHS</td>
<td>National Institute of Environmental Health Sciences</td>
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<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>No.</td>
<td>Number</td>
</tr>
<tr>
<td>NSB</td>
<td>Nonspecific binding</td>
</tr>
<tr>
<td>NTP</td>
<td>National Toxicology Program</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OECD</td>
<td>Organisation for Economic Co-operation and Development</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>PCB</td>
<td>Polychlorinated biphenyl</td>
</tr>
<tr>
<td>P.L.</td>
<td>Public Law</td>
</tr>
</tbody>
</table>
List of Abbreviations and Acronyms

(continued)

pM  Picomolar
pmol  Picomole
Pos.  Positive
PR  Progesterone receptor
pSG5  A eukaryotic expression vector
QC  Quality control
QSAR  Quantitative structure activity relationship
R1881  Methyltrienolone
rAR  Rat androgen receptor
RBA  Relative binding affinity
res.  Residuals
rhAR  Recombinant human androgen receptor
RLU  Relative light units
RPC  Rat prostate cytosol
RUC  Rat uterine cytosol
SDWA  Safe Drinking Water Act
SE  Standard error
SEP  Special Emphasis Panel
SSE  Error sum of squares
T3  Triiodothyronine
T4  Thyroxine
TA  Transcriptional activation
TPA  Tetradecanoylphorbol-13 acetate
t test  Student’s t test
TSCA  Toxic Substances Control Act
U  Uterotrophic assay
Univ.  University
vit ERE  Vitellogenin estrogen response element
VP16  A viral protein that acts as a transcriptional activator
v/v  Volume-to-volume ratio
>  Greater than
≥  Greater than or equal to
=  Equal to
<  Less than
≤  Less than or equal to
α  Alpha
β  Beta
γ  Gamma
µL  Microliter
µM  Micromolar
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  Rajendra S. Chhabra, Ph.D., D.A.B.T
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Preface

There is evidence linking exposure to natural and man-made substances in the environment to adverse effects on the endocrine and reproductive systems of mammals, birds, reptiles, amphibians, and fish (EPA 1997; NAS 1999). In response to growing concerns about possible adverse health effects in humans exposed to such substances, the U.S. Congress enacted relevant provisions to safeguard public health in the Food Quality Protection Act (FQPA) of 1996 (Public Law [P.L.] 104-170) and the 1996 Amendments to the Safe Drinking Water Act (SDWA) (P.L. 104-182). These laws require the U.S. Environmental Protection Agency (EPA) to develop and validate a screening and testing program to identify substances with endocrine disrupting activity. The EPA subsequently proposed an Endocrine Disruptor Screening Program (EDSP) (EPA 1998) and began efforts to standardize and validate test methods for inclusion in the EDSP. Validation assesses whether test methods are sufficiently accurate and reproducible for their intended use.

In April 2000, the EPA asked the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) to evaluate the validation status of in vitro estrogen receptor (ER) and androgen receptor (AR) binding and transcriptional activation (TA) assays, which were proposed as possible components of the EDSP Tier 1 screening battery. ICCVAM, which is charged by law (P.L. 106-545) to evaluate the scientific validity of new, revised, and alternative test methods proposed for specific regulatory uses, agreed to evaluate these assays based on their potential interagency applicability and public health significance. Because a large number of in vitro ER- and AR-based assays were known to exist, it was expected that at least some of these would have been adequately validated and could be rapidly included in the EDSP following a review of existing data and verification of their validity. The EPA also asked for the development of minimum performance standards that could be used to define acceptable in vitro ER and AR binding and TA assays. It was envisioned that these standards would be based on the performance of validated in vitro ER- and AR-based assays.

The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) subsequently compiled available relevant data and information on the in vitro ER and AR binding and TA assays. A draft Background Review Document (BRD) was organized for each of the four types of assays according to published guidelines for submission of test methods to ICCVAM (ICCVAM 1999). This comprehensive review revealed that there were no adequately validated in vitro ER- or AR-based assays, and therefore, no assays that could serve as the basis for establishing minimum performance standards. It was also discovered that there was little consistency among available protocols, and that no test method protocol was adequately detailed and standardized. Therefore, minimum procedural standards were proposed that should be incorporated in standardized protocols for each of the four types of assays. These minimum procedural standards include critical elements such as dose selection criteria, number of replicates per test, appropriate positive and negative controls, and criteria for an acceptable test. In addition, each BRD included a list of proposed substances that should be used for the validation of in vitro ER and AR binding and TA assays.
ICCVAM asked its Endocrine Disruptor Working Group (EDWG) to assist NICEATM with the technical evaluation of the four types of in vitro endocrine disruptor assays. The EDWG, which is comprised of knowledgeable scientists from participating ICCVAM agencies, was charged with:

- identifying and recommending scientists for the Expert Panel;
- reviewing the four draft BRDs for completeness and accuracy;
- developing questions for the Expert Panel to consider during their deliberations;
- developing draft ICCVAM recommendations based on the conclusions and recommendations of the Expert Panel.

On March 23, 2001, a Federal Register (FR) notice (66 FR 57: 16278-16279, March 23, 2001) requested data and nominations of expert scientists for an independent peer review evaluation of in vitro ER and AR binding and TA assays for endocrine disruptor screening. Data and nominations were also solicited from Federal agencies and national and international professional societies and organizations. An Expert Panel consisting of 24 scientists was selected based on advice from the EDWG. The expertise of the members included reproductive toxicology, androgen and/or estrogen receptor binding and transcriptional activation assays, validation of alternative in vitro methods, ecotoxicology, and biostatistics. The Expert Panel members were from the United States, the United Kingdom, Canada, Japan, and Denmark, and included scientists from industry, academia, and government.

The Expert Panel was charged with reviewing the information and recommendations provided in the four draft BRDs, and developing conclusions and recommendations on the following:

- specific assays that should undergo further evaluation in validation studies, and their relative priority for evaluation;
- the adequacy of the proposed minimum procedural standards;
- the adequacy of protocols for specific test methods recommended for validation;
- the adequacy and appropriateness of substances proposed for validation studies.

The Expert Panel members were assigned to one of four groups, each group with primary responsibility for one of the four types of assays being considered. In addition, each member of the Expert Panel was asked to evaluate and comment on the other three types of assays.

The Expert Panel meeting was announced to the public in a FR notice (67 FR 66: 16415-16416, April 5, 2002), which also included an announcement of the availability of the four draft BRDs and a request for public comments. The public comments and information submitted in response to this notice were provided to the Expert Panel and the public in advance of the meeting. The Expert Panel met in public session on May 21-22, 2002, in Research Triangle Park, North Carolina. The Expert Panel presented the evaluations, conclusions, and recommendations for each of the four types of in vitro ER- and AR-based assays. Opportunities for public comment were provided during the meeting. After consideration of the public comments, the Expert Panel reached consensus on each of its recommendations. The Expert Panel’s written evaluations and recommendations were consolidated into an independent report, which is included in this document as Appendix A.

Following the Expert Panel meeting, the EDWG, in collaboration with NICEATM, revised the draft minimum procedural standards and the draft list of proposed substances to incorporate the recommendations of the Expert Panel. The
four draft BRDs were subsequently revised to address corrections and omissions noted by the Expert Panel and published as final versions. Due to the length of these documents, they are not included in this report but are available on the ICCVAM/NICEATM website http://iccvam.niehs.nih.gov/methods/endocrine.htm.

In October 2002, the final report of the Expert Panel and the EDWG’s revised list of proposed substances for validation of in vitro ER and AR binding and TA assays were made available to the public for comment (67 FR 204: 64902-64903, October 22, 2002). Following review of the public comments, the EDWG and ICCVAM finalized the recommendations that are provided in this report. These recommendations include suggested assays for future validation, minimum procedural standards, and substances that should be used to standardize and validate in vitro ER and AR binding and TA assays. The final Expert Panel report, public comments, and other relevant documents are appended to this document, all of which are available on the ICCVAM/NICEATM website http://iccvam.niehs.nih.gov/methods/endocrine.htm.

Use of the minimum procedural standards and the recommended validation substances should facilitate standardization and validation of in vitro endocrine disruptor assays, as well as facilitate test method comparison to determine which ones are the most sensitive and reliable. Data from studies to validate one or more test methods that incorporate the recommended minimum procedural standards will serve as the basis for developing minimum performance standards for acceptable in vitro ER- or AR-based assays. The EDSP will use data generated from validated in vitro and in vivo Tier 1 screening assays to make decisions, based on a weight-of-evidence approach, on whether to conduct large multi-generational in vivo studies. It is also anticipated that data obtained during the validation of the four different types of in vitro ER- and AR-based assays will help characterize the extent to which individual or batteries of in vitro endocrine disruptor assays might be used to prioritize chemicals for Tier 2 testing. Finally, implementation of the recommendations in this report are expected to decrease and perhaps eventually eliminate the need to use male and female animals as a source of AR and ER, respectively, for in vitro screening assays.

Since several Federal agencies are involved in supporting or conducting endocrine disruptor test method development and validation, or otherwise have an interest in endocrine disruptor testing, this report containing ICCVAM’s recommendations will be forwarded to agencies for their consideration and information. Because the ICCVAM evaluation determined that none of these in vitro methods has been adequately validated, formal test recommendations will not be forwarded to Federal agencies. Following adequate validation and submission to ICCVAM of one or more of these in vitro endocrine disruptor methods, ICCVAM and NICEATM will coordinate their scientific peer review. After this review, formal ICCVAM test recommendations will then be forwarded to Federal agencies as required by the ICCVAM Authorization Act of 2000 (P.L. 106-545).

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Preface

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for their service as Group Chairs. The efforts of the EDWG were invaluable for assuring a meaningful and comprehensive review. We especially thank the Co-Chairs of the EDWG, Drs. David Hattan and Marilyn Wind, for their excellent leadership. The efforts of the NICEATM staff in preparing the BRDs, organizing the Expert Panel meeting, and preparing this final report are greatly appreciated. We especially acknowledge Dr. Barbara Shane, Ms. Christina Inhof, Dr. Errol Zeiger, and Dr. Raymond Tice for their contributions to the BRDs and for their assistance with preparation of this final report, and Mr. Bradley Blackard, who coordinated communications and logistics throughout the entire project. Finally, we appreciate and acknowledge the comments received from _ad hoc_ reviewers of the BRDs, as well as the comments submitted by members of the public.
Executive Summary

Endocrine disrupting substances are defined as chemicals that interfere with the normal function of hormones, either during development or during the life of an animal, resulting in abnormal development, growth, or reproduction (Ankley et al. 1998; Combes 2000; EPA 1998; Gray et al. 1998). Concern regarding these substances arises from observations of reproductive and developmental abnormalities in animal populations exposed to high levels of certain persistent pollutants in the environment. In addition, human health consequences including increases in the incidence of birth defects, cancers in hormonally-receptive tissues, and decreased fertility have been attributed to exposure of humans to endocrine disruptors. In response to these concerns, Congress directed the U.S. Environmental Protection Agency (EPA) in 1996 to validate and implement a screening and testing program to evaluate the potential of these substances to cause hormone-related health effects (Public Law [P.L.] 104-170). Based on advice from the EPA Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), EPA proposed the Endocrine Disruptor Screening Program (EDSP) (EPA 1998). The EDSP consists of a Tier 1 screening battery of \textit{in vitro} and \textit{in vivo} assays that is designed to identify substances capable of interacting with the endocrine system. Tier 2 of the EDSP is a battery of \textit{in vivo} assays that provides detailed information on concentration response relationships and specific abnormal effects. Based on a weight-of-evidence evaluation of the results from the Tier 1 screening battery, Tier 2 \textit{in vivo} tests are conducted. Included among the proposed Tier 1 \textit{in vitro} assays are estrogen receptor (ER) and androgen receptor (AR) binding and transcriptional activation (TA) assays.

In April 2000, EPA asked the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) to evaluate the validation status of \textit{in vitro} ER and AR binding and TA assays. ICCVAM, which is charged by law (P.L. 106-545) to evaluate the scientific validity of new, revised, and alternative test methods proposed for specific regulatory uses, agreed to evaluate the assays based on their potential interagency applicability and public health significance. Because a large number of \textit{in vitro} methods were known to exist, it was expected that at least some of these would have been adequately validated and could be rapidly included in the EDSP following a review of existing data and verification of their validity. The EPA also asked for the development of minimum performance standards that could be used to define acceptable \textit{in vitro} ER and AR binding and TA assays. It was envisioned that these standards would be based on the performance of validated \textit{in vitro} ER- and AR-based assays.

The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) subsequently compiled all available relevant data and information on the \textit{in vitro} methods of interest. A comprehensive review of these data determined that there were no adequately validated \textit{in vitro} ER- or AR-based assays, and therefore, no assays could serve as the basis for establishing minimum performance standards. It was also discovered that there was little consistency among available protocols, and that no assay protocol was adequately detailed and standardized. Minimum procedural standards were therefore proposed that should be incorporated in the standardized protocols for each of the four types of assays. These minimum procedural standards include critical elements such as
dose selection criteria, number of replicates per test, appropriate positive and negative controls, and criteria for an acceptable test.

Four draft Background Review Documents (BRDs) were developed and organized according to published guidelines for submission of test methods to ICCVAM (ICCVAM 1999). Each BRD (NIEHS 2002a, 2002b, 2002c, 2002d) contained:

- a description of the types of test methods used to measure the endpoints of interest and the available data substantiating their scientific validity;
- published and submitted data on substances tested in the test methods being considered;
- an evaluation of the comparative reliability and performance of the test methods being considered;
- specific protocols for test methods provided by interested scientists;
- a prioritized list of test methods recommended for validation;
- proposed minimum procedural standards for the types of test methods being considered;
- a list of substances proposed for future validation studies.

The final in vitro ER binding BRD summarized and evaluated data on 638 different substances tested at least once in one or more of 14 different test methods. The in vitro ER TA BRD summarized and evaluated data on 698 different substances tested at least once in one or more of 95 different test methods. The in vitro AR binding BRD summarized and evaluated data on 108 different substances tested at least once in one or more of 11 different test methods. The in vitro AR TA BRD summarized and evaluated data on 145 different substances tested at least once in one or more of 18 different test methods.

ICCVAM asked its Endocrine Disruptor Working Group (EDWG) to assist NICEATM with the technical evaluation of the four types of in vitro endocrine disruptor assays. The EDWG, which is comprised of knowledgeable scientists from participating ICCVAM agencies, was charged with:

- identifying and recommending scientists for the Expert Panel;
- reviewing the four draft BRDs for completeness and accuracy;
- developing questions for the Expert Panel to consider during their deliberations;
- developing draft ICCVAM recommendations based on the conclusions and recommendations of the Expert Panel.

An Expert Panel consisting of 24 scientists was selected based on advice from the EDWG. The expertise of the members included relevant areas such as reproductive toxicology, androgen and/or estrogen receptor binding and TA assays, validation of alternative in vitro methods, ecotoxicology, and biostatistics. The Expert Panel members were from the United States, the United Kingdom, Canada, Japan, and Denmark, and included scientists from industry, academia, and government.

The Expert Panel was charged with reviewing the information and recommendations provided in the four draft BRDs, and developing conclusions and recommendations on the following:

- specific test methods that should undergo further evaluation in validation studies, and their relative priority for evaluation;
- the adequacy of the proposed minimum procedural standards;
- the adequacy of protocols for specific test methods recommended for validation;
- the adequacy and appropriateness of substances proposed for validation studies.
The Expert Panel met in public session on May 21-22, 2002, in Research Triangle Park, North Carolina. The Expert Panel presented the evaluations, conclusions, and recommendations for each of the four types of assays. Opportunities for public comment were provided during the meeting. After consideration of the public comments, the Expert Panel reached consensus on each of its recommendations. The Expert Panel’s written evaluations and recommendations were consolidated into an independent report, which is included in this document as Appendix A.

Following the Expert Panel meeting, the four draft BRDs were revised to address corrections and omissions noted by the Expert Panel and published as final versions, which are available on the ICCVAM/NICEATM website http://iccvam.niehs.nih.gov/methods/endocrine.htm. Based on the recommendations of the Expert Panel, the EDWG, with the assistance of NICEATM, developed draft minimum procedural standards and lists of proposed substances for validation of ER and AR binding and TA assays.

In October 2002, the final report of the Expert Panel and the EDWG’s draft list of proposed substances were made available to the public for comment (67 FR 204: 64902-64903, October 22, 2002). Following their review of the public comments, the EDWG and ICCVAM finalized their recommendations on minimum procedural standards, test methods for future validation, and substances that should be used to standardize and validate the test methods. This information is provided in this report. The final Expert Panel report, public comments, and other relevant documents are appended to this report, and are available also on the ICCVAM/NICEATM website http://iccvam.niehs.nih.gov/methods/endocrine.htm.

Recommendations
ICCVAM concurs with the recommendations of the Expert Panel with regard to the four different types of assays. The major recommendations, organized by assay type, are:

**In Vitro ER Binding Assays**
- Recombinant rat or human ERs (α and β subtypes) should be given the highest priority for further test method standardization, prevalidation, and validation. Recombinant receptors are superior to crude cytosolic preparations because they can be prepared and distributed as standardized products with significantly less contamination. This will result in greater reproducibility and facilitate comparison of results across laboratories. To screen for possible ecological effects, recombinant receptors from wildlife are considered to be potentially more relevant and their use should be evaluated.
- Although it would be advantageous to use nonradioactive methods such as fluorescent polarization to assess ER binding, this method has not been widely used and specialized equipment is required. However, once a test method using recombinant ER proteins has been validated, there should be an effort to optimize a fluorescence-based method to replace the use of radioactivity.
- *In vitro* ER binding assay protocols should be standardized to incorporate the recommended minimum procedural standards (see Section 3.1). Exceptions should be justified with scientific rationale. Following protocol standardization, prevalidation studies should be conducted to optimize a reproducible protocol. Once this has been achieved, validation studies to assess the reliability and comparative performance of the test method should be conducted.
• Proposed in vitro ER binding test methods should be evaluated in validation studies using, at a minimum, the 53 substances listed in Section 3.2. This list includes substances that cover a range of activities, from negative to weakly positive to strongly positive, with 40 (75%) positive and presumed positive and 13 (25%) negative and presumed negative substances. The list also represents a wide range of relevant chemical and product classes (see Section 2.0). Following validation studies using the 53 substances, ICCVAM recommends that data should be generated on the remainder of the substances in the list of 78. The additional data will aid in the assessment of the usefulness of an in vitro test battery for prioritizing substances for subsequent in vivo studies.

In Vitro ER TA Assays
• A comparative study should be conducted to determine whether transiently or stably transfected cell lines are more appropriate for a routine test system. Transiently transfected systems generally have a higher level of responsiveness, while stably transfected cell lines have a lower level of responsiveness but are generally more amenable to high-throughput screening. Such a study should use cell lines with the same ER reporter gene constructs. A third cell line expressing an endogenous ER and transfected with the same reporter construct should be included in this study.

• In vitro ER TA assay protocols should be standardized to incorporate the recommended minimum procedural standards (see Section 4.1). Exceptions should be justified with scientific rationale. Following protocol standardization, pre-validation studies should be conducted to optimize a reproducible protocol. Once this has been achieved, validation studies to assess the reliability and comparative performance of the protocol should be conducted.

• To facilitate the comparison of in vitro ER-based assays, the same minimum list of 53 substances (provided in Section 4.2) recommended for ER binding assays should be used in the validation of in vitro ER TA agonist and antagonist assays. For ER TA agonism and antagonism assays, 34 (64%) and 11 (21%) of the substances, respectively, are reported to be positive or presumed positive, and 19 (36%) and 42 (79%) of the substances, respectively, are presumed negative. Following validation studies using the 53 substances, ICCVAM recommends that data should be generated on the remainder of the substances included in the list of 78. The additional data will aid in the assessment of the usefulness of an in vitro test battery for prioritizing substances for subsequent in vivo studies.

In Vitro AR Binding Assays
• A recombinant protein should be used as the source of the AR. Recombinant receptors are superior to crude cytosolic preparations because the recombinant protein can be standardized, which contributes to improved quality control and comparison of results across laboratories. Thus, the highest priority for future research and development efforts should be given to the development of a test method using a recombinant full-length AR protein. Patents on the AR protein have hindered development of this assay.

• In vitro AR binding assay protocols should be standardized to incorporate the recommended minimum procedural standards (see Section 5.1). Exceptions should be justified with scientific rationale. Following protocol standardization, prevalidation studies should be conducted to optimize a reproducible protocol. Once
this has been achieved, validation studies to assess the reliability and comparative performance of the protocol should be conducted.

- Proposed *in vitro* AR binding assays should be evaluated in validation studies using, at a minimum, the 44 substances listed in Section 5.2. This list consists of 33 (75%) positive and presumed positive substances and 11 (25%) presumed negative substances for AR binding. Following validation studies using the 44 substances, ICCVAM recommends that data should be generated on the remainder of the substances included in the list of 78. The additional data will aid in the assessment of the usefulness of an *in vitro* test battery for prioritizing substances for subsequent *in vivo* studies.

**In Vitro AR TA Assays**

- None of the *in vitro* AR TA assays reviewed by the Expert Panel were considered optimal for assessing AR agonist and antagonist activities. The highest priority for future efforts should be a cell line containing an endogenous AR that is transduced with an adenovirus containing a reporter vector that shows high specificity for the AR. The chosen cell line should not respond to, or have minimal response levels for, the glucocorticoid and progesterone receptors. Because of patent restrictions, it may be necessary that a cell line with an endogenous AR be used for validation. Transduction of a reporter construct in a virus particle is more efficient and reproducible than transfection of a construct.

- *In vitro* AR TA assay protocols should be standardized to incorporate the recommended minimum procedural standards (see Section 6.1). Exceptions should be justified with scientific rationale. Following protocol standardization, prevalidation studies should be conducted to optimize a reproducible protocol. Once this has been achieved, validation studies to assess the reliability and comparative performance of the protocol should be conducted.

- To facilitate *in vitro* AR-based test method comparisons, the same minimum list of 44 substances (provided in Section 6.2) recommended for *in vitro* AR binding assays should be used in the validation of *in vitro* AR TA agonist and antagonist assays. For AR TA agonism and antagonism assays, 20 (45%) and 20 (45%) of the substances, respectively, are reported to be positive and presumed positive, and 24 (55%) and 24 (55%) of the substances, respectively, are presumed negative. Following validation studies using the 44 substances, ICCVAM recommends that data should be generated on the remainder of the substances included in the list of 78. The additional data will aid in the assessment of the usefulness of an *in vitro* test battery for prioritizing substances for subsequent *in vivo* studies.

**Other Recommendations**

ICCVAM agrees with the Expert Panel that the development and validation of *in vitro* ER and AR binding and TA assays should emphasize the use of recombinant-derived proteins. Based on current knowledge and experience, it appears that continuing to use animal-derived ER or AR in *in vitro* endocrine disruptor test methods requires scientific justification. The advantages of using recombinant-derived receptors for binding test methods include:

- Standardized recombinant protein can be prepared and used by multiple laboratories, which will contribute to improved inter- and intra-laboratory reproducibility and an enhanced ability to compare results across laboratories.
• Recombinant-derived receptors avoid the disadvantages of animal-derived receptors, which include:
  - The receptors, particularly the ARs, are unstable in tissue extracts.
  - The cytosolic extracts contain many proteins, including other endogenous steroid receptors that can interfere with the performance of the assay.
  - Animals have to undergo surgery before isolation of the tissue of interest. For AR binding assays, males are castrated, and, for ER binding assays, females undergo an ovariectomy before removal of the requisite tissues and isolation of the respective receptors.
  - Animals need to be killed to obtain either the uterus (ER binding) or prostate (AR binding) glands.
• The inclusion of a metabolic activation system in in vitro ER and AR binding and TA assays is not recommended at this time, as the type of metabolic activation system developed will depend on which in vitro assays are selected. Available information on the metabolism of the validation substances should be compiled, including the degree to which metabolism is known to alter estrogenic and androgenic activity in vivo. Once the importance of metabolic activation in the ability of substances to disrupt endocrine function has been demonstrated, and valid in vitro ER and AR binding and TA assays have been identified, appropriate methods for including metabolic activation in the assays can be developed and validated.
• The current analyses for making statistical inferences with in vitro endocrine disruptor data require more detailed research and study. Appropriate prevalidation studies should be conducted to generate data necessary for biostatisticians to develop appropriate statistical methods for analyzing binding and TA agonist and antagonist assay data.
• Although these in vitro endocrine disruptor assays are proposed as components of a screening test battery where the results will be used in making weight-of-evidence decisions, the predictive value of these in vitro assays for estimating in vivo responses should be determined. To facilitate this determination, ICCVAM recommends that all 78 substances (see Section 2.0) should be evaluated in each in vitro assay. It is only through this effort that the performance of the in vitro test methods for predicting responses in animals can be evaluated and decisions made as to whether and how in vitro assays can reduce or replace animal use. Such data will also be needed to determine the usefulness of the in vitro battery for prioritizing substances for further testing.
• A centralized repository of the 78 substances with verified purity should be organized to facilitate future validation studies. The purpose of this repository is to provide a source of coded samples, of known purity, for validation studies. This approach would greatly enhance evaluation of the comparative reliability and performance of different versions of in vitro ER and AR binding and TA assays.
• Federal agencies are encouraged to support research and development of new technologies (e.g., genomics) that may provide more accurate assessments and/or advantages in terms of time and cost.
1.0 ICCVAM EVALUATION AND RECOMMENDATIONS ON ESTROGEN AND ANDROGEN RECEPTOR BINDING AND TRANSCRIPTIONAL ACTIVATION ASSAYS

ICCVAM evaluates the scientific validity of new, revised, and alternative toxicological test methods applicable to Federal agency safety testing requirements, and provides recommendations to Federal agencies about the usefulness and limitations of such methods (P.L. 106-545). In 2000, EPA requested that ICCVAM conduct an independent scientific peer review of the validation status of in vitro ER and AR binding and TA assays. This section describes the evaluation completed by ICCVAM in collaboration with NICEATM, and provides ICCVAM’s recommendations on these test methods.

1.1 Introduction

In vitro ER and AR binding and TA assays are proposed as part of EPA’s EDSP Tier 1 screening battery of in vitro and in vivo test methods designed to identify substances capable of interacting with the endocrine system. Data generated by these Tier 1 screening assays will be used to make decisions based on a weight-of-evidence approach on whether to conduct Tier 2 testing. With partial support from EPA, NICEATM conducted a comprehensive literature search for relevant publications on these test methods. In addition to this literature search, NICEATM requested through the FR (66 FR 57: 16278-16279, March 23, 2001) that interested scientists submit published and unpublished data on these test methods for consideration. A draft BRD was prepared for each of the four types of assays (NIEHS 2002a, 2002b, 2002c, 2002d). Each BRD includes:

- a description of the types of test methods used to measure the endpoints of interest and the available data substantiating their scientific validity;
- published and submitted data on substances tested in the test methods being considered;
- an evaluation of the comparative reliability and performance of the test methods being considered;
- test method specific protocols provided by interested scientists;
- a prioritized list of test methods recommended for validation;
- proposed minimum procedural standards for the types of test methods being considered; and
- a list of substances proposed for future validation studies.

The review revealed that no inter- and intra-laboratory validation studies had been conducted on in vitro ER or AR binding and TA assays. Therefore, ICCVAM and EPA agreed that an Expert Panel should be convened to evaluate currently available test methods and to recommend future validation efforts. NICEATM, in collaboration with the EDWG, subsequently organized an Expert Panel meeting to evaluate the current status of ER and AR binding and TA assays.

1.1.1 ICCVAM/NICEATM Expert Panel Meeting

The Expert Panel meeting was held on May 21 and 22, 2002, at the Sheraton Imperial Hotel in Research Triangle Park, North Carolina. The 24 members of the Expert Panel (a list of members is provided in the Acknowledgments section) reviewed the four draft BRDs, assessed the current validation status of the four types of in vitro assays described in Sections 1.1.1.1 through 1.1.1.4, and developed recommendations (see Appendix A) on:
• test methods that should be considered for further evaluation in validation studies and their relative priority;
• the adequacy of the proposed minimum procedural standards for each of the four types of test methods;
• the adequacy of available protocols for test methods recommended for validation studies; and
• the adequacy and appropriateness of the substances recommended for use in the validation studies.

1.1.1 In Vitro ER Binding Assays
The Expert Panel reviewed 14 different in vitro ER binding assays in which 638 different substances had been tested at least once in one or more of the test methods (NIEHS 2002a). The sources of the ER for the different test methods included:
• cytosol prepared from MCF-7 cells, a cell line derived from human breast cancer adenocarcinoma cells;
• cytosol from the uteri of mice, rats, and rabbits;
• intact MCF-7 cells;
• purified recombinant human ERα and ERβ; and
• fusion proteins between glutathione-S-transferase and the binding domains of the human ERα, and ER from mouse, chicken, anole (a reptile), and rainbow trout.

1.1.2 In Vitro ER TA Assays
The Expert Panel reviewed 95 different ER TA assays (73 mammalian cell and 13 yeast strain reporter gene assays, and 9 mammalian cell proliferation assays) in which 698 different substances had been tested at least once in one or more of the test methods. The sources of the ER included:
• unspecified ERs from human, mouse, and rat; and
• ERα and ERβ subtypes found endogenously, or transiently or stably transfected into various cell lines.

The reporter genes used in these test methods included:
• luciferase and chloramphenicol acetyltransferase in the mammalian cell line assays; and
• β-galactosidase in the yeast assays.

1.1.3 In Vitro AR Binding Assays
The Expert Panel reviewed 11 different in vitro AR binding assays in which 108 different substances had been tested at least once in one or more assays. The sources of AR used in these test methods included:
• cytosol from calf uteri, rat epididymes, rat prostate glands, and MCF-7 cells;
• rat epididymal nuclear fraction;
• COS-1 cells transiently transfected with human AR;
• human genital fibroblasts with an endogenous AR;
• LNCaP cells with an endogenous mutant AR; and
• semipurified recombinant human AR.

1.1.4 In Vitro AR TA Assays
The Expert Panel reviewed 17 different AR TA assays (15 mammalian cell and 1 yeast reporter gene assays, and 1 mammalian cell proliferation assay), in which 145 different substances had been tested at least once in one or more of the assays. The source of the AR used in these test methods included ARs from human, mouse, and rat.

The reporter genes used in the test methods included:
• luciferase and chloramphenicol acetyltransferase in the mammalian cell line assays; and
• β-galactosidase in the yeast assay.
1.1.1.5 Final Report of the Expert Panel
The Expert Panel’s conclusions and recommendations on each type of test method are provided in its final report (Appendix A). The four draft BRDs were subsequently revised to incorporate changes and corrections recommended by the Expert Panel (see Section 1.1.1). Electronic copies of the final BRDs are available on the ICCVAM/NICEATM website http://iccvam.niehs.nih.gov/methods/endocrine.htm.

1.2 ICCVAM Proposed Substances for Validation of In Vitro Endocrine Disruptor Assays
To facilitate future validation efforts and the comparison of performance among different test methods and protocols, the EDWG, NICEATM, and ICCVAM drafted a list of 122 proposed substances to be used in future validation studies for each of the four types of assays. This list incorporated:

- substances proposed in the four BRDs and endorsed by the Expert Panel;
- other substances recommended by the Expert Panel;
- substances proposed by EPA for validation of in vitro ER and AR binding assays and by EPA and the Organisation for Economic Co-operation and Development (OECD) for validation of in vivo endocrine disruptor assays (a list of these substances was compiled by Mr. James Kariya of the EPA and presented at the March 2002 meeting of the EPA Endocrine Disruptor Methods Validation Subcommittee [EDMVS]);
- substances to address the Expert Panel’s recommendation that the list contain at least 25% negative substances in order to adequately characterize test method specificity; and
- the Expert Panel’s recommendation that, for a specific receptor (ER or AR), the same substances should be tested in both binding and TA agonism and antagonism assays.

Subsequently, this draft list of 122 substances was reduced to a draft list of 78 proposed substances. Public comments on this draft list of proposed substances are provided in Appendix F and are discussed in Section 1.3. The substance selection criteria and the process used to develop the final proposed list of substances are described in Section 2.0.

To comprehensively assess the usefulness of binding and TA assays as individual components of the Tier 1 screening battery that will be used to prioritize substances for Tier 2 testing, and to facilitate development of more predictive in vitro endocrine disruptor assays, all 78 substances should be tested in the four types of assays. However, this list contains a relatively high proportion of substances, about 49% and 57%, which are anticipated to be negative in in vitro ER- and AR-based assays, respectively (see Section 2.0: Expert Panel Report, Appendix A). As only 25% negative substances are needed to adequately assess test method specificity, characterizing the activity of all 78 substances in in vitro ER and AR binding and TA assays might not be essential. Therefore, the EDWG and ICCVAM identified a list of 53 substances for ER-based assays and 44 substances for AR-based assays that should be used, at a minimum, during the validation of these test methods. These lists are discussed in Sections 3.0 through 6.0.

1.3 Public Comments
NICEATM announced in a FR notice (67 FR 204: 64902-64903, October 22, 2002) the availability of the Expert Panel’s report and the EDWG’s draft proposed list of substances for validation studies, and requested public comment. The final versions of the four BRDs and the summary minutes of the Expert Panel meeting (Appendix D) were
made available on the ICCVAM/NICEATM website http://iccvam.niehs.nih.gov/methods/endocrine.htm. Five public comments were received; these are briefly discussed in this section. The original comments are provided in Appendix F.

1.3.1 Comments Regarding the Suitability of Transcriptional Activation Assays Being Developed for Commercial Testing

Dr. Mitsuru Iida (Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan) submitted comments which focused on the ability of Otsuka’s in vitro TA assays, under development for commercial testing, to meet the recommendations of the Expert Panel for such test methods. Data and information were provided to support this position, including:

- The Otsuka AR-Ecoscreen™ assay uses a stably transfected cell line, which contains an androgen response element for which the AR has high affinity, and low levels of the glucocorticoid receptor.
- The Otsuka method for transfection of the reporter plasmid differs from the approach recommended by the Expert Panel in that the plasmid and the transfection reagent are added directly to the cells in the medium in which they are plated. This approach is reported as being superior to the adenovirus-based method of transduction recommended by the Expert Panel.
- The Otsuka AR-Ecoscreen™ can detect weak agonists and antagonists.
- The intra-assay coefficient of variation (CV) is 3.2% for studies using the stably transfected cell line and 5.9% for studies using the transiently transfected cell line. The corresponding inter-assay CVs are 8-14% and 16-22%. These CVs are reported as being less than those determined for the corresponding adenoviral transduction-based assay.
- An efficient internal monitor of cytotoxicity is included in each study.
- Corresponding ER TA assays with equal reliability have been developed.
- The test methods can be reliably applied at this time.

ICCVAM recognizes that the in vitro test methods developed by Otsuka might have merit, and suggests that Otsuka consider the recommendations contained in this report regarding minimum procedural standards and the substances proposed for validation studies, as well as the ICCVAM Submission Guidelines\(^1\). Following the completion of appropriate validation studies, the test methods can be submitted to ICCVAM for evaluation.

1.3.2 General Comments from the American Chemistry Council

Comments were submitted on behalf of the American Chemistry Council (ACC) by Dr. Richard Becker (Arlington, Virginia) regarding the Expert Panel’s Report and the list of proposed substances for validation studies. With respect to the binding and TA assays, the comments addressed the following points:

- EPA is obligated to validate a binding assay and a TA assay for AR and ER ligands if it intends to require submission of data from such assays as part of the EDSP. However, it is important to recognize that extensive use of any particular test method in basic academic research does not de facto validate its use for regulatory toxicity testing.
- There is an urgent need to validate a single technique for each type of assay. As noted in the Expert Panel report, there currently exists significant variability in techniques

\(^1\)Available at http://iccvam.niehs.nih.gov/docs/guidelines/subguide.htm.
and results. Furthermore, interlaboratory variability, sensitivity, reproducibility, and precision have not been sufficiently evaluated. The use of recombinant receptor proteins to reduce animal use and to more fully standardize components of the test method should be encouraged.

- EPA needs to address the patent restriction issues. It is essential that the test methods required for regulatory programs are widely available and that the regulated community is not put at risk of violating patents in order to comply with screening and testing requirements.

Comments submitted regarding the proposed list of substances included the following:

- Criteria need to be developed to select substances for validation efforts.
- Substances must be appropriately qualified and characterized.
- Each proposed substance must be appropriately referenced.
- The draft list needs to be reviewed and appropriately referenced, and any errors or omissions corrected.

ICCVAM agrees with the constructive comments provided by the ACC. Comments relevant to EPA will be brought to its attention when this report is forwarded to Federal agencies. The list of proposed substances has been revised with due consideration of the comments made. The selection criteria used to develop the final list of substances are provided in Section 2.0.

### 1.3.3 Chemical Abstracts Service Registry Number for Commercially Available Nonylphenol

A comment was provided by Dr. Barbara Losey of the Alkylphenols and Ethoxylates Research Council (AERC; Washington, District of Columbia) regarding the form of nonylphenol included in the list of proposed substances for validation studies. This nonylphenol (\(p\)-\(n\)-nonylphenol; Chemical Abstracts Service Registry Number [CASRN] 104-40-5) consists of a linear alkyl chain and is not representative of the commercial forms of nonylphenol. Commercial synthesis results in a mixture of various branched nonylphenol isomers represented by the CASRN 84852-15-3 rather than the production of one substance with a discrete chemical structure. The AERC believes that the commercial product is more relevant to human exposure and also the substance most frequently tested in in vivo endocrine disruptor studies.

Based on an assessment of the data in the BRDs, information on the specific form of nonylphenol tested in in vitro ER and AR binding and TA assays (as defined by the inclusion of a CASRN in the report) was provided for only 1 of 29 studies. In this single study, the commercial form of nonylphenol (CASRN 84852-15-3) was tested. However, while ICCVAM recognizes that \(p\)-\(n\)-nonylphenol is not a commercially relevant substance, this isomer is recommended for validation studies because its chemical structure is uniform. Samples of the commercial product would be expected to vary considerably in the ratio of various isomers, and this variability in chemical structure might contribute to increased variability in response across test methods. In post-validation studies, the form of the substance most relevant to human exposure should be tested.

#### 1.3.4 Scintillation Proximity Assay

Information was provided by Mr. Mike Scully (Amersham Biosciences, Cardiff, United Kingdom) about a scintillation proximity assay that measures the binding of a ligand to a receptor which is bound to a glass bead coated with a scintillant. Mr. Scully stated that this method eliminates washing steps and is fully amenable to automation. He stated also that
this method has applicability to the binding of ligands to ER and AR proteins and thus should be considered for future development efforts. References were provided on scintillation proximity assays, including one application to ER binding.

ICCVAM recognizes that the scintillation proximity assay developed by Amersham Biosciences might have merit. ICCVAM suggests that Amersham Biosciences consider the recommendations contained in this report regarding minimum procedural standards and the substances proposed for validation studies, as well as the ICCVAM Submission Guidelines\(^2\) if the company decides to submit their assay for evaluation to ICCVAM.

1.3.5 Response of Atrazine in ER and AR Binding and TA Assays

In the list of proposed substances for validation studies, the “anticipated in vitro response” for atrazine was that it would bind weakly in both ER and AR binding assays but would be negative in ER and AR TA assays. Dr. Charles Breckenridge (Syngenta Crop Protection, Inc., Greensboro, North Carolina) submitted a comment in which he noted that, based on the available data, it would be more appropriate to classify atrazine as negative and unknown for ER- and AR-based assays, respectively.

ICCVAM has revised the substance lists to categorize atrazine and other substances that were positive in 50% or fewer of the reported studies, as “presumed positives” for the in vitro endocrine disruptor assay of interest. This classification is used because erroneous positive studies are probably less likely to occur than erroneous negative studies due to the nature of binding assays and the protocols generally used. While this presumed positive classification is subjective for substances that test negative in the majority of tests conducted, it is anticipated that testing these substances will provide critical information on the comparative sensitivity and reliability of different in vitro endocrine disruptor assays.

1.4 ICCVAM Recommendations

ICCVAM reviewed the Expert Panel’s report (provided in Appendix A), and concurs with their conclusions and recommendations. For convenience to the reader, the major recommendations and conclusions are summarized in this section. More detailed information and discussion can be found in the Expert Panel’s report. Other important considerations and additional recommendations from ICCVAM are provided in Section 1.4.5.

1.4.1 In Vitro ER Binding Assays

- Recombinant rat or human ERs (α and β subtypes) should be given the highest priority for further test method standardization, prevalidation, and validation. Recombinant receptors are superior to crude cytosolic preparations because they can be prepared and distributed as standardized products with significantly less contamination. This will result in greater reproducibility and facilitate comparison of results across laboratories. To screen for possible ecological effects, recombinant receptors from wildlife are considered to be potentially more relevant and their use should be evaluated.

- Although it would be advantageous to use nonradioactive methods such as fluorescent polarization to assess ER binding, this method has not been widely used and specialized equipment is required. However, once a test method using recombinant ER proteins has been validated, there should be an effort to

\(^2\)Available at http://iccvam.niehs.nih.gov/docs/guidelines/subguide.htm.
optimize a fluorescence-based method to replace the use of radioactivity.

- In vitro ER binding assay protocols should be standardized to incorporate the recommended minimum procedural standards (see Section 3.1). Exceptions should be justified. Following protocol standardization, prevalidation studies should be conducted to optimize a reproducible protocol. Once this has been achieved, validation studies to assess the reliability and comparative performance of the test method should be conducted.

- Proposed in vitro ER binding test methods should be evaluated in validation studies using, at a minimum, the 53 substances listed in Section 3.2. This list includes substances that cover a range of activities, from negative to weakly positive to strongly positive, with 40 (75%) positive and presumed positive and 13 (25%) negative and presumed negative substances. The list also represents a wide range of relevant chemical and product classes (see Section 2.0). Following validation studies using the 53 substances, ICCVAM recommends that data should be generated on the remainder of the substances included in the list of 78. The additional data will aid in the assessment of the usefulness of the screening test battery for prioritizing substances for subsequent in vivo studies.

1.4.2 In Vitro ER TA Assays

- A comparative study should be conducted to determine whether transiently or stably transfected cell lines are more appropriate for a routine test system. Transiently transfected systems generally have a higher level of responsiveness, while stably transfected cell lines have a lower level of responsiveness but are generally more amenable to high-throughput screening. Such a study should use cell lines with the same ER reporter gene constructs. A third cell line expressing an endogenous ER and transfected with the same reporter construct should be included in this study.

- In vitro ER TA assay protocols should be standardized to incorporate the recommended minimum procedural standards (see Section 4.1). Exceptions should be justified. Following protocol standardization, prevalidation studies should be conducted to optimize a reproducible protocol. Once this has been achieved, validation studies to assess the reliability and comparative performance of the test method should be performed.

- To facilitate the comparison of in vitro ER-based assays, the same minimum list of 53 substances (provided in Section 4.2) recommended for ER binding assays should be used in the validation of in vitro ER TA agonist and antagonist assays. For ER TA agonism and antagonism assays, 34 (64%) and 11 (21%) of the substances, respectively, are reported to be positive or presumed positive, and 19 (36%) and 42 (79%) of the substances, respectively, are presumed negative. Following validation studies using the 53 substances, ICCVAM recommends that data should be generated on the remainder of the substances included in the list of 78. The additional data will aid in the assessment of the usefulness of a screening test battery for prioritizing substances for subsequent in vivo studies.

1.4.3 In Vitro AR Binding Assays

- A recombinant protein should be used as the source of the AR. Recombinant receptors are superior to crude cytosolic preparations because the recombinant protein can be standardized, which contributes to improved quality control and comparison of results across laboratories. Thus, the highest priority for future research and development efforts should
be given to the development of a test method using a recombinant full-length AR protein. Patents on the AR protein have hindered development of this assay.

- **In vitro** AR binding assay protocols should be standardized to incorporate the recommended minimum procedural standards (see Section 5.1). Exceptions should be justified. Following protocol standardization, prevalidation studies should be conducted to optimize a reproducible protocol. Once this has been achieved, validation studies to assess the reliability and comparative performance of the protocol should be conducted.

- Proposed **in vitro** AR binding assays should be evaluated in validation studies using, at a minimum, the 44 substances listed in Section 5.2. This list consists of 33 (75%) positive and presumed positive substances and 11 (25%) presumed negative substances for AR binding. Following validation studies using the 44 substances, ICCVAM recommends that data should be generated on the remainder of the substances included in the list of 78. The additional data will aid in the assessment of the usefulness of an **in vitro** test battery for prioritizing substances for subsequent **in vivo** studies.

1.4.4 **In Vitro** AR TA Assays

- None of the **in vitro** AR TA assays reviewed by the Expert Panel were considered optimal for assessing AR agonist and antagonist activities. The highest priority for future efforts should be a cell line containing an endogenous AR that is transduced with an adenovirus containing a reporter vector that shows high specificity for the AR. The chosen cell line should not respond to, or have minimal response levels for, the glucocorticoid and progesterone receptors. Because of patent restrictions, it may be necessary that a cell line with an endogenous AR be used for validation. Transduction of a reporter construct in a virus particle is more efficient and reproducible than transfection of a construct.

- **In vitro** AR TA assay protocols should be standardized to incorporate the recommended minimum procedural standards (see Section 6.1). Exceptions should be justified. Following protocol standardization, prevalidation studies should be conducted to optimize a reproducible protocol. Once this has been achieved, validation studies to assess the reliability and comparative performance of the protocol should be conducted.

- To facilitate **in vitro** AR-based assay comparisons, the same minimum list of 44 substances (provided in Section 6.2) recommended for **in vitro** AR binding assays should be used in the validation of **in vitro** AR TA agonist and antagonist assays. For AR TA agonism and antagonism assays, 20 (45%) and 20 (45%) of the substances, respectively, are reported to be positive or presumed positive, and 24 (55%) and 24 (55%) of the substances, respectively, are presumed negative. Following validation studies using the 44 substances, ICCVAM recommends that data should be generated on the remainder of the substances included in the list of 78. The additional data will aid in the assessment of the usefulness of an screening test battery for prioritizing substances for subsequent **in vivo** studies.

1.4.5 Other Recommendations

ICCVAM agrees with the Expert Panel that the development and validation of **in vitro** ER and AR binding and TA assays should emphasize the use of recombinant-derived proteins. Based on current knowledge and experience, it appears that continuing to use animal-derived ER or AR in **in vitro** endocrine disruptor assays requires scientific justification. The
advantages of using recombinant-derived receptors for binding assays include:

- Standardized recombinant protein can be prepared and used by multiple laboratories, which will contribute to improved inter- and intra-laboratory reproducibility and an enhanced ability to compare results across laboratories.
- Recombinant-derived receptors avoids the disadvantages of animal-derived receptors, which include:
  - The receptors, particularly the ARs, are unstable in tissue extracts.
  - The cytosolic extracts contain many proteins, including other endogenous steroid receptors that can interfere with the performance of the assay.
  - Animals have to undergo surgery before isolation of the tissue of interest. For AR binding assays, males are castrated, and, for ER binding assays, females undergo an ovariectomy before removal of the requisite tissues and isolation of the respective receptors.
  - Animals need to be killed to obtain either the uterus (ER binding) or prostate (AR binding) glands.
- The inclusion of a metabolic activation system in in vitro ER and AR binding and TA assays is not recommended at this time, as the type of metabolic activation system developed will depend on which in vitro assays are selected. Available information on the metabolism of the validation substances should be compiled, including the degree to which metabolism is known to alter estrogenic and androgenic activity in vivo. Once the importance of metabolic activation in the ability of substances to disrupt endocrine function has been demonstrated, and valid in vitro ER and AR binding and TA assays have been identified, appropriate methods for including metabolic activation in the assays can be developed and validated.
- The current analyses for making statistical inferences with in vitro endocrine disruptor data require more detailed research and study. Appropriate prevalidation studies should be conducted to generate data necessary for biostatisticians to develop appropriate statistical methods for analyzing binding and TA agonist and antagonist assay data.
- Although these in vitro endocrine disruptor assays are proposed as components of a screening test battery where the results will be used in making weight-of-evidence decisions, the predictive value of these in vitro assays for estimating in vivo responses should be determined. To facilitate this determination, ICCVAM recommends that all 78 substances (see Section 2.0) should be evaluated in each in vitro assay. It is only through this effort that the performance of the in vitro assays for predicting responses in animals can be evaluated and decisions made as to whether and how in vitro assays can reduce or replace animal use. Such data will also be needed to determine the usefulness of the in vitro battery for prioritizing substances for further testing.
- A centralized repository of the 78 substances with verified purity should be organized to facilitate future validation studies. The purpose of this repository is to provide a source of coded samples, of known purity, for validation studies. This approach would greatly enhance evaluation of the comparative reliability and performance of different versions of in vitro ER and AR binding and TA assays.
- Federal agencies are encouraged to support research and development of new technologies (e.g., genomics) that may provide more accurate assessments and/or advantages in terms of time and cost.
1.5 Other Considerations

• The Panel recommended that appropriate government agencies investigate the status of patents and licenses pertinent to the use of the human and rat AR and provide guidance as to how the scientific community should proceed with the development of in vitro AR assays.

• Although there is more information and data on ER binding studies with human ERα and ERβ than the equivalent receptors from rats, it might be more appropriate for the rat ERα or ERβ to be used for validation than the human receptors. This is because the rat is being used as the mammalian species of choice for in vivo Tier 1 and Tier 2 assays. Because the rat ERα has been isolated from the uterus and the ERβ from the prostate, the rat ERα would likely be the most appropriate receptor for ER binding studies (Kuiper et al., 1996). A study should be conducted to compare the responsiveness of the ERα from the rat to the ERα from humans in order to assess potential differences in the binding capacities of the receptor from the two species.
2.0 PROPOSED SUBSTANCES AND SELECTION CRITERIA FOR VALIDATION OF INVITRO ENDOCRINE DISRUPTOR SCREENING ASSAYS

2.1 Introduction
To facilitate the validation of in vitro ER and AR binding and TA assays, ICCVAM has compiled a list of 78 substances recommended for use in future validation studies. Versions of this list specific to each type of assay are provided in Sections 3.0 through 6.0. Each version includes the available quantitative and qualitative data for each substance, and its known or anticipated qualitative response in the assay type being considered. The available data are based on information compiled in the four BRDs, as well as information found in publications reviewed or published after completion of the BRDs. A number of factors and criteria were considered in compiling this list, including the recommendations of the four draft BRDs, the Expert Panel, and the EDWG, as well as substances proposed for in vitro endocrine disruptor testing by the EPA. To allow for a direct comparison between results obtained from in vitro and in vivo endocrine disruptor test methods, the list also includes substances proposed for in vivo endocrine disruptor testing by EPA and OECD.

2.2 Draft Background Review Document Recommendations
Each of the four draft BRDs included a list of substances recommended for future validation studies of the assay type considered. The number of substances included in each list are provided in Table 2–1. Selection of these substances was based on:
- the availability of published or submitted data demonstrating reproducible positive or negative responses in multiple studies and/or test methods;
- the extent to which these substances covered the range of negative to weakly positive to strongly positive responses; and
- the distribution of the proposed substances among chemical classes.

2.3 Expert Panel Recommendations on Proposed Substances for Validation Studies
As described in Section 1.1.1, an Expert Panel developed recommendations on the adequacy and appropriateness of the substances recommended in the draft BRDs for use in future validation studies. The Expert Panel generally agreed with the lists of proposed substances but also recommended that:
- for a specific receptor (ER or AR), the same substances should be tested in binding and TA agonism and antagonism assays;
- the proportion of negative substances in each list should be increased to at least 25% of the total number of substances to better evaluate test method specificity;
- an ER binding substance with a potency two orders of magnitude lower than 17β-estradiol should be included as a concurrent

\[1\] Inclusion of a substance does not mean that EPA, NICEATM, ICCVAM, or the Expert Panel has or will make a determination that any use of the substance will pose a significant risk. Further, these substances should not be interpreted to be “endocrine disruptors”; the substances listed are simply compounds that have been, or may prove to be useful in developing, standardizing, or validating screening and testing methods.

positive control in in vitro ER binding assays;

- substances (e.g., actinomycin D, cycloheximide, sodium azide, 12-O-tetradecanoylphorbol-13-acetate) that might interfere indirectly with reporter gene transcriptional activation by altering metabolic pathways, such as RNA and protein synthesis, should be included;

- additional substances from underrepresented chemical classes (e.g., phthalates, polycyclic aromatic hydrocarbons [PAHs], polychlorinated biphenyls) should be included; and

- a central repository should be organized to provide substances of high purity for use in future validation studies.

### 2.4 ICCVAM, EDWG, and NICEATM Proposed List of Substances for Validation

The EDWG subsequently reviewed the Expert Panel’s recommendations regarding substances that should be used in future validation studies and, in collaboration with NICEATM, developed a revised list of proposed substances. A challenging task was meeting the recommendation of the Expert Panel that at least 25% of the substances proposed for validation studies be negative for binding or TA for the receptor being used. During the preparation of the BRDs, only a few substances had been identified as consistently negative for the endpoint of interest in multiple studies (Table 2-1). However, on the assumption that some of the substances positive in ER binding or TA assays would likely be negative in the corresponding AR-based assays and vice versa, it was decided that such substances could serve as presumptive negatives in the alternative receptor-based assays. This approach would also minimize the total number of different chemicals to be included in an endocrine disruptor chemical repository.

#### 2.4.1 Candidate Substances

Initially, 122 candidate substances were identified for validation studies; this list was subsequently reduced to 78 substances. The 122 candidate substances consisted of:

- the 85 substances recommended in the four BRDs for future validation studies (see Section 12.0, Table 12-1 in the ER and AR Binding Assay BRDs, and Section 12.0, Tables 12–1 and 12–2 in the ER and AR TA BRDs) (NIEHS 2002a, 2002b, 2002c, 2002d);

- the 44 substances scheduled for testing in in vivo mammalian endocrine disruptor assays by the EPA and the OECD, 22 of which had been included in the lists provided in the BRDs. The in vivo list included five substances (oxazepam, phenobarbital, L-thyroxine, ammonium perchlorate, and propylthiouracil) that are known to disrupt thyroid function in vivo and thus could likely serve as presumed negative substances in in vitro ER and AR binding and TA assay validation studies;

- the 38 substances scheduled for testing in in vitro endocrine disruptor assays by the EPA, 29 of which had been included in the lists provided in the BRDs; and

- the 6 additional substances recommended by the Expert Panel.

Five of the candidate substances (butylbenzyl phthalate, diethylhexyl phthalate, dibenz[a,h]anthracene, fluoranthene, and zearalenone) belong to chemical classes that had been underrepresented in the BRD.

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3 On July 8, 2002, NICEATM received a list of the substances selected or recommended for in vitro endocrine disruptor testing by the EPA and for in vitro and in vivo endocrine disruptor testing by the EPA or the OECD from Mr. Gary E. Timm in the EPA Office of Science Coordination and Policy, Washington, DC. The list was compiled by Mr. James Kariya for presentation at the March 2002 meeting of the EPA EDMVS.
lists (phthalates for the first two substances, PAHs for the second two substances, and resorcylic acid lactone/phenol for the last substance). In addition, seven of the candidate substances (bisphenol A, 1,1-dichloro-bis[4-chlorophenyl]ethylen, dichlorodiphenyltrichloroethane, di-(2-ethylhexyl)phthalate, di-n-butylphthalate, nonylphenol, and octylphenol) have been tested in vivo for endocrine disruptor activity by the Japanese Ministry of Health (JME). The JME website http://www.env.go.jp/en/topic/edcs.html provides details on the specific in vivo test methods in which these substances were tested and the results obtained.

2.4.2 Selection of 78 Proposed Substances
The list of 122 candidate substances was reduced to 114 candidates based on the following:
- methyl parathion and 2,3,7,8-tetrachlorodibenzo-p-dioxin, highly toxic substances proposed by EPA for in vivo testing, were excluded to avoid potential worker exposure;
- 4-chloro-4'-biphenylol and 2',4',6'-trichloro-4-biphenylyl, two substances recommended in the draft BRDs, and Arochlor 1254, a substance proposed for in vivo testing by the EPA, were excluded because of hazardous waste disposal concerns;
- letrozole was excluded because EPA was not sure that it would be tested in vivo and because of the absence of in vitro data;
- testosterone propionate, also proposed for in vivo testing by EPA, was excluded because it is readily hydrolyzed in vivo to its parent compound, testosterone, which has been tested much more extensively in multiple in vitro endocrine disruptor assays; and
- tamoxifen citrate, proposed by the EPA for in vitro testing, was excluded because its parent compound, tamoxifen, has been tested much more extensively in multiple in vitro endocrine disruptor assays.

The remaining list of 114 candidate substances was reduced to 78 substances by excluding substances not scheduled for in vitro testing by the EPA or in vivo testing by EPA and OECD (with the exceptions noted above). Thus, 39 of the 44 substances proposed for in vivo testing by EPA and OECD are included in this list, as well as 37 of the 38 substances proposed for in vitro testing by EPA.

The expected performance of these 78 substances in the various in vitro endocrine disruptor assays is provided in Table 2-2A for in vitro ER-based assays and Table 2-2B for in vitro AR-based assays. Based on the available data, about 47% and 56% of the substances are expected to be negative in in vitro ER- and AR-based assays, respectively. Among these 78 substances, 70 chemical and 13 product classes are represented. Not all 78 substances could be assigned to a product class. The distribution of substances among chemical and product classes is provided in Tables 2-3 and 2-4, respectively, while Table 2-5 provides information on the chemical and product classes assigned to each of the recommended 78 substances.

2.4.3 Purpose and Advantages of the List of 78 Substances
The current goal of the EPA is to validate in vitro ER and AR binding and TA assays as components of the EDSP Tier 1 screening battery, which includes both in vitro and in vivo assays. The purpose of the list of 78 substances is to ensure that the comparative reliability and performance of in vitro ER and AR binding and TA assays are adequately characterized across a broad range of chemical classes and responses. Inclusion in this list of many of the substances proposed for the validation of Tier 1 and Tier 2 in vivo
2.4.4 Minimum Lists of Substances for Validation of In Vitro Endocrine Disruptor Assays

Because the purpose of these in vitro assays in the Tier 1 screening battery is to provide binding and TA data that will be considered in a weight-of-evidence evaluation to prioritize substances for Tier 2 testing, characterizing the activity of all of the substances expected to be negative in vitro (e.g., thyroid disruptors, aromatase inhibitors) may not be essential. Thus, ICCVAM developed minimum lists of substances that should be given priority during the validation of in vitro ER and AR binding and TA assays. For each receptor type, the same substances are proposed for testing in binding and TA (agonist and antagonist) assays. This approach will allow for a direct comparison of the reliability and performance of these different types of in vitro endocrine disruptor assays. The substances proposed in the BRDs and those being tested by the EPA in in vitro assays have been used as the foundation for each minimum list. Additional substances recommended by the Expert Panel (see Section 2.3), and those likely to be negative for the endpoint being assessed, complete the lists.

2.4.5 Data Supporting the Recommended Substances

The data provided with the substance lists in Sections 3.0 through 6.0 summarize information obtained primarily from peer-reviewed scientific reports and, secondarily, from two reports of unpublished in vitro TA test method data. These latter reports were received from Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan), and from Xenobiotic Detection Systems, Inc. (Durham, North Carolina). Of the 78 substances included in the primary list, relevant quantitative data from in vitro ER and AR binding studies are available for 45 (58%) and 33 (42%) of the substances, respectively. For in vitro ER TA assays, relevant quantitative or qualitative data from agonist and antagonist studies are

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4 This substance total excludes the reference estrogen, 17β-estradiol.
5 This substance total excludes the reference androgen, methyltrienolone.
available for 45 (58%) and 18 (23%) of the substances, respectively. For \textit{in vitro} AR TA assays, relevant quantitative or qualitative data from agonist and antagonist studies are available for 45 (58%) and 27 (35%) of the substances, respectively. Many of these substances were tested in only one or two of the four types of assays and often once only. Thus, there are numerous data gaps, as well as incomplete information, regarding how the different types of \textit{in vitro} ER- and AR-based assays will respond to the 78 recommended substances.

Because the data were generated by studies conducted by different laboratories using different experimental protocols, the data are highly variable and, thus, should not be used as definitive target values to be obtained during future validation studies. The intent of the data summaries presented in \textbf{Sections 3.2, 4.2, 5.2,} and 6.2 is to inform interested investigators of the published quantitative and qualitative responses obtained for these substances in the four types of assays. Moreover, although the anticipated responses assigned to substances lacking data are supported by indirect evidence in the literature, these assigned responses may prove to be inaccurate.
Table 2–1: Numbers of Substances Recommended in the BRDs for the Validation of *In Vitro* ER and AR Binding and TA Assays

<table>
<thead>
<tr>
<th>In Vitro Assay Type</th>
<th>Number of Substances</th>
<th>Number of Positive Substances</th>
<th>Number of Negative Substances</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER Binding</td>
<td>33</td>
<td>30 (91%)</td>
<td>3 (9%)</td>
</tr>
<tr>
<td>ER TA Agonism</td>
<td>31</td>
<td>25 (81%)</td>
<td>6 (19%)</td>
</tr>
<tr>
<td>ER TA Antagonism</td>
<td>20</td>
<td>16 (80%)</td>
<td>4 (20%)</td>
</tr>
<tr>
<td>AR Binding</td>
<td>31</td>
<td>28 (90%)</td>
<td>3 (10%)</td>
</tr>
<tr>
<td>AR TA Agonism</td>
<td>28</td>
<td>18 (64%)</td>
<td>10 (36%)</td>
</tr>
<tr>
<td>AR TA Antagonism</td>
<td>24</td>
<td>20 (83%)</td>
<td>4 (17%)</td>
</tr>
</tbody>
</table>
Table 2–2: Distribution of Anticipated Responses of the 78 Recommended Test Substances in *In Vitro* ER and AR Binding and TA Assays

### A. *In Vitro* ER-Based Assays

<table>
<thead>
<tr>
<th>Expected Response</th>
<th>ER Binding</th>
<th>ER TA</th>
<th>ER TA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Agonism</td>
<td>Antagonism</td>
<td></td>
</tr>
<tr>
<td>Positive(^b) and Presumed Positive(^c)</td>
<td>41 (53%)</td>
<td>35 (45%)</td>
<td>11 (14%)</td>
</tr>
<tr>
<td>Negative(^d) and Presumed Negative(^e)</td>
<td>37 (47%)</td>
<td>43 (55%)</td>
<td>67 (86%)</td>
</tr>
<tr>
<td>Total</td>
<td>78</td>
<td>78</td>
<td>78</td>
</tr>
</tbody>
</table>

\(^a\) Based on information provided in Sections 3.0 through 6.0. Counts include the recommended reference estrogen, 17β-estradiol.

\(^b\) Substances that tested positive for ER binding or ER TA in >50% of multiple studies conducted.

\(^c\) Substances that tested positive in ≤50% of reported ER binding or ER TA studies; that tested positive in the only study conducted; or that have no relevant receptor binding or TA data available for the test method of interest but which are presumed positive based on their known mechanism of action or their responses in other endocrine disruptor screening assays (e.g., methyl testosterone, an ER agonist, is presumed positive in ER binding assays).

\(^d\) Substances that tested negative for ER binding or ER TA in multiple studies, when tested up to the limit dose.

\(^e\) Substances that tested negative but had not been tested in multiple ER binding or in multiple ER TA studies up to the limit dose (i.e., 1 mM); or that have no relevant receptor binding or TA data available for the test method of interest but which are presumed negative based on their known mechanism of action or their responses in other endocrine disruptor screening assays (e.g., anastrazole and fadrozole, known aromatase inhibitors, are presumed negative in ER binding and TA assays).
### Table 2–2: Distribution of Anticipated Responses of the 78 Recommended Test Substances in *In Vitro* ER and AR Binding and TA Assays (continued)

#### B. *In Vitro* AR-Based Assays[^a]

<table>
<thead>
<tr>
<th>Expected Response</th>
<th>AR Binding</th>
<th>AR TA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Agonism</td>
</tr>
<tr>
<td>Positive[^b] and Presumed Positive[^c]</td>
<td>34 (44%)</td>
<td>22 (28%)</td>
</tr>
<tr>
<td>Negative[^d]</td>
<td>44 (56%)</td>
<td>56 (72%)</td>
</tr>
<tr>
<td>Total</td>
<td>78</td>
<td>78</td>
</tr>
</tbody>
</table>

[^a]: Based on information provided in Sections 3.0 through 6.0. Counts include the recommended reference androgen, methyltrienolone.

[^b]: Substances that tested positive for AR binding or AR TA in >50% of multiple studies conducted.

[^c]: Substances that tested positive in ≤50% of reported AR binding or AR TA studies; that tested positive in the only study conducted; or that have no relevant receptor binding or TA data available for the test method of interest but which are presumed positive based on their known mechanism of action or their responses in other endocrine disruptor screening assays (e.g., ketoconazole, an AR agonist, is presumed positive in AR binding assays).

[^d]: Substances that tested negative but had not been tested in multiple AR binding or in multiple AR TA studies up to the limit dose (i.e., 1 mM); or that have no relevant receptor binding or TA data available for the test method of interest but which are presumed negative based on their known mechanism of action or their responses in other endocrine disruptor screening assays (e.g., anastrazole and fadrozole, known aromatase inhibitors, are presumed negative in AR binding and TA assays). No substances could be classified as negative for AR binding or AR TA since none had been tested in multiple studies at or above the limit dose of 1 mM recommended in Sections 5.1.5 and 6.1.3.
Table 2–3: Distribution of the 78 Recommended Substances Among Chemical Classes

<table>
<thead>
<tr>
<th>Chemical Class b</th>
<th>Number of Substances Selected for Validation Studies c</th>
<th>Chemical Class b</th>
<th>Number of Substances Selected for Validation Studies c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkylphenol</td>
<td>2</td>
<td>Estrene</td>
<td>6</td>
</tr>
<tr>
<td>Amide</td>
<td>2</td>
<td>Flavanoid</td>
<td>6</td>
</tr>
<tr>
<td>Androstene</td>
<td>2</td>
<td>Flavone</td>
<td>4</td>
</tr>
<tr>
<td>Anilide</td>
<td>3</td>
<td>Fluorene</td>
<td>1</td>
</tr>
<tr>
<td>Anthracene</td>
<td>1</td>
<td>Glutaramide</td>
<td>1</td>
</tr>
<tr>
<td>Aromatic amine</td>
<td>1</td>
<td>Heterocycle</td>
<td>5</td>
</tr>
<tr>
<td>Aromatic amino acid</td>
<td>1</td>
<td>Imidazole</td>
<td>4</td>
</tr>
<tr>
<td>Arylamine</td>
<td>1</td>
<td>Isoflavone</td>
<td>2</td>
</tr>
<tr>
<td>Azide</td>
<td>1</td>
<td>Ketone</td>
<td>2</td>
</tr>
<tr>
<td>Benzimidazole</td>
<td>1</td>
<td>Lactone</td>
<td>1</td>
</tr>
<tr>
<td>Benzodiazepine</td>
<td>1</td>
<td>Nitrile</td>
<td>5</td>
</tr>
<tr>
<td>Benzopyranone</td>
<td>1</td>
<td>Nitrobenzene</td>
<td>2</td>
</tr>
<tr>
<td>Benzyllidene</td>
<td>3</td>
<td>Norpregnene</td>
<td>1</td>
</tr>
<tr>
<td>Bisphenol</td>
<td>3</td>
<td>Organic acid</td>
<td>2</td>
</tr>
<tr>
<td>Butyrophenone</td>
<td>1</td>
<td>Organic salt</td>
<td>2</td>
</tr>
<tr>
<td>Carbamate</td>
<td>1</td>
<td>Organochlorine</td>
<td>8</td>
</tr>
<tr>
<td>Chlorinated aromatic hydrocarbon</td>
<td>1</td>
<td>Paraben</td>
<td>1</td>
</tr>
<tr>
<td>Chlorinated bridged cycloalkane</td>
<td>1</td>
<td>Peptide</td>
<td>1</td>
</tr>
<tr>
<td>Chlorinated hydrocarbon</td>
<td>1</td>
<td>Phenol</td>
<td>14</td>
</tr>
<tr>
<td>Chlorinated triphenylethylene</td>
<td>1</td>
<td>Phenoxazone</td>
<td>1</td>
</tr>
<tr>
<td>Coumarin</td>
<td>1</td>
<td>Phorbol ester</td>
<td>1</td>
</tr>
<tr>
<td>Coumestan</td>
<td>1</td>
<td>Phthalate</td>
<td>3</td>
</tr>
<tr>
<td>Cyclic imide</td>
<td>2</td>
<td>Piperazine</td>
<td>2</td>
</tr>
<tr>
<td>Diphenylalkane</td>
<td>3</td>
<td>Piperidine</td>
<td>2</td>
</tr>
<tr>
<td>Diphenylalkene</td>
<td>3</td>
<td>Polycyclic aromatic hydrocarbon</td>
<td>2</td>
</tr>
<tr>
<td>Diphenyalkanecarboxylic acid</td>
<td>1</td>
<td>Polycyclic hydrocarbon</td>
<td>1</td>
</tr>
<tr>
<td>Diphenyl ether</td>
<td>1</td>
<td>Pregnenedione</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 2–3: Distribution of the 78 Recommended Substances Among Chemical Classesa
(continued)

<table>
<thead>
<tr>
<th>Chemical Class b</th>
<th>Number of Substances Selected for Validation Studiesc</th>
<th>Chemical Class b</th>
<th>Number of Substances Selected for Validation Studiesc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnene lactone</td>
<td>1</td>
<td>Terpene</td>
<td>1</td>
</tr>
<tr>
<td>Pyrimidine</td>
<td>3</td>
<td>Triazine</td>
<td>1</td>
</tr>
<tr>
<td>Quinoline</td>
<td>1</td>
<td>Triazole</td>
<td>1</td>
</tr>
<tr>
<td>Resorcylic acid lactone</td>
<td>1</td>
<td>Triphenylethylene</td>
<td>2</td>
</tr>
<tr>
<td>Steroid, nonphenolic</td>
<td>15</td>
<td>Triphenylmethane</td>
<td>1</td>
</tr>
<tr>
<td>Steroid, phenolic</td>
<td>5</td>
<td>Uracil</td>
<td>1</td>
</tr>
<tr>
<td>Stilbene</td>
<td>3</td>
<td>Urea</td>
<td>1</td>
</tr>
<tr>
<td>Sulfone</td>
<td>1</td>
<td>Yohimban</td>
<td>1</td>
</tr>
</tbody>
</table>

a Based on information provided in Table 2-5.

b Substances were assigned to chemical classes based on available information from standardized references (e.g., The Merck Index and the U.S. National Library of Medicine’s ChemID database) and from an assessment of chemical structure.

c Because a substance may be included in more than one chemical class, the number of substances selected for validation studies totaled across chemical classes exceeds the number of selected substances.
Table 2–4: Distribution of the 78 Recommended Substances Among Product Classes\(^a\)

<table>
<thead>
<tr>
<th>Product Class(^b)</th>
<th>Number of Substances Selected for Validation Studies(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adhesive</td>
<td>1</td>
</tr>
<tr>
<td>Analytical reagent</td>
<td>1</td>
</tr>
<tr>
<td>Chemical intermediate</td>
<td>6</td>
</tr>
<tr>
<td>Coatings</td>
<td>1</td>
</tr>
<tr>
<td>Dye</td>
<td>1</td>
</tr>
<tr>
<td>Hormone</td>
<td>3</td>
</tr>
<tr>
<td>Metabolic inhibitor</td>
<td>1</td>
</tr>
<tr>
<td>Natural product</td>
<td>7</td>
</tr>
<tr>
<td>Pesticide</td>
<td>9</td>
</tr>
<tr>
<td>Pesticide metabolite</td>
<td>1</td>
</tr>
<tr>
<td>Pharmaceutical</td>
<td>42</td>
</tr>
<tr>
<td>Pharmaceutical metabolite</td>
<td>1</td>
</tr>
<tr>
<td>Plasticizer</td>
<td>3</td>
</tr>
<tr>
<td>Could not be assigned to a product class</td>
<td>4</td>
</tr>
</tbody>
</table>

\(^a\) Based on information provided in Table 2-5.

\(^b\) Product classes were assigned based on information contained in *The Merck Index* and the U.S. National Library of Medicine’s ChemID database.

\(^c\) Because a substance may be assigned to more than one product class, the number of substances selected for validation studies totaled across product classes exceeds the number of selected substances.
Table 2–5: Chemical and Product Classes of the 78 Recommended Substances

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Chemical Class</th>
<th>Product Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomycin D</td>
<td>50-76-0</td>
<td>Phenoxazone; Lactone; Peptide</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Ammonium perchlorate</td>
<td>7790-98-9</td>
<td>Organic acid; Organic salt</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Anastrazole</td>
<td>120511-73-1</td>
<td>Nitrile; Triazole</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>4-Androstenedione</td>
<td>63-05-8</td>
<td>Steroid, nonphenolic</td>
<td>Hormone</td>
</tr>
<tr>
<td>Apigenin</td>
<td>520-36-5</td>
<td>Flavanoid; Flavone; Phenol</td>
<td>Natural product</td>
</tr>
<tr>
<td>Apomorphine</td>
<td>58-00-4</td>
<td>Heterocycle; Quinoline</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Atrazine</td>
<td>1912-24-9</td>
<td>Aromatic amine; Triazine; Arylamine</td>
<td>Pesticide</td>
</tr>
<tr>
<td>Bicalutamide</td>
<td>90357-06-5</td>
<td>Anilide; Nitrile; Sulfone</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Bisphenol A</td>
<td>80-05-7</td>
<td>Diphenylalkane; Bisphenol; Phenol</td>
<td>Chemical intermediate</td>
</tr>
<tr>
<td>Bisphenol B</td>
<td>77-40-7</td>
<td>Diphenylalkane; Bisphenol; Phenol</td>
<td>Adhesive, Chemical intermediate, Coatings</td>
</tr>
<tr>
<td>Butylbenzyl phthalate</td>
<td>85-68-7</td>
<td>Phthalate</td>
<td>Plasticizer</td>
</tr>
<tr>
<td>2-sec-Butylphenol</td>
<td>89-72-5</td>
<td>Phenol</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>CGS 18320B</td>
<td>112808-99-8</td>
<td>Nitrile; Imidazole</td>
<td>Metabolic inhibitor</td>
</tr>
<tr>
<td>Clomiphene citrate</td>
<td>50-41-9</td>
<td>Chlorinated triphenylethylene; Benzylidene; Stilbene</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>50-22-6</td>
<td>Steroid, nonphenolic</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Coumestrol</td>
<td>479-13-0</td>
<td>Coumestan; Ketone Benzopyranone; Coumarin</td>
<td>Natural product</td>
</tr>
<tr>
<td>4-Cumylphenol</td>
<td>599-64-4</td>
<td>Phenol</td>
<td>Chemical intermediate</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>66-81-9</td>
<td>Piperidine; Glutaramide</td>
<td>Pharmaceutical</td>
</tr>
</tbody>
</table>
Table 2–5: Chemical and Product Classes of the 78 Recommended Substances

(continued)

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Chemical Class</th>
<th>Product Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyproterone acetate</td>
<td>427-51-0</td>
<td>Nitrile; Diphenyl ether; Organochlorine</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Daidzein</td>
<td>486-66-8</td>
<td>Flavanoid; Isoflavone; Phenol</td>
<td>Natural product</td>
</tr>
<tr>
<td>p,p’-DDE</td>
<td>72-55-9</td>
<td>Organochlorine; Diphenylalkene</td>
<td>Pesticide metabolite</td>
</tr>
<tr>
<td>o,p’-DDT</td>
<td>789-02-6</td>
<td>Organochlorine; Diphenylalkene</td>
<td>Pesticide</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>50-02-2</td>
<td>Steroid, nonphenolic</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Dibenzo[a,h]anthracene</td>
<td>53-70-3</td>
<td>Polycyclic aromatic hydrocarbon; Anthracene</td>
<td>None</td>
</tr>
<tr>
<td>Di-n-butyl phthalate</td>
<td>84-74-2</td>
<td>Phthalate</td>
<td>Plasticizer</td>
</tr>
<tr>
<td>Diethylhexyl phthalate</td>
<td>117-81-7</td>
<td>Phthalate</td>
<td>Plasticizer</td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>56-53-1</td>
<td>Stilbene; Benzyllidene; Diphenylalkene</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>521-18-6</td>
<td>Steroid, nonphenolic</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>17α-Estradiol</td>
<td>57-91-0</td>
<td>Steroid, phenolic; Estrene</td>
<td>None</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>50-28-2</td>
<td>Steroid, phenolic; Estrene</td>
<td>Hormone</td>
</tr>
<tr>
<td>Estrone</td>
<td>53-16-7</td>
<td>Steroid, phenolic; Estrene</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>17α-Ethinyl estradiol</td>
<td>57-63-6</td>
<td>Steroid, phenolic</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Ethyl paraben</td>
<td>120-47-8</td>
<td>Paraben; Organic acid</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Fadrozole</td>
<td>102676-47-1</td>
<td>Imidazole; Nitrile</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Fenarimol</td>
<td>60168-88-9</td>
<td>Heterocycle; Pyrimidine</td>
<td>Pesticide</td>
</tr>
<tr>
<td>Finasteride</td>
<td>98319-26-7</td>
<td>Steroid, nonphenolic; Androstene</td>
<td>Pharmaceutical</td>
</tr>
</tbody>
</table>
### Table 2–5: Chemical and Product Classes of the 78 Recommended Substances

(continued)

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Chemical Class</th>
<th>Product Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavone</td>
<td>525-82-6</td>
<td>Flavanoid; Flavone</td>
<td>Natural product</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>206-44-0</td>
<td>Polycyclic aromatic hydrocarbon; Fluorene</td>
<td>None</td>
</tr>
<tr>
<td>Fluroxymestrone</td>
<td>76-43-7</td>
<td>Steroid, nonphenolic</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Flutamide</td>
<td>13311-84-7</td>
<td>Amide; Anilide; Nitrobenzene</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Genistein</td>
<td>446-72-0</td>
<td>Flavanoid; Isoflavone; Phenol</td>
<td>Natural product</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>52-86-8</td>
<td>Butyrophenone; Ketone; Piperazine</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>meso-Hexestrol</td>
<td>84-16-2</td>
<td>Diphenylalkane; Bisphenol; Phenol</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Hydroxyflutamide</td>
<td>52806-53-8</td>
<td>Amide; Anilide; Nitrobenzene</td>
<td>Pharmaceutical, Metab.</td>
</tr>
<tr>
<td>4-Hydroxytamoxifen</td>
<td>68047-06-3</td>
<td>Triphenylethylene; Phenol; Benzyldene; Stilbene</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>ICI 182,780</td>
<td>129453-61-8</td>
<td>Steroid, phenolic</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>520-18-3</td>
<td>Flavanoid; Flavone; Phenol</td>
<td>Natural product</td>
</tr>
<tr>
<td>Kepone</td>
<td>143-50-0</td>
<td>Organochlorine; Chlorinated bridged cycloalkane</td>
<td>Pesticide</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>65277-42-1</td>
<td>Imidazole; Piperazine</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Linuron</td>
<td>330-55-2</td>
<td>Urea</td>
<td>Pesticide</td>
</tr>
<tr>
<td>Medroxyprogesterone acetate</td>
<td>71-58-9</td>
<td>Steroid, nonphenolic; Polycyclic hydrocarbon</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>p, p’-Methoxychlor</td>
<td>72-43-5</td>
<td>Organochlorine; Chlorinated hydrocarbon</td>
<td>Pesticide</td>
</tr>
<tr>
<td>Methyl testosterone</td>
<td>58-18-4</td>
<td>Steroid, nonphenolic; Androstene</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Substance</td>
<td>CASRN</td>
<td>Chemical Class</td>
<td>Product Class</td>
</tr>
<tr>
<td>-----------------------</td>
<td>---------</td>
<td>-----------------------------------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Methyltrienolone</td>
<td>965-93-5</td>
<td>Steroid, nonphenolic; Estrene</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Mifepristone</td>
<td>84371-65-3</td>
<td>Steroid, nonphenolic; Estrene</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Morin</td>
<td>480-16-0</td>
<td>Flavanoid; Flavone; Phenol</td>
<td>Dye</td>
</tr>
<tr>
<td>Nilutamide</td>
<td>63612-50-0</td>
<td>Heterocycle; Imidazole</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>p-n-Nonylphenol</td>
<td>104-40-5</td>
<td>Alkylphenol; Phenol</td>
<td>Chemical intermediate</td>
</tr>
<tr>
<td>Norethynodrel</td>
<td>68-23-5</td>
<td>Steroid, nonphenolic; Norpregnene</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>4-tert-Octylphenol</td>
<td>140-66-9</td>
<td>Alkylphenol; Phenol</td>
<td>Chemical intermediate</td>
</tr>
<tr>
<td>Oxazepam</td>
<td>604-75-1</td>
<td>Benzodiazepine</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>57-30-7</td>
<td>Heterocycle; Pyrimidine</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Phenolphthalin</td>
<td>81-90-3</td>
<td>Triphenylmethane; Diphenyalkane carboxylic acid</td>
<td>Analytical reagent</td>
</tr>
<tr>
<td>Pimozide</td>
<td>2062-78-4</td>
<td>Piperidine; Benzimidazole</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Procymidone</td>
<td>32809-16-8</td>
<td>Organochlorine; Cyclic imide</td>
<td>Pesticide</td>
</tr>
<tr>
<td>Progesterone</td>
<td>57-83-0</td>
<td>Steroid, nonphenolic; pregnenedione</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Propylthiouracil</td>
<td>51-52-5</td>
<td>Pyrimidine; Uracil</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Reserpine</td>
<td>50-55-5</td>
<td>Heterocycle; Yohimban</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>26628-22-8</td>
<td>Organic salt; Azide</td>
<td></td>
</tr>
<tr>
<td>Spironolactone</td>
<td>52-01-7</td>
<td>Steroid, nonphenolic; pregnene lactone</td>
<td>Pharmaceutical</td>
</tr>
</tbody>
</table>
### Table 2-5: Chemical and Product Classes of the 78 Recommended Substances\(^a\)

(continued)

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Chemical Class</th>
<th>Product Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tamoxifen</td>
<td>10540-29-1</td>
<td>Triphenylethylene; Benzylidene; Stilbene</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>Testosterone</td>
<td>58-22-0</td>
<td>Steroid, nonphenolic</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>12-O-Tetradecanoyl-phorbol-13-acetate</td>
<td>16561-29-8</td>
<td>Phorbol ester; Terpene</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>L-Thyroxine</td>
<td>51-48-9</td>
<td>Aromatic amino acid</td>
<td>Hormone</td>
</tr>
<tr>
<td>17β-Trenbolone</td>
<td>10161-33-8</td>
<td>Steroid, nonphenolic; Estrene</td>
<td>Pharmaceutical</td>
</tr>
<tr>
<td>2,4,5-Trichlorophenoxyacetic acid</td>
<td>93-76-5</td>
<td>Organochlorine; Chlorinated aromatic hydrocarbon</td>
<td>Pesticide</td>
</tr>
<tr>
<td>Vinclozolin</td>
<td>50471-44-8</td>
<td>Organochlorine; Cyclic imide; Carbamate</td>
<td>Pesticide</td>
</tr>
<tr>
<td>Zearalenone</td>
<td>17924-92-4</td>
<td>Resorcylic acid lactone; Phenol</td>
<td>Chemical intermediate, Natural product</td>
</tr>
</tbody>
</table>

\(^a\) Substances were assigned to chemical and product classes based on available information from standardized references (e.g., *The Merck Index* and the U.S. National Library of Medicine’s ChemID database) and from an assessment of chemical structure.

**Abbreviations:**

\(p,p'\)-DDE = 1,1-Dichloro-2,2-di\((p\)-chlorophenyl\)ethylene; \(o,p'\)-DDT = 1,1,1-Trichloro-2-\((o\)-chlorophenyl\)-2-\((p\)-chlorophenyl\)ethane; \(p,p'\)-DDT = 1,1-Trichloro-2,2-di\((4\)-chlorophenyl\)ethane
3.0 IN VITRO ESTROGEN RECEPTOR BINDING ASSAYS

3.1 Minimum Procedural Standards

More than 14 different in vitro assays have been used to evaluate the ER binding ability of various substances (NIEHS 2002a). Of the 14 ER binding assays evaluated in the BRD, four used cytosolic proteins, four used recombinant proteins, five used glutathione-S-transferase protein constructs, and one used intact cells. No validation studies have been conducted to assess the performance and reliability of these test methods and very few substances have been tested multiple times using either the same test method or different test methods. Although there was insufficient information available to thoroughly assess the comparative performance of these 14 ER binding assays, the Expert Panel recommended that future validation efforts be directed to test methods using a recombinant receptor protein (see Appendix A). To assist in the development, standardization, and validation of in vitro ER binding assays, NICEATM and the EDWG developed proposed minimum procedural standards for consideration by the Expert Panel (NIEHS 2002a). Although a non-radioactive-based test method (the fluorescent polarization assay) has been developed to measure ER binding activity (NIEHS 2002a), these minimum procedural standards focused on test methods that used a radiolabeled reference estrogen to detect substances that could bind to the ER. The purpose of minimum procedural standards is to specify information essential for maximizing test method intra- and inter-laboratory reproducibility while minimizing the likelihood of erroneous results. Also, adherence to such standards will enhance any assessment of the comparative performance of in vitro ER binding assays. The minimum procedural standards provided here have been revised to incorporate recommendations and comments of the Expert Panel, the EDWG, and the public. Except as noted, all in vitro ER binding assays should incorporate these minimum procedural standards in their protocols, and scientific justification should be provided for any deviations.

3.1.1 Animal Studies

All studies requiring animal tissues should have animal use procedures approved by an Institutional Animal Care and Use Committee (IACUC) or its equivalent.

Rationale: An IACUC review will help ensure that animals needed as sources of tissue for isolation of the ER will be used in a humane manner. The review will also ensure consideration of alternative test methods that do not require animal tissues and appropriate justification if animal tissues are used.

3.1.2 Reference Estrogen

The displacement of a radiolabeled reference estrogen from the ER in a competitive binding study is used to identify substances that bind to the ER. 17\(\beta\)-Estradiol (CASRN 50-28-2) should be used as the reference estrogen in all ER binding assays; the hexa-tritium-labeled form (i.e., \([2,3,6,7,16,17-\text{H}]\) 17\(\beta\)-estradiol) is recommended. The relative binding affinity (RBA), a measure of relative activity, of a test substance is equal to the IC_{50} of the unlabeled reference estrogen divided by the IC_{50} of the test substance, multiplied by 100. The IC_{50} is the (calculated) concentration that inhibits the binding of the radiolabeled reference estrogen to the ER by 50%, and is determined by simultaneously incubating the ER with a saturating amount of the radiolabeled estrogen and a range of concentrations of the test substance or the unlabeled reference estrogen. The concentration range used for the unlabeled estrogen should be 1 nM to 1 \(\mu\)M. IC_{50} and RBA values should be calculated and presented for all in vitro ER binding assays.
**Rationale:** 17β-Estradiol is recommended because it is the most potent naturally occurring estrogen in the human body. The commercially available hexa-tritium-labeled form offers the highest specific radioactivity, which increases the sensitivity of competitive binding assays.

### 3.1.3 Dissociation Constant of the Reference Estrogen

Prior to conducting studies to evaluate the ER-binding ability of test substances, the dissociation constant ($K_d$) of the reference estrogen and the total number of receptors in the ER preparation ($B_{\text{max}}$, which is expressed as fmol/mg protein) should be determined using a saturation binding experiment. To determine the $K_d$ and $B_{\text{max}}$, the ER should be exposed to the radiolabeled reference estrogen at seven to ten concentrations, spaced across a three to four log interval. The ligand binding array of Raffelsberger and Wittliff (1997)\(^1\) has the advantage of determining simultaneously in each study the $K_d$ of the radiolabeled reference estrogen, the $B_{\text{max}}$ at different concentrations of the ER (if desired, but not required), and the $IC_{50}$ values of the unlabeled reference estrogen and the test substance. Thus, the Expert Panel recommended this method for determining the $K_d$ of the reference estrogen.

**Rationale:** The purpose of determining $B_{\text{max}}$ is to demonstrate that a finite number of receptors are saturated with the reference estrogen, which ensures that the test system is optimized with respect to receptor and ligand concentrations. The purpose of determining the $K_d$ is to identify the appropriate concentration of the radiolabeled reference estrogen to be used in competitive binding studies. Furthermore, the ability to obtain $K_d$ and $B_{\text{max}}$ values that are within the accepted limits for a specific test method (i.e., reference estrogen and ER protein) is a critical measure of the robustness of the procedure.

### 3.1.4 Preparation of Test Substances and Volume of Administered Solvent

Test substances should be dissolved in a solvent that is miscible with an aqueous solution. Water, ethanol (95 to 100%), or dimethyl sulfoxide (DMSO) is the preferred solvent. Preference should be given to the solvent that allows testing of the test substance at the maximum concentration possible, but without exceeding the limit dose (see Section 3.1.5). However, in testing situations where more than one solvent could be used, preference should be given to water, followed by ethanol (95 to 100%), and then DMSO. Other solvents may be used if it can be demonstrated that they do not interact or otherwise interfere with the test system. The volume of the solvent included in the reaction mixture generally has ranged from 0.1 to 1% of the total volume. For any solvent, it should be demonstrated that the maximum volume used does not interfere with the test system. This can be accomplished by comparing the $K_d$ obtained for the radiolabeled reference estrogen in the presence of the highest volume of the solvent with the $K_d$ of the reference estrogen in the absence of the solvent. The stability of the dissolved test substance should be determined prior to testing. In the absence of stability information, the stock solution should be prepared fresh prior to use.

**Rationale:** Selection of water, ethanol (95 to 100%), or DMSO as solvents is based on historical usage. Members of the Expert Panel stated that water or ethanol (95 to 100%) is
preferred to DMSO because some substances, when dissolved in DMSO, appear to bind with lower affinity to the receptor. For this reason, most investigators have not used DMSO at a final concentration greater than 0.1%. Because of possible differences in receptor protein sensitivity, the maximal concentration of a solvent that does not interfere with the performance should be determined for each test method.

### 3.1.5 Concentration Range of Test Substances

In the absence of solubility constraints, the maximum test substance concentration (i.e., the limit dose) should be 1 mM. Seven test substance concentrations spaced at log intervals up to the limit dose (i.e., 1 nM, 10 nM, 100 nM, 1 µM, 10 µM, 100 µM, 1 mM) should be tested.

**Rationale:** Most test method guidelines include a limit dose to ensure that all substances are tested over the same dose range while avoiding excessive amounts of a test substance that can perturb the test system through physicochemical mechanisms. An established limit dose also helps to minimize the effort and cost of screening and testing. Based on the range of published IC\textsubscript{50} values for ER binding (NIEHS 2002a), a limit dose of 1 mM, unless precluded by solubility constraints, was deemed suitable by the Expert Panel, the EDWG, and ICCVAM for assessing the ability of test substances to bind to the ER.

The seven recommended test substance concentrations, spaced at log intervals, should be sufficient to determine an IC\textsubscript{50} value with sufficient accuracy because, currently, the experimental results will be used in a semi-quantitative manner only (i.e., RBA values should not be used to rank substances regarding possible in vivo potency). If a lower maximum concentration is tested because of solubility constraints, the number of concentrations tested should remain the same by adding intermediate concentrations within the adjusted range.

### 3.1.6 Negative, Solvent, and Positive Controls

Controls are required for the development of a saturation binding curve to determine the B\textsubscript{max} and K\textsubscript{d}, and in subsequent competitive binding studies to evaluate the ER binding ability of test substances (see NIEHS 2002a, Appendix B5). For the saturation binding curve, a control set of tubes containing the ER and the radiolabeled reference estrogen is required to determine total (maximum) binding of the radiolabeled reference estrogen to the ER. A set of tubes containing unlabeled reference estrogen at a concentration that will saturate the ER, the radiolabeled reference estrogen, and the ER is required to measure nonspecific binding. A set of tubes containing the radiolabeled estrogen alone is required to determine the total radioactivity of the reference estrogen added to each tube. In addition, a set of negative control tubes containing the ER, the radiolabeled estrogen, and a negative control substance (i.e., a substance such as methyltrienolone [R1881] that does not bind to the ER) is included to demonstrate the specificity of the interaction between the ER and the reference estrogen.

For a competitive binding assay, a set of solvent control tubes containing the ER, the radiolabeled reference estrogen, and the solvent used to dissolve the test substance is required to determine total (maximum) binding of the radiolabeled reference estrogen to the ER. The solvent control should be added at the highest volume used to administer the test substance to the reaction mixture. A set of tubes to measure nonspecific binding and those containing a negative control substance,
as described above, are also included in each study. In addition to the unlabeled reference estrogen, another positive control substance (e.g., norethynodrel, 4-tert-octylphenol) with a binding affinity that is between two and three orders of magnitude lower than the reference estrogen should be included in each study, and its IC$_{50}$ and RBA values reported.

**Rationale:** In *in vitro* competitive ER binding assays, the binding of a test substance to the ER is demonstrated by its ability to reduce the amount of radiolabeled reference estrogen bound to the receptor at the end of the incubation period. Thus, the control response in each study is the total (maximum) binding of radiolabeled reference estrogen to the ER that occurs in the absence of the test substance. The inclusion of the various sets of control and negative substance control tubes are to ensure that the saturation binding and the competitive binding studies are performed properly. The inclusion in each study of an additional positive control substance with an RBA value two to three orders of magnitude lower than the reference estrogen provides another quality control (QC) measure by which to judge the sensitivity and acceptability of a test method for detecting substances that bind weakly to the receptor, and by which to evaluate the intralaboratory reproducibility of the test method. The usefulness of an additional positive control estrogen with an RBA value that is two to three times lower than that of the reference estrogen in each study should be evaluated during the validation process.

### 3.1.7 Within-Test Replicates

All concentration levels of the various controls, the reference estrogen, and the test substance should be tested in triplicate.

**Rationale:** The purpose of triplicate assay tubes for each concentration of the various controls, the reference estrogen, and the test substance is to ensure robust data and the ability to evaluate interreplicate variability. The most appropriate number of replicate tubes, however, should be evaluated after sufficient data have been collected using an optimized assay protocol.

#### 3.1.8 Data Analysis

The first step in determining the IC$_{50}$ value for the test substance is to determine the $B_{\text{max}}$ and $K_d$ values of the radiolabeled reference estrogen in the ER preparation. These parameters are obtained from a saturation binding experiment which is usually analyzed using a non-linear regression model (see Section 3.1.3). Several different software programs (e.g., Compete® and OneSite® [Lundon Software, Inc., Cleveland Heights, Ohio], GraphPad Prism® [GraphPad Software, Inc., San Diego, California], and LIGAND [Munson and Rodbard, 1980]) have been used to compute the $K_d$ and $B_{\text{max}}$ values of the radiolabeled reference estrogen in a particular ER preparation. Once these parameters are known, the IC$_{50}$ values of the unlabeled reference estrogen and the test substance can be determined using either a conventional competitive binding assay or the ligand binding array (Raffelsberger and Wittliff, 1997). The experimental design differs between the two methodologies and, thus, the most appropriate methods for data analyses will differ also. Although stating that the more frequently used competitive binding assay is acceptable, the Expert Panel recommended the ligand binding array for future validation studies. The IC$_{50}$ values for the unlabeled reference estrogen and the test substance are used to calculate the RBA value of the test substance.

The statistical methods used to calculate the $B_{\text{max}}$, $K_d$, and IC$_{50}$ values should be justified. This includes a formal assessment of the nature of the statistical characteristics of the data (distribution, variance patterns, specific
nonlinear models, etc.) and how the models fit the data. Confidence limits should be calculated and provided for these values. In addition, the corresponding historical mean and confidence intervals for the $K_d$ value for the radiolabeled reference estrogen, the $B_{\text{max}}$ for the ER preparation, and the $IC_{50}$ values for the unlabeled reference estrogen and the additional positive control (if used) should be calculated and presented. For those test substances that significantly reduce the extent of binding of the radiolabeled reference substance (as determined using an appropriate statistical test) but without achieving an $IC_{50}$, it might be useful to determine whether inhibition is via a competitive or noncompetitive mechanism. In the former case, the test substance binds to the ER at the same amino acid sequence (cognate sequence) as 17β-estradiol, the natural ligand, whereas, in the latter case, the test substance binds to an amino acid sequence different from the binding domain and acts allosterically to prevent receptor binding.

**Rationale:** The different statistical methods for calculating the $K_d$, $B_{\text{max}}$ and $IC_{50}$ values or methods for determining a statistically significant decrease in ER binding of the radiolabeled reference estrogen that does not achieve a 50% reduction have not been formally evaluated for their appropriateness. Data generated from a prevalidation study are needed for this purpose.

### 3.1.9 Good Laboratory Practice Compliance

Studies should be performed in compliance with Good Laboratory Practice (GLP) guidelines (EPA 2001, 2002; FDA 2002; OECD 1998).

**Rationale:** Conducting studies in compliance with GLP guidelines increases confidence in the quality and reliability of test data. Furthermore, if data using these test methods are to be submitted to the EPA in response to Federal testing requirements, then compliance with appropriate GLP guidelines will be required.

### 3.1.10 Study Acceptance Criteria

- The $IC_{50}$ value for the unlabeled reference estrogen should be approximately equal to the molar concentration of the radiolabeled reference estrogen plus the $K_d$ value.
- The $K_d$ and $IC_{50}$ values for the reference estrogen should be within the 95% confidence limits for historical data.
- The ratio of total binding in the absence of a competitor to the amount of the radiolabeled reference estrogen added per assay tube should not be greater than 10%.
- The $IC_{50}$ and RBA values for the concurrent additional positive control, if used, should be within the 95% confidence limits for historical data.
- The solvent control, at the concentration used, should not alter the performance of the assay.
- The limit dose should be 1 mM, unless precluded by solubility constraints.
- The study should comply with GLP guidelines.

**Rationale:** Established study acceptance criteria are required to ensure that each study is conducted appropriately.

### 3.1.11 Interpretation of Results

A substance is classified as positive for binding to the ER if an $IC_{50}$ value can be calculated. In general, the test substance should induce a sigmoid-shaped dose response curve over at least a few log concentrations. If a precipitous decrease in binding of the radiolabeled reference estrogen to the ER occurs over a narrow concentration range (i.e., over a one log increment), the response might reflect precipitation of the ER rather than competitive binding by the test substance. If a substance does not bind to the ER after
testing to the limit dose or to the maximum concentration possible based on its solubility (while not exceeding the limit dose), the test substance is classified as “negative” for binding to the ER under the conditions of the test. Test substances that induce a statistically significant reduction, but less than 50%, in binding of the radiolabeled reference estrogen to the ER, are classified as “equivocal”.

**Rationale:** Until information becomes available about the biological relevance of studies in which the test substance induces a significant but less than 50% reduction in binding of the radiolabeled reference estrogen to the ER, such responses should be noted and the substances classified as equivocal. The inability of a substance to decrease binding by at least 50% might be due to its relative insolubility, or its nonspecific binding to proteins other than the ER.

### 3.1.12 Repeat Studies
Generally, in a validation study, repeat studies would be conducted in order to evaluate intralaboratory repeatability and reproducibility. In contrast, in screening studies, repeat studies are not needed except to clarify equivocal results. If a study is repeated, the use of test substance concentrations more closely distributed in the range of interest might facilitate a more accurate analysis of the dose-response relationship for the test substance.

**Rationale:** Repeat studies are used in a validation study to demonstrate the intralaboratory repeatability and reproducibility of a test method. However, for a screening study, if the acceptance criteria are met and a clear negative or positive response is obtained, a repeat study to verify the original result usually is not considered necessary. In studies where an accurate IC$_{50}$ value cannot be calculated or where an equivocal response is obtained, a repeat study using adjusted dose levels might be needed to ensure a reliable conclusion.

### 3.1.13 Study Report
At a minimum, the study report should include the following information.

**Reference Estrogen**
- name, CASRN, purity, and supplier or source of the reference estrogen (radiolabeled and unlabeled), and specific activity of the radiolabeled reference estrogen
- concentrations and volumes used

**Additional Positive Control (if used)**
- name, CASRN, purity, and supplier or source
- concentrations and volumes used

**Negative Binding Control Substance**
- name, CASRN, purity, and supplier or source
- concentrations and volumes used

**Test Substance**
- name, chemical structure (if known), CASRN (if known), and supplier or source
- physical nature (solid or liquid) and purity, if known (every attempt should be made to determine the purity)
- physicochemical properties relevant to the study (e.g., solubility, pH, stability, volatility)
- concentrations and volumes used

**Solvent**
- name, CASRN, purity, and supplier or source
- justification for choice of solvent
- information on the solubility of the test substance in all solvents in which it was tested
ICCVAM Review of In Vitro Endocrine Disruptor Screening Assays

In Vitro ER Binding Assays

- information to demonstrate that the solvent, at the maximum volume used, does not interact or otherwise interfere with the assay

**Estrogen Receptor**

- type and source of ER and the supplier
- if the ER is isolated from animal tissues, information on species, strain, age, and gender of the animals used, the surgical procedure used to remove the tissue, and the method used to isolate the ER
- if a recombinant ER protein is used, information on the cloning procedure used, the methods used to express the protein, and the procedures used for isolation of the protein
- protein concentration of ER preparation
- method used to measure protein concentration
- method for storage of ER, if applicable

**Study Conditions**

- $K_d$ of the reference estrogen and $B_{max}$ of the ER
- rationale for the concentration of the radiolabeled reference estrogen in the binding assay
- protein concentration of ER used in the binding assay
- name(s) and concentration(s) of protease inhibitor(s) included in the animal tissue isolation buffer, if used
- composition of buffers used
- concentration range of the test substance, with justification
- volume of the solvent used to dissolve the test substance and the volume added to the reaction mixture
- incubation volume, duration, and temperature
- description of the solvent control
- type and composition of metabolic activation system, if used

- description of the method used to separate ER-bound and -unbound radiolabeled reference estrogen
- method used to analyze concentration of receptor-ligand complexes
- statistical method used to determine $K_d$, $B_{max}$, and $IC_{50}$ values
- any other statistical method(s) used to assess the ability of the test substance to inhibit the binding of the radiolabeled reference estrogen

**Results**

- observations for and extent of any test substance precipitation
- the IC data for each replicate at each concentration of the test substance, along with confidence levels or other measure of intradose repeatability
- graphically presented dose-response curves for the unlabeled reference estrogen, the positive control, and the test substance
- $IC_{50}$ values and confidence limits for the unlabeled reference estrogen, the additional positive control (if used), and the test substance
- calculated RBA values for the additional positive control, if used, and the test substance

**Discussion of Results**

- reproducibility of the $K_d$ of the reference estrogen and $B_{max}$ of the ER, compared to historical data
- historical $IC_{50}$ values for the unlabeled reference estrogen, including ranges, means, standard deviations, and confidence intervals
- reproducibility of the $IC_{50}$ values of the unlabeled reference estrogen, compared to historical data
- historical $IC_{50}$ and RBA values for the additional positive control substance, if used, with ranges, means, standard deviations, and confidence intervals
• reproducibility of the IC$_{50}$ and RBA values for the additional positive control substance compared to historical data
• the test substance dose-response relationship for inhibition of binding of the radiolabeled reference estrogen to the ER

Conclusion
• classification of the test substance with regard to in vitro ER binding activity

Rationale: Minimum reporting standards are needed to ensure that a study report contains the level of information and detail that would be required if the study results are reviewed by the applicable regulatory agency, or for independent replication of the study, if deemed necessary.

3.2 Recommended Substances for Validation of In Vitro Estrogen Receptor Binding Assays

To facilitate validation of in vitro ER binding assays, ICCVAM has compiled a list of 78 recommended substances for use in future validation studies. The 78 substances are presented in Table 3-1, with a summary of available quantitative in vitro ER binding data for each substance. Section 2.0 provides a detailed account of how these substances were selected. RBA data are available for 38 (49%) of these 78 recommended substances. Although 17β-estradiol is included in the list of recommended substances, it was not included in the count of substances for validation as it is the reference standard against which all test substances are compared. Quantitative in vitro ER binding data are provided for substances that induced a positive response in at least one study. This includes the median RBA value and the range of RBA values where more than one positive study had been conducted, and the number of studies and assays in which each substance was tested. In situations where only one positive study was reported, the RBA value obtained in that study is reported. The substances with RBA data are listed first, sorted by potency from strongest to weakest, based on the median or single RBA value of each substance across all positive studies. The median or single RBA values range from 2.34 to 0.0002, extending over seven orders of magnitude. Positive and “presumed positive” substances have been grouped into six RBA categories in log decrements: $>10$, $<10$ to 1, $<1$ to 0.1, $<0.1$ to 0.01, $<0.01$ to 0.001, and $<0.001$. Presumed positive substances induced a positive response in 50% or fewer of the ER binding studies in which they were tested. Substances were classified as negative if they did not induce at least a 50% reduction in the binding of the radiolabeled reference estrogen to the ER in multiple studies when tested up to the limit dose as defined in this document (i.e., 1 mM). Substances reported as negative for ER binding were classified as “presumed negative” if they had not been tested to the limit dose in multiple studies (i.e., reproducibility for a negative response had not been demonstrated at a test substance concentration up to 1 mM). Diethylhexylphthalate is the only substance that had been reported as negative when tested to the limit dose in multiple studies. The negative and presumed negative substances are listed below the sixth RBA category (<0.001) and include the highest dose tested (HDT) used among studies, if available, in addition to the number of studies and assays in which the substance was tested. No effort was made to assess the validity and quality of

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2 Inclusion of a substance in this list does not mean that EPA, NICEATM, ICCVAM, or the Expert Panel has or will make a determination that any use of the substance will pose a significant risk. Further, these substances should not be interpreted to be “endocrine disruptors”; the substances listed are simply compounds that have been or may prove to be useful in developing, standardizing, or validating screening and testing methods.
each negative or positive response reported for each substance in each study. Following the presumed negative substances are those that have not been tested for ER binding activity. These substances have been assigned a presumed positive or negative response in in vitro ER binding assays based on the substances’ anticipated or known mechanism of action and their response in in vitro ER TA assays. Presumed positive substances are listed first, followed by presumed negative substances that have been selected for the minimal list of substances (see below and Section 2.4.4). Both categories are sorted alphabetically by substance name. The other substances that are presumed negative are sorted alphabetically at the end of the list.

Substances have been classified as presumed positive even when they were reported as positive for ER binding in less than 50% of the studies conducted. This classification is because erroneous positive studies are probably less likely than erroneous negative studies due to the nature of ER binding assays and the protocols generally used. For example, in many negative studies, the HDT was below the IC_{50} value obtained in positive studies reported for the same substance. The classification of a substance as positive (and its ranking), presumed positive, or presumed negative in this list is based sometimes on the results of a single study and, therefore, the accuracy of the classification is questionable. However, it is anticipated that testing these presumed positive and negative substances will provide critical information on the comparative sensitivity and reproducibility of different in vitro ER binding assays, when such methods are standardized and conducted using the recommended minimum procedural standards.

As described in Section 2.4.4, a subset of 53 substances has been identified that, at a minimum, should be used in any validation of in vitro ER binding assays. Of these substances, 75% (40) are classified as positive (22) or presumed positive (18) for ER binding, and 25% (13) are classified as negative (1) or presumed negative (12).
<table>
<thead>
<tr>
<th>RBA Range b (Classification)</th>
<th>Substance</th>
<th>CASRN</th>
<th>Median RBA Value and RBA Range c</th>
<th>No. Positive Responses/No. Times Tested</th>
<th>No. ER Binding Assays in Which Tested</th>
<th>Completed/Anticipated In Vivo Testing d</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;10 (Positive)</td>
<td>meso-Hexestrol e</td>
<td>84-16-2</td>
<td>234 (58 - 302)</td>
<td>7/7</td>
<td>6</td>
<td>Strong ER agonist</td>
<td>Diphenylalkane; Bisphenol; Phenol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4-Hydroxytamoxifen e</td>
<td>68047-06-3</td>
<td>172 (2.9 - 400)</td>
<td>18/18</td>
<td>13</td>
<td>ER antagonist</td>
<td>Triphenylethylene; Benzylidene; Stilbene; Phenol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17α-Ethinyl estradiol e</td>
<td>57-63-6</td>
<td>148 (100 - 867)</td>
<td>10/10</td>
<td>7</td>
<td>U; 407; F-PA</td>
<td>Strong ER agonist</td>
<td>Steroid, phenolic</td>
</tr>
<tr>
<td></td>
<td>Diethylstilbestrol e</td>
<td>56-53-1</td>
<td>124 (0.003 - 5000)</td>
<td>38/38</td>
<td>14</td>
<td>IUL</td>
<td>Strong ER agonist</td>
<td>Stilbene; Benzylidene; Diphenylalkene</td>
</tr>
<tr>
<td></td>
<td>17β-Estradiol e,f</td>
<td>50-28-2</td>
<td>100 (Reference estrogen)</td>
<td>82/82</td>
<td>14</td>
<td>IM; IUL; FRS</td>
<td>Strong ER agonist; AR agonist and antagonist</td>
<td>Steroid, phenolic; Estrene</td>
</tr>
<tr>
<td></td>
<td>Estrone e</td>
<td>53-16-7</td>
<td>41 (0.22 - 100)</td>
<td>18/18</td>
<td>13</td>
<td>Strong ER agonist; AR agonist</td>
<td>Steroid, phenolic; Estrene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ICI 182,780 e</td>
<td>129453-61-8</td>
<td>37.5 (25 - 500)</td>
<td>5/5</td>
<td>4</td>
<td>IM</td>
<td>ER antagonist</td>
<td>Steroid, phenolic</td>
</tr>
<tr>
<td></td>
<td>Zearalenone e</td>
<td>17924-92-4</td>
<td>15 (5 - 82)</td>
<td>12/12</td>
<td>10</td>
<td>ER agonist</td>
<td>Resorcyclic acid lactone; Phenol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Coumestrol e</td>
<td>479-13-0</td>
<td>12 (0.24 - 185)</td>
<td>15/15</td>
<td>11</td>
<td>IM</td>
<td>ER agonist</td>
<td>Coumestan; Benzopyranone; Coumarin; Ketone</td>
</tr>
<tr>
<td></td>
<td>17α- Estradiol e</td>
<td>57-91-0</td>
<td>11 (0.22 - 1000)</td>
<td>9/9</td>
<td>7</td>
<td>ER agonist</td>
<td>Steroid, phenolic; Estrene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tamoxifen e</td>
<td>10540-29-1</td>
<td>4 (0.017 - 25)</td>
<td>21/21</td>
<td>14</td>
<td>ER antagonist</td>
<td>Triphenylethylene; Benzylidene; Stilbene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Genistein e</td>
<td>446-72-0</td>
<td>1.45 (0.33 - 87)</td>
<td>18/18</td>
<td>11</td>
<td>U; 407</td>
<td>Weak ER agonist and antagonist</td>
<td>Flavanoid; Isoflavone; Phenol</td>
</tr>
<tr>
<td></td>
<td>Apigenin e</td>
<td>520-36-5</td>
<td>1.15 (0.028 - 6)</td>
<td>4/4</td>
<td>3</td>
<td>IUL</td>
<td>ER agonist</td>
<td>Flavanoid; Flavone; Phenol</td>
</tr>
<tr>
<td></td>
<td>Kaempferol e</td>
<td>520-18-3</td>
<td>1.05 (0.025 - 3)</td>
<td>4/4</td>
<td>3</td>
<td>ER agonist</td>
<td>Flavanoid; Flavone; Phenol</td>
<td></td>
</tr>
</tbody>
</table>

a | b | c | d | e

Section 3

ICCVAM Review of In Vitro Endocrine Disruptor Screening Assays

Table 3-1: ICCVAM Recommended Substances for Validation of *In Vitro* ER Binding Assays
<table>
<thead>
<tr>
<th>RBA Range</th>
<th>Substance</th>
<th>CASRN</th>
<th>Median RBA Value and RBA Range</th>
<th>No. Positive Responses/No. Times Tested</th>
<th>No. ER Binding Assays in Which Tested</th>
<th>Completed/Anticipated In Vivo Testing</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1 to 0.1 (Positive)</td>
<td>Clomiphene citrate&lt;sup&gt;e&lt;/sup&gt;</td>
<td>50-41-9</td>
<td>0.72</td>
<td>1/1</td>
<td>1</td>
<td></td>
<td>ER agonist; Chlorinated triphenylethylene; Benzylidine; Stilbene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Norethynodrel&lt;sup&gt;e&lt;/sup&gt;</td>
<td>68-23-5</td>
<td>0.23 (0.2 - 0.7)</td>
<td>4/4</td>
<td>3</td>
<td></td>
<td>ER agonist; Steroid, nonphenolic; Norpregnene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4-tert-Octylphenol&lt;sup&gt;e&lt;/sup&gt;</td>
<td>140-66-9</td>
<td>0.17 (0.01 - 3.2)</td>
<td>11/11</td>
<td>9 J(U,H,1G,F,A)</td>
<td></td>
<td>ER agonist; Alkylphenol; Phenol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bisphenol B&lt;sup&gt;e&lt;/sup&gt;</td>
<td>77-40-7</td>
<td>0.118 (0.086 - 0.15)</td>
<td>2/2</td>
<td>1</td>
<td></td>
<td>ER agonist; Diphenylalkane; Bisphenol; Phenol</td>
<td></td>
</tr>
<tr>
<td>&lt;0.1 to 0.01 (Positive)</td>
<td>5α-Dihydrotestosterone</td>
<td>521-18-6</td>
<td>0.037 (0.001 - 0.38)</td>
<td>10/11</td>
<td>10 H</td>
<td></td>
<td>Weak ER agonist; Strong AR agonist; Steroid, nonphenolic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bisphenol A&lt;sup&gt;e&lt;/sup&gt;</td>
<td>80-05-7</td>
<td>0.033 (0.0013 - 1.0)</td>
<td>22/22</td>
<td>14 U; F-PA; J(1G,F,A)</td>
<td></td>
<td>Weak ER agonist; Diphenylalkane; Bisphenol; Phenol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p-n-Nonylphenol&lt;sup&gt;e,h&lt;/sup&gt;</td>
<td>104-40-5</td>
<td>0.033 (0.0025 - 0.5)</td>
<td>14/14</td>
<td>4 U; 407; J(H,U,1G,F,A)</td>
<td></td>
<td>ER and AR antagonist; ER agonist; Alkylphenol; Phenol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kepone (Chlordecone)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>143-50-0</td>
<td>0.03 (0.0035 - 0.2)</td>
<td>11/12</td>
<td>10 H</td>
<td></td>
<td>Binds to ER and AR; Organochlorine; Chlorinated bridged cycloalkane</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Testosterone&lt;sup&gt;e,d&lt;/sup&gt;</td>
<td>58-22-0</td>
<td>0.025 (0.01 - 0.04)</td>
<td>2/9</td>
<td>7 IM</td>
<td></td>
<td>Strong AR agonist; Steroid, nonphenolic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p,p'-DDE&lt;sup&gt;e,d&lt;/sup&gt;</td>
<td>72-55-9</td>
<td>0.021 (0.0003 - 0.042)</td>
<td>2/11</td>
<td>9 H; 407; M-PA; IM; J(1G,F,A)</td>
<td></td>
<td>Weak AR agonist and antagonist; Organochlorine; Diphenylalkene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Daidzein&lt;sup&gt;e&lt;/sup&gt;</td>
<td>486-66-8</td>
<td>0.02 (0.022 - 1.11)</td>
<td>8/8</td>
<td>4 H</td>
<td></td>
<td>Weak ER agonist; Flavanoid; Isoflavone; Phenol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>o,p'-DDT&lt;sup&gt;e&lt;/sup&gt;</td>
<td>789-02-6</td>
<td>0.02 (0.00031 - 0.43)</td>
<td>15/17</td>
<td>12 U; J(1G,F,A)</td>
<td></td>
<td>Weak ER and AR antagonist; weak ER agonist; Organochlorine; Diphenylalkene</td>
<td></td>
</tr>
</tbody>
</table>
Table 3-1: ICCVAM Recommended Substances for Validation of In Vitro ER Binding Assays\(^a\) (continued)

<table>
<thead>
<tr>
<th>RBA Range (^b) (Classification)</th>
<th>Substance</th>
<th>CASRN</th>
<th>Median RBA Value and RBA Range (^c)</th>
<th>No. Positive Responses/No. Times Tested</th>
<th>No. ER Binding Assays in Which Tested</th>
<th>Completed/ Anticipated In Vivo Testing (^d)</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.01 to 0.001 (Presumed Positive)</td>
<td>4-Androstenedione (^e)</td>
<td>63-05-8</td>
<td>0.007 (0.00062 - 0.95)</td>
<td>1/4</td>
<td>4</td>
<td></td>
<td>Strong AR agonist</td>
<td>Steroid, nonphenolic</td>
</tr>
<tr>
<td>p,p'-Methoxychlor (^e)</td>
<td>72-43-5</td>
<td>0.0067</td>
<td>8/18</td>
<td>13</td>
<td>U; F&amp;M-PA; IUL; IM; FRS; 2G(avian)</td>
<td></td>
<td>Weak ER agonist; AR antagonist</td>
<td>Organochlorine; Chlorinated hydrocarbon</td>
</tr>
<tr>
<td>4-Cumylphenol (^e)</td>
<td>599-64-4</td>
<td>0.005</td>
<td>1/1</td>
<td>1</td>
<td></td>
<td></td>
<td>Weak ER agonist</td>
<td>Phenol</td>
</tr>
<tr>
<td>Di-n-butyl phthalate (^e)</td>
<td>84-74-2</td>
<td>0.0027 (0.0026 - 0.0028)</td>
<td>2/5</td>
<td>3</td>
<td>U; M-PA; 1G; J(U,H,1G,F,A)</td>
<td></td>
<td>ER agonist</td>
<td>Phthalate</td>
</tr>
<tr>
<td>Butylbenzyl phthalate (^e)</td>
<td>85-68-7</td>
<td>0.0018 (0.000036 - 0.012)</td>
<td>4/13</td>
<td>8</td>
<td>IUL</td>
<td></td>
<td>ER agonist</td>
<td>Phthalate</td>
</tr>
<tr>
<td>Ethyl paraben (^e)</td>
<td>120-47-8</td>
<td>0.0006</td>
<td>1/1</td>
<td>1</td>
<td></td>
<td></td>
<td>Binds weakly to ER</td>
<td>Paraben; Organic acid</td>
</tr>
<tr>
<td>Morin (^e)</td>
<td>480-16-0</td>
<td>0.0005</td>
<td>1/1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>Flavanoid; Flavone; Phenol</td>
</tr>
<tr>
<td>Progesterone (^e)</td>
<td>57-83-0</td>
<td>0.0003</td>
<td>1/8</td>
<td>5</td>
<td>IM</td>
<td></td>
<td></td>
<td>Steroid, nonphenolic; Pregnenedione</td>
</tr>
<tr>
<td>Atrazine (^e,j)</td>
<td>1912-24-9</td>
<td>0.0003</td>
<td>1/8</td>
<td>7</td>
<td>M-PA; IUL</td>
<td></td>
<td></td>
<td>Aromatic amine; Triazine; Arylamine</td>
</tr>
<tr>
<td>Hydroxyflutamide (^e)</td>
<td>52806-53-8</td>
<td>0.0003</td>
<td>1/2</td>
<td>1</td>
<td></td>
<td></td>
<td>AR agonist and antagonist</td>
<td>Amide; Anilide; Nitrobenzene</td>
</tr>
<tr>
<td>Vinclozolin (^e)</td>
<td>50471-44-8</td>
<td>0.0003</td>
<td>1/2</td>
<td>1</td>
<td>H; M-PA; IM; IUL; 1G; FRS</td>
<td></td>
<td>AR antagonist</td>
<td>Organochlorine; Cyclic imide; Carbamate</td>
</tr>
<tr>
<td>2-sec-Butylphenol (^e)</td>
<td>89-72-5</td>
<td>0.0003</td>
<td>1/1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>Phenol</td>
</tr>
<tr>
<td>Phenolphthalein (^e)</td>
<td>81-90-3</td>
<td>0.0002</td>
<td>1/1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>Triphenylmethane; Diphenylalkane; Carboxylic acid</td>
</tr>
<tr>
<td>No RBA Value (Negative)</td>
<td>Diethylhexyl phthalate (^e,k)</td>
<td>117-81-7</td>
<td>HDT - 5000 µM</td>
<td>0/3</td>
<td>1</td>
<td>J(U,H,1G,F,A)</td>
<td></td>
<td>Phthalate</td>
</tr>
</tbody>
</table>
### Table 3-1: ICCVAM Recommended Substances for Validation of *In Vitro* ER Binding Assays (continued)

<table>
<thead>
<tr>
<th>RBA Range (Classification)</th>
<th>Substance</th>
<th>CASRN</th>
<th>Median RBA Value and RBA Range</th>
<th>No. Positive Responses/No. Times Tested</th>
<th>No. of Assays in Which Tested</th>
<th>Completed/Anticipated In Vivo Testing</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>No RBA Value (Presumed Negative)</td>
<td>Dibenzo[a,h]-anthracene</td>
<td>53-70-3</td>
<td>HDT - 5 µM</td>
<td>0/1</td>
<td>1</td>
<td></td>
<td></td>
<td>Polycyclic aromatic hydrocarbon; Anthracene</td>
</tr>
<tr>
<td></td>
<td>Fluoranthene</td>
<td>206-44-0</td>
<td>HDT - 5 µM</td>
<td>0/1</td>
<td>1</td>
<td></td>
<td>AR antagonist</td>
<td>Polycyclic aromatic hydrocarbon; Fluorene</td>
</tr>
<tr>
<td></td>
<td>Corticosterone</td>
<td>50-22-6</td>
<td>HDT - 100 µM</td>
<td>0/3</td>
<td>2</td>
<td></td>
<td>Binds weakly to AR</td>
<td>Steroid, nonphenolic</td>
</tr>
<tr>
<td></td>
<td>Dexamethasone</td>
<td>50-02-2</td>
<td>HDT - 100 µM</td>
<td>0/1</td>
<td>1</td>
<td></td>
<td>AR agonist</td>
<td>Steroid, nonphenolic</td>
</tr>
<tr>
<td></td>
<td>Flavone</td>
<td>525-82-6</td>
<td>HDT - 100 µM</td>
<td>0/3</td>
<td>3</td>
<td>M-PA; IM</td>
<td>Weak ER antagonist</td>
<td>Flavanoid; Flavone</td>
</tr>
<tr>
<td></td>
<td>2,4,5-Trichlorophenoxyacetic acid</td>
<td>93-76-5</td>
<td>HDT - 1000 µM</td>
<td>0/1</td>
<td>1</td>
<td></td>
<td>Weak ER agonist</td>
<td>Organochlorine; Chlorinated aromatic hydrocarbon</td>
</tr>
</tbody>
</table>

#### ANTICIPATED RESPONSES

<table>
<thead>
<tr>
<th>RBA Data Not Available (Presumed Negative)</th>
<th>Substance</th>
<th>CASRN</th>
<th>Response</th>
<th>RBA Data Not Available (Presumed Negative)</th>
<th>F-PA</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fenarimol</td>
<td>60168-88-9</td>
<td>Pos.</td>
<td></td>
<td>Heterocycle; Pyrimidine</td>
<td>F-PA</td>
<td>Aromatase inhibitor; Weak ER agonist</td>
<td></td>
</tr>
<tr>
<td>Methyl testosterone</td>
<td>58-18-4</td>
<td>Pos.</td>
<td>H; 407; M-PA; IUL; FRS</td>
<td>Steroid, nonphenolic; Androstene</td>
<td></td>
<td>ER and AR agonist</td>
<td></td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>50-76-0</td>
<td>Neg.</td>
<td>RNA synthesis inhibitor</td>
<td>Phenozaone; Lactone; Peptide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fadrozole</td>
<td>102676-47-1</td>
<td>Neg.</td>
<td>F-PA; IM; FRS</td>
<td>Imidazole; Nitrile</td>
<td></td>
<td>Aromatase inhibitor</td>
<td></td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>57-30-7</td>
<td>Neg.</td>
<td>F&amp;M-PA; IM</td>
<td>Heterocycle; Pyrimidine</td>
<td></td>
<td>Enhances thyroid hormone excretion</td>
<td></td>
</tr>
<tr>
<td>Propylthiouracil</td>
<td>51-52-5</td>
<td>Neg.</td>
<td>407; F&amp;M-PA; IM; IUL; 2G</td>
<td>Pyrimidine; Uracil</td>
<td></td>
<td>Inhibits T3/T4 synthesis</td>
<td></td>
</tr>
<tr>
<td>Sodium azide</td>
<td>26628-22-8</td>
<td>Neg.</td>
<td>IUL</td>
<td>Organic salt; Azide</td>
<td></td>
<td>Cytotoxic</td>
<td></td>
</tr>
<tr>
<td>12-O-Tetradecanoylphorbol-13-acetate</td>
<td>16561-29-8</td>
<td>Neg.</td>
<td></td>
<td>Phenol ester; Terpene</td>
<td></td>
<td>Activates ligand independent cell division</td>
<td></td>
</tr>
<tr>
<td>Ammonium perchlorate</td>
<td>7790-98-9</td>
<td>Neg.</td>
<td></td>
<td>Organic acid; Organic salt</td>
<td></td>
<td>Thyroid disruptor</td>
<td></td>
</tr>
</tbody>
</table>

# Interview:

**Q1:** What is the significance of the median RBA value in the table?

**A1:** The median RBA value represents the midpoint of the RBA range, indicating the central tendency of the responses obtained from the assays. This value is crucial for assessing the effectiveness of the substances in binding to the estrogen receptor.

**Q2:** How are the substances classified in terms of their chemical class?

**A2:** The substances are classified into different chemical classes, such as Polycyclic aromatic hydrocarbons, Steroids, nonphenolic, Flavanoids, and others, which help in understanding their structural and functional similarities.

**Q3:** What does the presence of an asterisk (*) indicate in the table?

**A3:** An asterisk (*) indicates additional information or notes that are crucial for interpreting the results, such as the need for further testing or specific comments about the substances' effects.

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**Section 3:**

**In Vitro ER Binding Assays**

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**ICCVAM Review of In Vitro Endocrine Disruptor Screening Assays**

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**Page 39**
Table 3-1: ICCVAM Recommended Substances for Validation of In Vitro ER Binding Assays

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Median RBA Value and RBA Range</th>
<th>No. Positive Responses/No. Times Tested</th>
<th>No. ER Binding Assays in Which Tested</th>
<th>Completed/Anticipated In Vivo Testing</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anastrazole</td>
<td>120511-73-1</td>
<td>Neg.</td>
<td>IM</td>
<td></td>
<td></td>
<td>Aromatase inhibitor</td>
<td>Nitrile; Triazole</td>
</tr>
<tr>
<td>Apomorphine</td>
<td>58-00-4</td>
<td>Neg.</td>
<td>IM</td>
<td></td>
<td></td>
<td>Dopamine D1/D2 receptor agonist</td>
<td>Heterocycle; Quinoline</td>
</tr>
<tr>
<td>Bicalutamide</td>
<td>90357-06-5</td>
<td>Neg.</td>
<td>AR antagonist</td>
<td></td>
<td></td>
<td>Anilide; Nitrile; Sulfone</td>
<td></td>
</tr>
<tr>
<td>CGS 18320B</td>
<td>112808-99-8</td>
<td>Neg.</td>
<td>407</td>
<td></td>
<td></td>
<td>Aromatase inhibitor</td>
<td>Nitrile; Imidazole</td>
</tr>
<tr>
<td>Cyloheximide</td>
<td>66-81-9</td>
<td>Neg.</td>
<td>IM</td>
<td></td>
<td></td>
<td>Protein synthesis inhibitor</td>
<td>Piperidine; Glutaramide</td>
</tr>
<tr>
<td>Cycproterone acetate</td>
<td>427-51-0</td>
<td>Neg.</td>
<td>IM</td>
<td></td>
<td></td>
<td>AR agonist and antagonist</td>
<td>Nitrile; Diphenyl ether; Organochlorine</td>
</tr>
<tr>
<td>Finasteride</td>
<td>98319-26-7</td>
<td>Neg.</td>
<td>H; M-PA; IM</td>
<td></td>
<td></td>
<td>5α-reductase inhibitor</td>
<td>Steroid, nonphenolic; Androstenone</td>
</tr>
<tr>
<td>Fluoxymestrone</td>
<td>76-43-7</td>
<td>Neg.</td>
<td>H; 407; M-PA; IM</td>
<td></td>
<td></td>
<td>Weak AR agonist</td>
<td>Steroid, nonphenolic</td>
</tr>
<tr>
<td>Flutamide</td>
<td>13311-84-7</td>
<td>Neg.</td>
<td>H; 407; M-PA; IM</td>
<td></td>
<td></td>
<td>AR antagonist</td>
<td>Amide; Anilide; Nitrobenzene</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>52-86-8</td>
<td>Neg.</td>
<td>IM</td>
<td></td>
<td></td>
<td>Dopamine D2 receptor antagonist</td>
<td>Butyrophenone; Ketone; Piperazine</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>65277-42-1</td>
<td>Neg.</td>
<td>F&amp;M-PA; IM</td>
<td></td>
<td></td>
<td>Weak AR agonist</td>
<td>Imidazole; Piperazine</td>
</tr>
<tr>
<td>Linuron</td>
<td>330-55-2</td>
<td>Neg.</td>
<td>M-PA</td>
<td></td>
<td></td>
<td>Weak AR agonist and antagonist</td>
<td>Urea</td>
</tr>
<tr>
<td>Medroxyprogesterone acetate</td>
<td>71-58-9</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Weak AR agonist</td>
<td>Steroid, nonphenolic; Polycyclic hydrocarbon</td>
</tr>
<tr>
<td>Methyltrienolone</td>
<td>965-93-5</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>AR agonist</td>
<td>Steroid, nonphenolic; Estrene</td>
</tr>
<tr>
<td>Mifepristone</td>
<td>84371-65-3</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>AR agonist and antagonist</td>
<td>Steroid, nonphenolic; Estrene</td>
</tr>
<tr>
<td>Nilutamide</td>
<td>63612-50-0</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>AR agonist</td>
<td>Heterocycle; Imidazole</td>
</tr>
</tbody>
</table>
### Table 3-1: ICCVAM Recommended Substances for Validation of In Vitro ER Binding Assays

<table>
<thead>
<tr>
<th>RBA Range</th>
<th>Substance</th>
<th>CASRN</th>
<th>Median RBA Value and RBA Range</th>
<th>No. Positive Responses/No. Times Tested</th>
<th>No. ER Binding Assays in Which Tested</th>
<th>Completed/ Anticipated In Vivo Testing</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neg.</td>
<td>Oxazepam</td>
<td>604-75-1</td>
<td>Neg.</td>
<td>IM</td>
<td>1</td>
<td>407</td>
<td>Enhances thyroid hormone excretion</td>
<td>Benzodiazepine</td>
</tr>
<tr>
<td>Neg.</td>
<td>Pimozide</td>
<td>2062-78-4</td>
<td>Neg.</td>
<td>F&amp;M-PA; IM</td>
<td></td>
<td></td>
<td>Dopamine receptor antagonist</td>
<td>Piperidine; Benzimidazole</td>
</tr>
<tr>
<td>Neg.</td>
<td>Procymidone</td>
<td>32809-16-8</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>AR antagonist</td>
<td>Organochlorine; Cyclic imide</td>
</tr>
<tr>
<td>Neg.</td>
<td>Reserpine</td>
<td>50-55-5</td>
<td>Neg.</td>
<td>IM</td>
<td></td>
<td></td>
<td>Depletes dopamine</td>
<td>Heterocycle; Yohimban</td>
</tr>
<tr>
<td>Neg.</td>
<td>Spironolactone</td>
<td>52-01-7</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>AR agonist and antagonist</td>
<td>Steroid, nonphenolic; Pregnen lactone</td>
</tr>
<tr>
<td>Neg.</td>
<td>L-Thyroxine</td>
<td>51-48-9</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Thyroid hormone</td>
<td>Aromatic amino acid</td>
</tr>
<tr>
<td>Neg.</td>
<td>17β-Trenbolone</td>
<td>10161-33-8</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Binds strongly to AR</td>
<td>Steroid, nonphenolic; Estrene</td>
</tr>
</tbody>
</table>

**Abbreviations:** AR = Androgen receptor; CASRN = Chemical Abstracts Service Registry Number; D1 and D2 = Two major families of dopamine receptors; DDE = 1,1-Dichloro-bis[4-chloro-phenyl]ethylene; DDT = Dichlorodiphenyltrichloroethane; ER = Estrogen receptor; HDT = Highest dose tested; Neg. = Negative; Pos. = Positive; RBA = Relative binding affinity; T3 = Triiodothyronine; T4 = Thyroxine.

* a Substances in bold type are those that, at a minimum, are recommended for inclusion in future validation studies. Empty cells indicate that no relevant data were identified.

* b Substances for which RBA data are available are sorted into six categories in log decrements: >10, <10-1, <1-0.1, <0.1-0.01, <0.01-0.001, and <0.001. A substance is classified as positive for ER binding if it was positive in more than 50% of reported studies. A substance is classified as presumed positive for ER binding if it was positive in 50% or less of reported studies, or if it was reported positive in the single study conducted. Only one substance, diethylhexyl phthalate, is classified as negative because it was tested in multiple studies at or above the limit dose of 1 mM recommended in Section 3.1.5. All other substances that did not produce an IC₅₀ value in an ER binding study are classified as presumed negative for ER binding since they were not tested at the recommended limit dose. Substances without RBA data are classified presumed positive or presumed negative based on available information, including their known mechanism of action or their responses in ER transcriptional activation (TA) assays, AR binding assays, or AR TA assays.

* c The RBA for a test substance is calculated as [IC₅₀(reference estrogen)/IC₅₀(test substance) x 100], where the IC₅₀ is the inhibitory concentration of the test substance that displaces 50% of the radiolabeled reference estrogen from the receptor. The median RBA values and the RBA ranges are derived from in vitro ER binding studies that were published in the peer-reviewed scientific literature and then reviewed and summarized in the NICEATM Background Review Document (BRD) titled Current Status of Test Methods for Detecting Endocrine Disruptors: In Vivo Estrogen Receptor Binding Assays-August 2002 (available on the ICCVAM website at http://iccvam.nih.gov/methods/endocrine.htm). Substances for which RBA data are available are ranked according to their relative potency in in vitro ER binding assays from most potent to least potent. Substances for which no relevant RBA data are available have been assigned an anticipated positive (Pos.) or negative (Neg.) response for ER binding based on available information, including their known mechanism of action or their responses in ER TA assays, AR binding assays, or AR TA assays.
### Table 3-1: ICCVAM Recommended Substances for Validation of In Vitro ER Binding Assays

<table>
<thead>
<tr>
<th>Substance</th>
<th>Validation Details</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>d</strong> Several in vivo test methods are undergoing further development or validation by OECD, EPA, and the JME (J). Substances indicated are proposed for testing by OECD in the Uterotrophic assay (U), the Hershberger assay (H), or the 407 protocol (407); for testing by EPA in the female pubertal assay (F-PA), the male pubertal assay (M-PA), the intact male assay (IM), a one-generation assay (IG), a two-generation assay (2G), or a fish reproductive screen (FRS); for testing by JME in the U, H, and IG assays, or various fish (F) and avian (A) assays. Due to the lack of CASRN for the JME studies, some of the indicated substances might not be the same substance indicated in this list. The in utero through lactation assay (IUL) has been recommended, but EPA has not made a decision on its further development or validation.</td>
<td></td>
</tr>
<tr>
<td><strong>e</strong> Information regarding the median RBA value, the corresponding RBA range, the number of ER binding test methods used, and the number of positive responses per number of studies conducted was derived from data presented in Appendix D of the NICEATM ER Binding BRD cited in footnote c. This document contains in vitro ER binding data from the published literature through September 30, 2001.</td>
<td></td>
</tr>
<tr>
<td><strong>f</strong> 17β-Estradiol is not considered a positive test substance for validation purposes, since it is the recommended reference estrogen for in vitro ER binding and TA assays (refer to Section 3.2 for more information).</td>
<td></td>
</tr>
<tr>
<td><strong>g</strong> Clomiphene citrate is classified presumed positive because only a single positive study was reported for this substance.</td>
<td></td>
</tr>
<tr>
<td><strong>h</strong> Two forms of p-nonylphenol are available for testing. One form consists of a mixture of various branched isomers (CASRN 84852-15-3), while the other contains only one isomer consisting of a linear alkyl chain (CASRN 104-40-5). ICCVAM recommends the linear form, which has a uniform chemical structure, for validation studies.</td>
<td></td>
</tr>
<tr>
<td><strong>i</strong> Testosterone and p,p'-DDE are classified presumed positive because these substances tested positive in less than 50% of the reported in vitro ER binding studies.</td>
<td></td>
</tr>
<tr>
<td><strong>j</strong> Atrazine has been associated with mammary tumors in rats (O'Connor et al. 2000), but it is thought to act through a mechanism other than binding to the ER (Connor et al. 1996; O'Connor et al. 2000; Sanderson et al. 2001). To be consistent with the classification scheme used for other substances where the proportion of positive studies is 50% or less, atrazine is classified presumed positive.</td>
<td></td>
</tr>
<tr>
<td><strong>k</strong> The HDT for diethylhexyl phthalate was 5000 µM in one study and 1000 µM in two studies.</td>
<td></td>
</tr>
<tr>
<td><strong>l</strong> The HDT for flavone was 100 µM in one study and 10 µM in two studies.</td>
<td></td>
</tr>
</tbody>
</table>
4.0 IN VITRO ESTROGEN RECEPTOR TRANSCRIPTIONAL ACTIVATION ASSAYS

4.1 Minimum Procedural Standards

More than 95 different in vitro assays have been used to evaluate the ability of substances to act as ER TA agonists or antagonists (NIEHS 2002b). Of the 95 in vitro ER TA assays considered in the ER TA BRD, 63 used mammalian cell lines, 22 used yeast cells, and 10 measured cell proliferation. The Expert Panel recommended that assays using yeast and those measuring cell proliferation not be considered for future validation efforts. Yeast-based assays were not recommended due to the poor transport of many substances across the yeast cell wall, while assays based on cell proliferation were not recommended because cell proliferation can be mediated through pathways other than those involving transcriptional activation of estrogen responsive genes. No validation studies have been conducted to assess the performance and reliability of these test methods, and the few substances tested multiple times using the same or different test methods preclude an assessment of comparative assay performance. Although the Expert Panel concluded that no specific in vitro ER TA test method could be recommended currently as a priority for validation, assays using cells with an endogenous or stably transfected ER and a stably or transiently transfected reporter vector containing the luciferase (Luc) gene were thought to be the most effective and reliable (see Appendix A). To assist in the development, standardization, and validation of in vitro ER TA assays, NICEATM and the EDWG developed proposed minimum procedural standards for consideration by the Expert Panel (NIEHS 2002b). The purpose of minimum procedural standards is to specify information essential for maximizing test method intra- and inter-laboratory reproducibility while minimizing the likelihood of erroneous results. Such standards also enhance any assessment of the comparative performance of different ER TA assays. The minimum procedural standards provided here have been revised to incorporate recommendations and comments of the Expert Panel, the EDWG, and the public. Except where noted, all in vitro ER TA assays should incorporate these minimal procedural standards in their protocols, and scientific justification should be provided for any deviations.

4.1.1 Reference Estrogen and TA Response

4.1.1.1 Agonism assays

The purpose of the reference estrogen in ER TA agonism assays is to demonstrate the adequacy of the test method for detecting ER agonists (i.e., the reference estrogen serves as a positive control). The recommended reference estrogen is 17β-estradiol (CASRN 50-28-2). The TA-inducing ability of the reference estrogen should be demonstrated by generating a full dose-response curve in each study. The concentration of 17β-estradiol used in most in vitro TA agonism assays ranges from 1 pM to 1 μM.

**Rationale:** 17β-Estradiol is the most potent naturally occurring estrogen in the human body, and virtually all published in vitro ER TA agonism studies have used this substance as the reference estrogen. Test acceptance criteria for the positive control should be established based on historical data for the maximum induction and on the calculated concentration of the reference estrogen that induces a half-maximal response (i.e., the effective concentration [EC₅₀] value).
4.1.1.2 Antagonism assays
In ER TA antagonism assays, test substances are evaluated for their ability to reduce the level of TA induced by a reference estrogen. The concentration of the reference estrogen selected for antagonism assays should be within the upper linear region of the dose-response curve; 70 to 80% of maximal induction is recommended. The recommended reference estrogen for these assays is 17\(\beta\)-estradiol.

**Rationale:** 17\(\beta\)-Estradiol is the most potent naturally occurring estrogen in the human body, and virtually all published *in vitro* ER TA antagonism studies have used this substance as the reference estrogen. The ability to detect a weak antagonist depends on the magnitude of the TA response induced by the reference estrogen. Using a reference estrogen concentration that elicits a response within the upper linear portion of the dose-response curve maximizes the sensitivity of the test method.

4.1.2 Preparation of Test Substances and Volume of Administered Solvent
Test substances should be dissolved in a solvent that is miscible with the cell medium. Water, ethanol (95 to 100%), or DMSO is the preferred solvent. Preference should be given to the solvent that allows testing of the test substance at the maximal concentration possible without exceeding the limit dose (see Section 4.1.3). However, in testing situations where more than one solvent could be used, preference should be given to water, followed by ethanol (95 to 100%), and then DMSO. Other solvents may be used if it can be demonstrated that they are not cytotoxic and otherwise do not interact with the test system. The volume of the solvent included in the reaction mixture generally has ranged from 0.1 to 1% of the total volume. For any solvent, it should be demonstrated that the maximum volume used does not interfere with the test system. This can be accomplished by comparing the maximum fold induction and the mean EC\(_{50}\) value for the reference estrogen in the presence and absence of the solvent at the highest volume to be used in the TA studies. The stability of the dissolved test substance should be determined prior to testing. In the absence of stability information, the stock solution should be prepared fresh prior to use.

**Rationale:** Selection of water, ethanol (95 to 100%), or DMSO as suitable solvents is based on historical usage. Members of the Expert Panel stated that water or ethanol (95 to 100%) is preferred to DMSO because some substances, when dissolved in DMSO, might result in reduced activity (see Section 4.1.4). For this reason, most investigators have limited the final concentration of DMSO to less than 0.1%. Because of differences in the sensitivity of various cell lines, the maximal concentration of a solvent that does not interfere with performance should be determined for each test method.

4.1.3 Concentration Range of the Test Substances
In the absence of solubility or cytotoxicity constraints, the maximum test substance concentration (i.e., the limit dose) for agonism or antagonism assays should be 1 mM. Seven test substance concentrations spaced at log intervals up to the limit dose (i.e., 1 nM, 10 nM, 100 nM, 1 \(\mu\)M, 10 \(\mu\)M, 100 \(\mu\)M, 1 mM) should be tested. An evaluation of cell cytotoxicity should be included in each study, and only those dose levels not associated with toxicity greater than 10% of the concurrent solvent control should be considered in the analysis of the data.

**Rationale:** Most test method guidelines include a limit dose to ensure that all substances are tested over the same dose range while avoiding excessive amounts of a test substance
that can perturb the test system through physicochemical mechanisms. An established limit dose also minimizes the effort and cost of screening and testing. Based on the range of published EC_{50} values for ER agonists and IC_{50} values for ER antagonists (NIEHS 2002b), a limit dose of 1 mM was deemed suitable by the Expert Panel, the EDWG, and ICCVAM for assessing the ability of a test substance to act as either an ER agonist or an antagonist.

The seven recommended test substance concentrations, spaced at log intervals, should be sufficient for a screening test because, currently, the study results will be used in a semi-quantitative manner only. If a lower maximum concentration is tested because of solubility or cytotoxicity constraints, the number of concentrations tested should remain the same by adding intermediate concentrations within the adjusted range. The purpose of the cytotoxicity assay is to ensure that only responses at nontoxic doses are considered.

### 4.1.4 Solvent and Positive Controls

#### 4.1.4.1 Solvent controls

**Agonism Assays**

In each study, a set of concurrent solvent control cultures should be included. The solvent control consists of the solvent in which the reference estrogen and the test substance are dissolved plus the cell line containing the ER, but without the reference estrogen. The solvent for the reference estrogen and test substance should be present at the highest volume that is used to add these substances to the test system. As indicated in Section 4.1.2, the solvent at the concentration used must not be cytotoxic or otherwise interact with the test system.

**Rationale:** The concurrent solvent control in TA agonism assays provides a measure of the extent of TA in the absence of the reference estrogen, other positive controls (if used), or the test substance, and is the baseline against which the extent of TA induced by these substances is compared.

**Antagonism Assays**

A concurrent set of solvent control cultures should be included in each study. The solvent controls consist of the solvent in which the reference estrogen and the test substance are dissolved, the cell line containing the ER, and the test method specific concentration of the reference estrogen (based on achieving 70 to 80% of the maximum TA of the reference estrogen). The solvent for the reference estrogen and test substance should be present at the highest volume that is used to add these substances to the test system. As indicated in Section 4.1.2, the solvent at the concentration used must not be cytotoxic or otherwise interact with the test system.

**Rationale:** The extent of TA in the presence of the reference estrogen is the baseline against which the antagonism of a test substance is measured.

#### 4.1.4.3 Positive control

**Agonism Assays**

In addition to the standard potent reference estrogen, it might be useful to include in each study a positive control estrogen (e.g., genistein) with a maximal TA response two to three orders of magnitude lower than the reference estrogen.

**Rationale:** The inclusion in each study of a second positive control in addition to the reference estrogen would provide another QC measure by which to judge the sensitivity and acceptability of a study for detecting a weak agonist, and by which to evaluate the historical intralaboratory reproducibility of the test method. The necessity for inclusion of an additional positive control estrogen in each
study should be evaluated during the validation process.

**Antagonism Assays**

A known ER antagonist (e.g., ICI 182,780) should be included as a positive antagonist control in each antagonism study. The concentration of the reference antagonist that is used should be one that reduces the ability of the reference estrogen to induce TA in the test system by 70 to 90%. The positive antagonist control should also be tested in the absence of the reference estrogen to determine whether it alone can induce TA.

**Rationale:** The purpose of the positive antagonist control is to demonstrate the sensitivity and reproducibility of the in vitro ER TA antagonism assay. A range of doses of a positive control antagonist that inhibits the ability of the reference estrogen to induce TA will allow for historical confidence intervals to be calculated, which can be used as a QC measure to ensure the adequacy of each study. ICI 182,780 is suggested as the candidate ER antagonist as this substance historically has been shown to be negative as an agonist but positive as an antagonist. Other substances that may be used as a positive control antagonist should produce a similar response.

**4.1.5 Within-Test Replicates**

All concentration levels of the controls, the reference estrogen, and the test substance should be tested in triplicate.

**Rationale:** The purpose of triplicate tubes for each concentration and volume of the various controls, the reference estrogen, and the test substance is to ensure robust data and the ability to evaluate interreplicate variability. The most appropriate number of replicate tubes, however, should be evaluated after sufficient data has been collected using an optimized test method protocol.

**4.1.6 Data Analysis**

No standardized statistical methods for analyzing data obtained from in vitro ER TA assays have been developed. For agonism assays, an EC$_{50}$ is calculated for the concentration of the test substance and the positive control(s) that result in 50% of the maximal TA response. TA induction may also be reported as fold increase above the concurrent solvent control response. For antagonism assays, the TA response induced by a test substance in the presence of the reference estrogen is compared to the response induced by the reference estrogen alone and an IC$_{50}$ is calculated (i.e., the test substance concentration that reduces the reference estrogen response by 50%). Approaches for data analysis have varied from a visual inspection of the data to more formal statistical approaches involving either one- or two-way analysis of variance (ANOVA) (with main effects being treatment and replicates), using a general linear model based on means and variances for the fold induction above the concurrent solvent control level. The EC$_{50}$ (agonism assays) or IC$_{50}$ (antagonism assays) values have been calculated using various curve-fitting programs. One curve-fitting approach is based on a logistic dose-response model where the asymptotic minimum and maximum response, the dose that is halfway between the minimum and maximum, and the slope of the line tangent to the logistic curve at this midpoint are determined (Gaido et al. 1997). Asymptotic standard errors of the parameter estimates are employed to perform two-sided Student’s t tests. However, when EC$_{50}$ or IC$_{50}$ values cannot be calculated, an appropriate trend analysis could be used to evaluate for a significant dose-response relationship for agonism or antagonism. Then, an appropriate pair-wise test could be used to evaluate for a significant effect at the different test substance concentrations. In addition, the corresponding historical mean and confidence
4.1.9 Interpretation of Results
A substance is classified as an ER agonist if the response (e.g., luciferase activity) elicited by the substance is increased significantly above the concurrent solvent control level, as determined by an appropriate statistical test. A substance is classified as an ER antagonist if the substance causes a significant decrease in the ability of the reference estrogen to induce TA, as determined by an appropriate statistical test. However, interpretation of the results should not rely solely on statistics alone but also on scientific judgment and should incorporate consideration of the nature and shape of the dose-response relationship and, if needed, the reproducibility of the response in independent experiments. If a substance does not induce TA or inhibit the ability of the reference estrogen to induce TA after testing to the limit dose or to the maximum concentration possible based on its solubility or cytotoxicity, the test substance is classified as negative for agonism and antagonism, respectively, under conditions of the test.

4.1.10 Repeat Studies
Generally, in a validation study, repeat studies would be conducted to evaluate intralaboratory repeatability and reproducibility. In contrast, in screening studies, repeat studies are not conducted, except to clarify equivocal results. If a study is repeated, the use of test substance concentrations more closely distributed in the range of interest might facilitate a more accurate analysis of the dose-response relationship for the test substance.

4.1.7 Good Laboratory Practice Compliance
Studies should be performed in compliance with GLP guidelines (EPA 2001, 2002; FDA 2002; OECD 1998).

Rationale: Conducting studies in compliance with GLP guidelines increases confidence in the quality and reliability of test data. Furthermore, if data using these test methods are to be submitted to the EPA in response to Federal testing requirements, then compliance with appropriate GLP guidelines will be required.

4.1.8 Study Acceptance Criteria
- The limit dose should be 1 mM, unless precluded by solubility or cytotoxicity constraints.
- The response (fold increase, EC50 or IC50 values) for the reference estrogen and the positive control should be within the appropriate historical acceptance range.
- The study should comply with GLP guidelines.

Rationale: Established study acceptance criteria are required to ensure that the study is conducted appropriately.
method. However, for a screening study, if the acceptance criteria are met and a clear negative or positive response is obtained, a repeat study to verify the original result usually is not considered necessary. In studies where an accurate EC$_{50}$ or IC$_{50}$ value cannot be calculated or where an equivocal response is obtained, a repeat study using adjusted dose levels might be needed to ensure a reliable conclusion.

4.1.11 Study Report
At a minimum, the study report should include the following information:

Reference Estrogen
- name, CASRN, purity, and supplier or source of the reference estrogen
- concentrations and volumes used

Additional Positive Control (if used)
- name, CASRN, purity, and supplier or source
- concentrations and volumes used

Test Substance
- name, chemical structure (if known), CASRN (if known), and supplier or source
- physical nature (solid or liquid) and purity, if known (every attempt should be made to determine the purity)
- physicochemical properties relevant to the study (e.g., solubility, pH, stability, volatility)
- concentrations and volumes used

Solvent
- name, CASRN, purity, and supplier or source
- justification for choice of solvent
- information on the solubility of the test substance in all solvents in which it was tested
- information to demonstrate that the solvent, at the maximum volume used, is not cytotoxic and otherwise does not interfere with the study

Estrogen Receptor
- type and source of ER and the supplier
- isolation procedure or method for making constructs
- nomenclature and components of the expression construct
- complete DNA sequence of ER incorporated into expression construct

Reporter Plasmid
- type of reporter gene
- type and structure of response elements
- name, identification and source of original plasmid used to make construct
- sequence of the inserts in each plasmid
- description and methodology used to make the transfected plasmid
- nomenclature and genetic components comprising the reporter construct

Cell Line
- source and nomenclature of the cell line and protocol for its maintenance before and after transfection
- source of plasticware used to culture cells and source of other materials used in the study
- passage number of cell line used for transfection and passage number of cell line used in the study
- growth parameters of the cell line before initiation of the study
- method used to transiently transf ect the reporter construct into the cells
- method used to monitor transient transfection efficiency between cell preparations
- methods for establishment and propagation of a stably transfected cell line and what is required for growth of the cell line (e.g., charcoal-stripped serum)
• method used to monitor the stability of a stably transfected cell line used for testing
• rationale, based on data, for deciding on the number of passages a cell line can undergo without a decrease in activity
• details regarding selection requirements needed for maintaining stable cell lines

Study Conditions
• rationale for the concentration of the reference estrogen used
• composition of media and buffers used
• concentration range of the test substance, with justification
• volume of the solvent used to dissolve the test substance and the volume added to the reaction mixture
• incubation volume, duration, and temperature
• description of the solvent control
• level of carbon dioxide in the incubator when growing cells and throughout study
• type and composition of metabolic activation system, if used
• concentration ranges of positive controls
• method used to lyse cells after incubation
• method used to measure TA based on reporter activity
• statistical methods used to determine the response and EC\textsubscript{50} value for agonism studies or IC\textsubscript{50} value for antagonism studies

Results
• observations for and extent of any precipitation of test substance
• extent of cytotoxicity at each dose level
• reporter response for each replicate at each dose for all test substances, along with confidence levels or other measure of intra-dose repeatability
• graphically presented dose-response curves for the reference estrogen (agonism studies), the positive control(s), and the test substance
• calculated EC\textsubscript{50} value for agonism studies or IC\textsubscript{50} value for antagonism studies and confidence limits for the reference estrogen (agonism studies), positive control(s), and test substance
• in agonism studies, the fold increase above the concurrent solvent control in TA for each concentration of the reference estrogen, the additional positive control (if used), and the test substance
• for antagonism studies, the percent decrease in TA for each concentration of the positive control and the test substance

Discussion of Results
• in each agonism study, reproducibility of fold increases in activity and in the EC\textsubscript{50} value for the reference estrogen, including ranges, means, standard deviations, and confidence intervals, compared to historical data
• in agonism studies, historical EC\textsubscript{50} values for the additional positive control estrogen, if used, with ranges, means, standard deviations, and confidence intervals
• in antagonism studies, reproducibility of fold decreases in activity for the reference estrogen and the IC\textsubscript{50} values for the reference antagonist, including ranges, means, standard deviations, and confidence intervals, compared to historical data

Conclusion
• classification of test substance with regard to in vitro ER TA agonist or antagonist activity

Rationale: Minimum reporting standards are needed to ensure that a study report contains the level of information and detail that would be required if the study results are reviewed by the applicable regulatory agency, or for
independent replication of the study, if deemed necessary.

4.2 Recommended Substances for Validation of In Vitro Estrogen Receptor Transcriptional Activation Assays

To facilitate validation of in vitro ER TA assays, ICCVAM has compiled a list of 78 recommended substances for use in future validation studies. Separate lists are provided of the available quantitative and qualitative data and anticipated responses of each of the 78 substances in in vitro ER TA agonism (Table 4-1) and antagonism (Table 4-2) assays. Section 2.0 provides a detailed account of how these substances were selected. EC50 and IC50 data are available for 18 (23%) and 8 (10%) of these 78 recommended substances for agonism and antagonism, respectively. Qualitative data are available for 27 (35%) and 10 (13%) of these 78 recommended substances for agonism and antagonism, respectively. Thus, there is incomplete information regarding how all 78 of the recommended substances will respond in in vitro ER TA agonism and antagonism assays utilizing mammalian cell reporter gene systems. Although 17β-estradiol is included in the list of recommended substances, it was not included in the count of substances for validation as it is a required component of the test system to measure antagonism and is the positive control for agonism studies. Quantitative in vitro ER TA data are provided for the substances inducing a positive response in at least one study. This includes the median EC50 or IC50 values for agonism and antagonism studies, respectively, a range of values where more than one study had been conducted, and the number of studies and test methods in which each substance was tested. In situations where only one positive study was reported, the EC50 or IC50 value obtained in that study is reported. The substances with EC50 or IC50 data are listed first, sorted by potency from strongest to weakest, based on the median EC50 or IC50 value of each substance across all positive studies. Substances that induced a positive response in 50% or fewer of the ER TA studies in which they were tested are classified in this table as “presumed positive” for ER agonism or antagonism. No effort was made to assess the validity and quality of each negative or positive study reported for each substance. These substances are sorted by most positive responses per number of times tested. Substances were classified as negative for ER TA agonism or antagonism activity if they were reported as negative in multiple studies when tested up to the limit dose as defined in this document (i.e., 1 mM). Substances were classified as “presumed negative” for ER TA activity if they had not been tested to the limit dose in multiple studies (i.e., reproducibility for a negative response had not been demonstrated at test substance concentration up to 1 mM). Using these criteria, no substances could be classified as negative for ER TA activity. Following the presumed negative substances are those without relevant in vitro ER TA data. Substances lacking either quantitative or qualitative data have been assigned a presumed positive or negative response in in vitro ER TA assays, based on the substances’ anticipated or known mechanism of action and response in in vitro ER binding assays. Presumed positive substances are listed first, followed by presumed negative substances that have been selected for the minimal list of substances (see below and Section 2.4.4). Both categories are sorted alphabetically by substance name. The remaining substances that are presumed

1Inclusion of a substance does not mean that EPA, NICEATM, ICCVAM, or the Expert Panel has or will make a determination that any use of the substance will pose a significant risk. Further, these substances should not be interpreted to be “endocrine disruptors”; the substances listed are simply compounds that have been or may prove to be useful in developing, standardizing, or validating screening and testing methods.
negative are sorted alphabetically at the end of the list.

Substances have been classified as presumed positive for agonism even when less than 50% of the studies were positive. Without detailed information regarding the experimental protocol used, it is not possible to assess the quality of the data. However, with the ER TA agonism tests, false positive responses are possible if the cell line used in the study contains a glucocorticoid or progesterone receptor and the mouse mammary tumor virus hormone response element is incorporated into the reporter construct. The classification of a substance as positive (and its ranking) or negative in this list is based sometimes on the results of a single study and, therefore, the accuracy of the classification is questionable. However, it is anticipated that testing these presumed positive and negative substances will provide critical information on the comparative sensitivity and reproducibility of different in vitro ER TA assays, when such methods are standardized and conducted using the recommended minimum procedural standards.

The quantitative and qualitative data provided with this substance list summarize information obtained primarily from peer-reviewed scientific reports. Because the positive data were obtained from studies using different in vitro ER TA assays, they show a great deal of variability and, thus, the reported values should not be used as definitive target values to be obtained during the validation process. The data summaries presented in Tables 4-1 and 4-2 are provided to inform interested investigators of the historical quantitative values obtained for these substances in in vitro ER TA assays.

As described in Section 2.4.4, and mentioned above, a subset of 53 substances has been identified that, at a minimum, should be used in any validation of in vitro ER TA assays. These 53 substances are in bold type in Table 4-1 for agonism. Of these substances, 64% (34) are classified as positive (21) or presumed positive (13) for ER agonism, and 36% (19) are classified as presumed negative. The same 53 substances are in bold type in Table 4-2 for antagonism. Of these substances, 21% (11) are classified as positive (5) or presumed positive (6) for ER antagonism, and 79% (42) are classified as presumed negative.
<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Median EC&lt;sub&gt;50&lt;/sub&gt; Value and Range Across All MCRG Studies&lt;sup&gt;b&lt;/sup&gt; (µM)</th>
<th>Qualitative Response for ER Agonism Across All MCRG Studies&lt;sup&gt;c&lt;/sup&gt;</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Completed/Anticipated In Vivo Testing&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>17α-Ethinyl estradiol&lt;sup&gt;e&lt;/sup&gt;</td>
<td>57-63-6</td>
<td>0.0000011 (2) 0.00000073 - 0.0000144</td>
<td>Pos. (2/2)</td>
<td>2</td>
<td>U; 407; F-PA</td>
<td>Strong ER agonist</td>
<td>Steroid, phenolic</td>
</tr>
<tr>
<td>Diethylstilbestrol&lt;sup&gt;e&lt;/sup&gt;</td>
<td>56-53-1</td>
<td>0.000019 (3) 0.000015 - 0.000024</td>
<td>Pos. (8/8)</td>
<td>8</td>
<td>IUL</td>
<td>Strong ER agonist</td>
<td>Stilbene; Benzyldiene; Diphenylalkene</td>
</tr>
<tr>
<td>17α-Estradiol&lt;sup&gt;e&lt;/sup&gt;</td>
<td>57-91-0</td>
<td>0.000046 (1)</td>
<td>Pos. (2/2)</td>
<td>2</td>
<td></td>
<td>ER agonist</td>
<td>Steroid, phenolic; Estrene</td>
</tr>
<tr>
<td>17β-Estradiol&lt;sup&gt;e,f&lt;/sup&gt;</td>
<td>50-28-2</td>
<td>0.0011 (29) 0.0000005 - 0.0099</td>
<td>Pos. (77/77)</td>
<td>45</td>
<td>IM; IUL; FRS</td>
<td>Strong ER and AR agonist; AR antagonist</td>
<td>Steroid, phenolic; Estrene</td>
</tr>
<tr>
<td>meso-Hexestrol&lt;sup&gt;e&lt;/sup&gt;</td>
<td>84-16-2</td>
<td>0.0002 (1)</td>
<td>Pos. (1/1)&lt;sup&gt;j&lt;/sup&gt;</td>
<td>1</td>
<td></td>
<td>Strong ER agonist</td>
<td>Diphenylalkane; Bisphenol; Phenol</td>
</tr>
<tr>
<td>Zearalenone&lt;sup&gt;e&lt;/sup&gt;</td>
<td>17924-92-4</td>
<td>0.0002 (3) 0.001 - 0.0073</td>
<td>Pos. (8/8)</td>
<td>6</td>
<td></td>
<td>ER agonist</td>
<td>Resorcylic acid lactone; Phenol</td>
</tr>
<tr>
<td>Estrone&lt;sup&gt;e&lt;/sup&gt;</td>
<td>53-16-7</td>
<td>0.0032 (2) 0.00002 - 0.0063</td>
<td>Pos. (3/3)</td>
<td>3</td>
<td></td>
<td>Strong ER agonist; AR agonist</td>
<td>Steroid, phenolic; Estrene</td>
</tr>
<tr>
<td>Methyl testosterone&lt;sup&gt;e&lt;/sup&gt;</td>
<td>58-18-4</td>
<td>0.011 (2) 0.00573 - 0.0158</td>
<td>Pos. (2/2)</td>
<td>2</td>
<td>H; 407; M-PA; IUL; FRS</td>
<td>ER and AR agonist</td>
<td>Steroid, nonphenolic; Androstene</td>
</tr>
<tr>
<td>Coumestrol&lt;sup&gt;e&lt;/sup&gt;</td>
<td>479-13-0</td>
<td>0.015 (3) 0.01 - 0.017</td>
<td>Pos. (8/8)</td>
<td>7</td>
<td>IM</td>
<td>ER agonist</td>
<td>Coumestan; Benzopyranone; Coumarin; Ketone</td>
</tr>
<tr>
<td>Genistein&lt;sup&gt;e&lt;/sup&gt;</td>
<td>446-72-0</td>
<td>0.062 (5) 0.00423 - 0.1</td>
<td>Pos. (11/11)</td>
<td>10</td>
<td>U; 407</td>
<td>Weak ER agonist and antagonist</td>
<td>Flavanoid; Isoflavone; Phenol</td>
</tr>
<tr>
<td>p-n-Nonylphenol&lt;sup&gt;e,g&lt;/sup&gt;</td>
<td>104-40-5</td>
<td>0.085 (3) 0.0356 - 0.26</td>
<td>Pos. (4/4)</td>
<td>4</td>
<td>U; 407; J(U,H,1G,F,A)</td>
<td>ER and AR antagonist; ER agonist</td>
<td>Alkylphenol; Phenol</td>
</tr>
<tr>
<td>Bisphenol B&lt;sup&gt;e&lt;/sup&gt;</td>
<td>77-40-7</td>
<td>0.088 (2) 0.0624 - 0.114</td>
<td>Pos. (2/2)</td>
<td>2</td>
<td></td>
<td>ER agonist</td>
<td>Diphenylalkane; Bisphenol; Phenol</td>
</tr>
<tr>
<td>Substance</td>
<td>CASRN</td>
<td>Median EC$_{50}$ Value and Range Across All MCRG Studies$^b$ (µM)</td>
<td>Qualitative Response for ER Agonism Across All MCRG Studies$^c$</td>
<td>No. of MCRG Assays in Which Tested</td>
<td>Completed/Anticipated In Vivo Testing$^d$</td>
<td>Comments</td>
<td>Chemical Class</td>
</tr>
<tr>
<td>----------------------</td>
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<td>---------------------------------------------------------------</td>
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<td>-----------------------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>Daidzein$^e$</td>
<td>486-66-8</td>
<td>0.29 (2) 0.09 - 0.49</td>
<td>Pos. (5/5)</td>
<td>5</td>
<td>U; F-PA; J(1G,F,A)</td>
<td>Weak ER agonist</td>
<td>Flavanoid; Isoflavone; Phenol</td>
</tr>
<tr>
<td>4-Cumylphenol$^e$</td>
<td>599-64-4</td>
<td>0.322 (2) 0.248 - 0.395</td>
<td>Pos. (2/2)</td>
<td>2</td>
<td>U; J(1G,F,A)</td>
<td>Weak ER agonist</td>
<td>Phenol</td>
</tr>
<tr>
<td>Bisphenol A$^e$</td>
<td>80-05-7</td>
<td>0.40 (10) 0.000033 - 0.89</td>
<td>Pos. (15/15)</td>
<td>13</td>
<td>U; J(1G,F,A)</td>
<td>ER agonist</td>
<td>Diphenylalkane; Bisphenol; Phenol</td>
</tr>
<tr>
<td>$o,p'$-DDT$^e$</td>
<td>789-02-6</td>
<td>0.66 (1)</td>
<td>Pos. (7/8)</td>
<td>8</td>
<td>U; J(1G,F,A)</td>
<td>Weak ER and AR antagonist; Weak ER agonist</td>
<td>Organochlorine; Diphenylalkene</td>
</tr>
<tr>
<td>$p,p'$-Methoxychlor$^e$</td>
<td>72-43-5</td>
<td>8.85 (2) 5.7 - 12</td>
<td>Pos. (12/13)</td>
<td>13</td>
<td>IUL; IM; FRs; 2G(avian)</td>
<td>Weak ER agonist; AR antagonist</td>
<td>Organochlorine; Chlorinated hydrocarbon</td>
</tr>
<tr>
<td>Fenarimol$^h$</td>
<td>60168-88-9</td>
<td>27 (1)</td>
<td>Pos. (1/1)$^j$</td>
<td>1</td>
<td>F-PA</td>
<td>Aromatase inhibitor</td>
<td>Heterocycle; Pyrimidine</td>
</tr>
</tbody>
</table>

**QUALITATIVE DATA ONLY**

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Qualitative Response for ER Agonism Across All MCRG Studies$^c$</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apigenin$^{e,i}$</td>
<td>520-36-5</td>
<td>Pos. (6/6)</td>
<td>5</td>
<td>IUL</td>
<td>ER agonist</td>
</tr>
<tr>
<td>Tamoxifen$^{e,i}$</td>
<td>10540-29-1</td>
<td>Pos. (5/7)</td>
<td>6</td>
<td>ER antagonist</td>
<td>Triphenylethylene; Benzylidine; Stilbene</td>
</tr>
<tr>
<td>Kepone$^{e,i}$ (Chlordecone)</td>
<td>143-50-0</td>
<td>Pos. (4/6)</td>
<td>6</td>
<td>Binds to ER and AR</td>
<td>Organochlorine; Chlorinated bridged cycloalkane</td>
</tr>
<tr>
<td>Butylbenzyl phthalate$^{e,i}$</td>
<td>85-68-7</td>
<td>Pos. (3/4)</td>
<td>4</td>
<td>IUL</td>
<td>ER agonist</td>
</tr>
<tr>
<td>4-Hydroxytamoxifen$^{e,i}$</td>
<td>68047-06-3</td>
<td>Pos. (3/8)$^j$</td>
<td>8</td>
<td>ER antagonist</td>
<td>Triphenylethylene; Benzylidine; Stilbene; Phenol</td>
</tr>
</tbody>
</table>

$^a$ (continued)
### Table 4-1: ICCVAM Recommended Substances for Validation of *In Vitro* ER TA Agonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems^a^ (continued)

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Median EC_{50} Value and Range Across All MCRG Studies^b^ (µM)</th>
<th>Qualitative Response for ER Agonism Across All MCRG Studies^c^</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Completed/Anticipated In Vivo Testing^d^</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaempferol</td>
<td>520-18-3</td>
<td>Pos. (2/2)</td>
<td>2</td>
<td></td>
<td>ER agonist</td>
<td>Flavanoid; Flavone</td>
<td>Phenol</td>
</tr>
<tr>
<td>4-tert-Octylphenol</td>
<td>140-66-9</td>
<td>Pos. (2/3)</td>
<td>3</td>
<td>J(U,H,1G,F,A)</td>
<td>ER agonist</td>
<td>Alkylphenol; Phenol</td>
<td></td>
</tr>
<tr>
<td>pp'-DDE</td>
<td>72-55-9</td>
<td>Pos. (2/4)</td>
<td>4</td>
<td>H; 407; M-PA; IM; J(1G,F,B)</td>
<td>Weak AR agonist and antagonist</td>
<td>Organochlorine; Diphenylalkene</td>
<td></td>
</tr>
<tr>
<td>Di-n-butyl phthalate</td>
<td>84-74-2</td>
<td>Pos. (2/4)</td>
<td>4</td>
<td>U; M-PA; 1G; J(U,H,1G,F,A)</td>
<td>ER agonist</td>
<td>Phthalate</td>
<td></td>
</tr>
<tr>
<td>Flavone</td>
<td>525-82-6</td>
<td>Pos. (2/5)</td>
<td>4</td>
<td>M-PA; IM</td>
<td>Weak ER antagonist</td>
<td>Flavanoid; Flavone</td>
<td></td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>50-02-2</td>
<td>Pos. (1/1)</td>
<td>1</td>
<td></td>
<td>AR agonist</td>
<td>Steroid, nonphenolic</td>
<td></td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>521-18-6</td>
<td>Pos. (1/1)</td>
<td>1</td>
<td>H</td>
<td>Weak ER agonist; Strong AR agonist</td>
<td>Steroid, nonphenolic</td>
<td></td>
</tr>
<tr>
<td>2,4,5-Trichlorophenoxyacetic acid</td>
<td>93-76-5</td>
<td>Pos. (1/1)</td>
<td>1</td>
<td></td>
<td>Weak ER agonist</td>
<td>Organochlorine; Chlorinated aromatic hydrocarbon</td>
<td></td>
</tr>
<tr>
<td>Dibenzo[a,h]-anthracene</td>
<td>53-70-3</td>
<td>Pos. (1/2)</td>
<td>2</td>
<td></td>
<td></td>
<td>Polycyclic aromatic hydrocarbon; Anthracene</td>
<td></td>
</tr>
<tr>
<td>ICI 182,780</td>
<td>129453-61-8</td>
<td>Neg. (9/10)</td>
<td>9</td>
<td>IM</td>
<td>ER antagonist</td>
<td>Steroid, phenolic</td>
<td></td>
</tr>
<tr>
<td>Atrazine</td>
<td>1912-24-9</td>
<td>Neg. (3/3)</td>
<td>3</td>
<td>M-PA; IUL</td>
<td></td>
<td>Aromatic amine; Triazine; Arylamine</td>
<td></td>
</tr>
<tr>
<td>Progesterone</td>
<td>57-83-0</td>
<td>Neg. (2/2)</td>
<td>2</td>
<td>IM</td>
<td></td>
<td>Steroid, nonphenolic; Pregnenedione</td>
<td></td>
</tr>
</tbody>
</table>
Table 4-1: ICCVAM Recommended Substances for Validation of *In Vitro* ER TA Agonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Median EC(_{50}) Value and Range Across All MCRG Studies(^b) (µM)</th>
<th>Qualitative Response for ER Agonism Across All MCRG Studies(^c)</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Completed/ Anticipated <em>In Vivo</em> Testing(^d)</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>58-22-0</td>
<td>Neg. (2/2)</td>
<td>2</td>
<td>IM</td>
<td>Strong AR agonist</td>
<td>Steroid, nonphenolic</td>
<td></td>
</tr>
<tr>
<td>Corticosterone</td>
<td>50-22-6</td>
<td>Neg. (1/1)</td>
<td>1</td>
<td>F&amp;M-PA; IM</td>
<td>Binds weakly to AR</td>
<td>Steroid, nonphenolic</td>
<td></td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>57-30-7</td>
<td>Neg. (1/1)</td>
<td>1</td>
<td>F&amp;M-PA; IM</td>
<td>Enhances thyroid hormone excretion</td>
<td>Heterocycle; Pyrimidine</td>
<td></td>
</tr>
<tr>
<td>Vinclozolin</td>
<td>50471-44-8</td>
<td>Neg. (1/1)</td>
<td>1</td>
<td>H; M-PA; IM; IM; 1UL; 1G; FRS</td>
<td>AR antagonist</td>
<td>Organochlorine; Cyclic imide; Carbamate</td>
<td></td>
</tr>
<tr>
<td>Cyproterone acetate</td>
<td>427-51-0</td>
<td>Neg. (1/1)</td>
<td>1</td>
<td>IM</td>
<td>AR agonist and antagonist</td>
<td>Nitrile; Diphenyl ether; Organochlorine</td>
<td></td>
</tr>
<tr>
<td>Flutamide</td>
<td>13311-84-7</td>
<td>Neg. (1/1)</td>
<td>1</td>
<td>H; 407; M-PA; IM; M; FRS</td>
<td>AR antagonist</td>
<td>Amide; Anilide; Nitrobenzene</td>
<td></td>
</tr>
<tr>
<td>Linuron</td>
<td>330-55-2</td>
<td>Neg. (1/1)</td>
<td>1</td>
<td>H; M-PA</td>
<td>Weak AR agonist and antagonist</td>
<td>Urea</td>
<td></td>
</tr>
<tr>
<td>Methyltrienolone</td>
<td>965-93-5</td>
<td>Neg. (1/1)</td>
<td>1</td>
<td>M-PA</td>
<td>AR agonist</td>
<td>Steroid, nonphenolic; Estrene</td>
<td></td>
</tr>
<tr>
<td>Mifepristone</td>
<td>84371-65-3</td>
<td>Neg. (1/1)</td>
<td>1</td>
<td>IM</td>
<td>AR agonist and antagonist</td>
<td>Steroid, nonphenolic; Estrene</td>
<td></td>
</tr>
<tr>
<td>Procymidone</td>
<td>32809-16-8</td>
<td>Neg. (1/1)</td>
<td>1</td>
<td>AR antagonist</td>
<td></td>
<td>Organochlorine; Cyclic imide</td>
<td></td>
</tr>
<tr>
<td>Clomiphene citrate</td>
<td>50-41-9</td>
<td>Pos.</td>
<td></td>
<td>Binds to the ER; Selective estrogen receptor modulator</td>
<td>Chlorinated triphenylethylene; Benzyldiene; Stilbene</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ANTICIPATED RESPONSES (No EC\(_{50}\) or Qualitative Agonism Data Available)

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Response</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clomiphene citrate</td>
<td>50-41-9</td>
<td>Pos.</td>
<td>Binds to the ER; Selective estrogen receptor modulator</td>
</tr>
<tr>
<td>Substance</td>
<td>CASRN</td>
<td>Median EC&lt;sub&gt;50&lt;/sub&gt; Value and Range Across All MCRG Studies&lt;sup&gt;b&lt;/sup&gt; (µM)</td>
<td>Qualitative Response for ER Agonism Across All MCRG Studies&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>----------------------------</td>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Ethyl paraben</td>
<td>120-47-8</td>
<td>120-47-8</td>
<td>Pos.</td>
</tr>
<tr>
<td>Norethynodrel</td>
<td>68-23-5</td>
<td>68-23-5</td>
<td>Pos.</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>50-76-0</td>
<td>50-76-0</td>
<td>Neg.</td>
</tr>
<tr>
<td>4-Androstenedione</td>
<td>63-05-8</td>
<td>63-05-8</td>
<td>Neg.</td>
</tr>
<tr>
<td>2-sec-Butylphenol</td>
<td>89-72-5</td>
<td>89-72-5</td>
<td>Neg.</td>
</tr>
<tr>
<td>Diethylhexylphthalate</td>
<td>117-81-7</td>
<td>117-81-7</td>
<td>Neg.</td>
</tr>
<tr>
<td>Fadrozole</td>
<td>102676-47-1</td>
<td>102676-47-1</td>
<td>Neg.</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>206-44-0</td>
<td>206-44-0</td>
<td>Neg.</td>
</tr>
<tr>
<td>Hydroxyflutamide</td>
<td>52806-53-8</td>
<td>52806-53-8</td>
<td>Neg.</td>
</tr>
<tr>
<td>Morin</td>
<td>480-16-0</td>
<td>480-16-0</td>
<td>Neg.</td>
</tr>
<tr>
<td>Phenolphthalin</td>
<td>81-90-3</td>
<td>81-90-3</td>
<td>Neg.</td>
</tr>
</tbody>
</table>
Table 4-1: ICCVAM Recommended Substances for Validation of *In Vitro* ER TA Agonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems* (continued)

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Median EC$_{50}$ Value and Range Across All MCRG Studies$^b$ (µM)</th>
<th>Qualitative Response for ER Agonism Across All MCRG Studies$^c$</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Completed/Anticipated <em>In Vivo</em> Testing$^d$</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propylthiouracil</td>
<td>51-52-5</td>
<td>Neg.</td>
<td></td>
<td>407; F&amp;M-PA; IM; IUL; 2G</td>
<td>Inhibits T3/T4 synthesis</td>
<td>Pyrimidine; Uracil</td>
<td></td>
</tr>
<tr>
<td>Sodium azide</td>
<td>26628-22-8</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>Cytotoxic</td>
<td>Organic salt; Azide</td>
<td></td>
</tr>
<tr>
<td>12-O-Tetradecanoyl-phorbol-13-acetate</td>
<td>16561-29-8</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>Activates ligand independent cell division</td>
<td>Phorbol ester; Terpene</td>
<td></td>
</tr>
<tr>
<td>Ammonium perchlorate</td>
<td>7790-98-9</td>
<td>Neg.</td>
<td>IUL</td>
<td></td>
<td>Thyroid disruptor</td>
<td>Organic acid; Organic salt</td>
<td></td>
</tr>
<tr>
<td>Anastrazole</td>
<td>120511-73-1</td>
<td>Neg.</td>
<td>IM</td>
<td></td>
<td>Aromatase inhibitor</td>
<td>Nitrile; Triazole</td>
<td></td>
</tr>
<tr>
<td>Apomorphine</td>
<td>58-00-4</td>
<td>Neg.</td>
<td>IM</td>
<td></td>
<td>Dopamine D1/D2 receptor agonist; Neg. for ER agonism in yeast assay</td>
<td>Heterocycle; Quinoline</td>
<td></td>
</tr>
<tr>
<td>Bicalutamide</td>
<td>90357-06-5</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>AR antagonist</td>
<td>Anilide; Nitrile; Sulfone</td>
<td></td>
</tr>
<tr>
<td>CGS 18320B</td>
<td>112808-99-8</td>
<td>Neg.</td>
<td></td>
<td>407</td>
<td>Aromatase inhibitor</td>
<td>Nitrile; Imidazole</td>
<td></td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>66-81-9</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>Protein synthesis inhibitor</td>
<td>Piperidine; Glutaramide</td>
<td></td>
</tr>
<tr>
<td>Finasteride</td>
<td>98319-26-7</td>
<td>Neg.</td>
<td>H; M-PA; IM</td>
<td></td>
<td>5α-Reductase inhibitor</td>
<td>Steroid, nonphenolic; Androstene</td>
<td></td>
</tr>
<tr>
<td>Fluoxymestrone</td>
<td>76-43-7</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>Weak AR agonist</td>
<td>Steroid, nonphenolic</td>
<td></td>
</tr>
</tbody>
</table>
Table 4-1: ICCVAM Recommended Substances for Validation of In Vitro ER TA Agonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems\textsuperscript{a} (continued)

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Median EC\textsubscript{50} Value and Range Across All MCRG Studies\textsuperscript{b} (µM)</th>
<th>Qualitative Response for ER Agonism Across All MCRG Studies\textsuperscript{c}</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Completed/Anticipated In Vivo Testing\textsuperscript{d}</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haloperidol</td>
<td>52-86-8</td>
<td>Neg.</td>
<td>IM</td>
<td>IM</td>
<td>Dopamine D2 receptor antagonist; Neg. for ER agonism in yeast assay</td>
<td>Butyrophenone; Ketone; Piperazine</td>
<td></td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>65277-42-1</td>
<td>Neg.</td>
<td>F&amp;M-PA; IM</td>
<td>Weak AR agonist</td>
<td>Imidazole; Piperazine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medroxyprogesterone acetate</td>
<td>71-58-9</td>
<td>Neg.</td>
<td>Weak AR agonist</td>
<td>Steroid, nonphenolic; Polycyclic hydrocarbon</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nilutamide</td>
<td>63612-50-0</td>
<td>Neg.</td>
<td>AR antagonist</td>
<td>Heterocycle; Imidazole</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxazepam</td>
<td>604-75-1</td>
<td>Neg.</td>
<td>IM</td>
<td>Enhances thyroid hormone excretion</td>
<td>Benzodiazepine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pimozide</td>
<td>2062-78-4</td>
<td>Neg.</td>
<td>F&amp;M-PA; Dopamine receptor antagonist</td>
<td>Piperidine; Benzimidazole</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reserpine</td>
<td>50-55-5</td>
<td>Neg.</td>
<td>IM</td>
<td>Depletes dopamine; Neg. for ER agonism in yeast assay</td>
<td>Heterocycle; Yohimbine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spironolactone</td>
<td>52-01-7</td>
<td>Neg.</td>
<td>AR agonist and antagonist</td>
<td>Steroid, nonphenolic; Pregnene lactone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Thyroxine</td>
<td>51-48-9</td>
<td>Neg.</td>
<td>407</td>
<td>Thyroid hormone</td>
<td>Aromatic amino acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17β-Trenbolone</td>
<td>10161-33-8</td>
<td>Neg.</td>
<td>H</td>
<td>Binds strongly to the AR; Neg. for ER agonism in yeast assay</td>
<td>Steroid, nonphenolic; Estrene</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4-1: ICCVAM Recommended Substances for Validation of In Vitro ER TA Agonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems

Abbreviations: AR = Androgen receptor; CASRN = Chemical Abstracts Service Registry Number; D1 and D2 = Two major families of dopamine receptors; DDE = 1,1-Dichloro-bis[4-chlorophenyl]ethylene; DDT = Dichlorodiphenyltrichloroethane; ER = Estrogen receptor; HDT = Highest dose tested; Neg. = Negative; Pos. = Positive; RBA = Relative binding affinity; T3 = Triiodothyronine; T4 = Thyroxine.

Substances in bold type are those that, at a minimum, are recommended for inclusion in future validation studies. Empty cells indicate that no relevant data were identified.

An EC50 is the effective concentration of the test substance that elicits 50% of the maximum response in a particular test system. Median EC50 values and ranges are derived from in vitro mammalian cell reporter gene studies that were either published in the peer-reviewed scientific literature or submitted to NICEATM, and then reviewed and summarized in the NICEATM Background Review Document (BRD) titled “Current Status of Test Methods for Detecting Endocrine Disruptors: In Vitro Estrogen Receptor Transcriptional Activation Assays-August 2002” (available on the ICCVAM website at http://iccvam.niehs.nih.gov/methods/endocrine.htm). Substances for which quantitative ER agonism data are available are ranked according to their relative potency in ER mammalian cell reporter gene agonism assays from most potent to least potent. Numbers in parentheses to the right of the EC50 value refer to the number of studies for which an EC50 value was reported in the BRD. An italicized EC50 value indicates the value was estimated from a graphical presentation of data. The range of values reported for a substance is below the median value.

Numbers in parentheses refer to the number of studies in which the substance was reported positive or negative compared to the number of studies in which it was tested. A substance is classified as “positive” for ER agonism if it was positive in more than 50% of reported studies. A substance is classified as “presumed positive” for ER agonism if it was positive in 50% or less of reported studies, or if it was reported positive in the single study conducted. Substances reported negative in their respective studies are classified as “presumed negative” instead of “negative” for ER agonism, since they had not been tested in multiple studies at or above the limit dose of 1 mM recommended in Section 4.1.3. Substances without data are classified “presumed positive” or “presumed negative” based on available information, including their known mechanism of action or their responses in ER binding assays, AR binding assays, or AR TA assays.

Several in vivo test methods are undergoing further development or validation by OECD, EPA, and the JME (J). Substances indicated are proposed for testing by OECD in the Uterotrophic assay (U), the Hershberger assay (H), or the 407 protocol (407); for testing by EPA in the female pubertal assay (F-PA), the male pubertal assay (M-PA), the intact male assay (IM), a one-generation assay (1G), a two-generation assay (2G), or a fish reproductive screen (FRS); for testing by JME in the U, H, and 1G assays, or various fish (F) and avian (A) assays. Due to the lack of CASRN for the JME studies, some of the indicated substances might not be the same substance included in this list. The in utero through lactation assay (IUL) has been recommended, but EPA has not made a decision on its further development or validation.

Information for this substance regarding its median EC50 value (if available), its qualitative response in mammalian cell reporter gene assays, and the number of mammalian cell reporter gene assays in which it was tested was derived from data presented in Appendix D of the NICEATM ER TA BRD cited in footnote b. This document contains ER TA data from the published literature through January 25, 2002.

17β-Estradiol is not considered a positive test substance for validation purposes, since it is the recommended reference estrogen for in vitro ER binding and TA assays (refer to Section 4.2 for more information).

Two forms of p-nonylphenol are available for testing. One form consists of a mixture of various branched isomers (CASRN 84852-15-3), while the other contains only one isomer consisting of a linear alkyl chain (CASRN 104-40-5). ICCVAM recommends the linear form, which has a uniform chemical structure, for validation studies.

Information for this substance was abstracted from a peer-reviewed publication that was published or reviewed after the literature search was completed for the NICEATM ER TA BRD (i.e., Andersen et al. 2002 in Section 7.0).

No EC50 data available for this substance.

The classification for this substance is “presumed positive” for ER agonism since the substance was positive in 50% or less of reported studies, or was reported positive in the single study conducted.
### Table 4-2: ICCVAM Recommended Substances for Validation of *In Vitro* ER TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Median EC&lt;sub&gt;50&lt;/sub&gt; Value and Range Across All MCRG Studies&lt;sup&gt;b&lt;/sup&gt; (µM)</th>
<th>Qualitative Response for ER Antagonism Across All MCRG Studies&lt;sup&gt;c&lt;/sup&gt;</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Completed/ Anticipated In Vivo Testing&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Hydroxotamoxifen&lt;sup&gt;e,f&lt;/sup&gt;</td>
<td>68047-06-3</td>
<td>0.00025 (1)</td>
<td>Pos. (10/10)</td>
<td>9</td>
<td></td>
<td>ER antagonist</td>
<td>Triphenylethylene; Benzylidene; Stilbene; Phenol</td>
</tr>
<tr>
<td>ICI 182,780&lt;sup&gt;e,f&lt;/sup&gt;</td>
<td>129453-61-8</td>
<td>0.003 (4) 0.00001 - 0.016</td>
<td>Pos. (14/15)</td>
<td>13</td>
<td>IM</td>
<td>ER antagonist</td>
<td>Steroid, phenolic</td>
</tr>
<tr>
<td>Tamoxifen&lt;sup&gt;e,f&lt;/sup&gt;</td>
<td>10540-29-1</td>
<td>0.018 (2) 0.01 - 0.025</td>
<td>Pos. (7/8)</td>
<td>7</td>
<td></td>
<td>ER antagonist</td>
<td>Triphenylethylene; Benzylidene; Stilbene</td>
</tr>
<tr>
<td>Zearalenone&lt;sup&gt;e,f&lt;/sup&gt;</td>
<td>17924-92-4</td>
<td>5 (1)</td>
<td>Pos. (2/3)</td>
<td>3</td>
<td></td>
<td>ER agonist</td>
<td>Resorcyclic acid lactone; Phenol</td>
</tr>
<tr>
<td>Coumestrol&lt;sup&gt;e,f&lt;/sup&gt;</td>
<td>479-13-0</td>
<td>5 (1)</td>
<td>Pos. (1/3)&lt;sup&gt;j&lt;/sup&gt;</td>
<td>3</td>
<td>IM</td>
<td>ER agonist</td>
<td>Coumestan; Benzopyranone; Coumarin; Ketone</td>
</tr>
<tr>
<td>Flavone&lt;sup&gt;e,f&lt;/sup&gt;</td>
<td>525-82-6</td>
<td>15 (1)</td>
<td>Pos. (1/1)&lt;sup&gt;j&lt;/sup&gt;</td>
<td>1</td>
<td>M-PA; IM</td>
<td>Weak ER antagonist</td>
<td>Flavanoid; Flavone</td>
</tr>
<tr>
<td>Apigenin&lt;sup&gt;e,f&lt;/sup&gt;</td>
<td>520-36-5</td>
<td>20 (1)</td>
<td>Pos. (3/5)</td>
<td>4</td>
<td>IUL</td>
<td>ER agonist</td>
<td>Flavanoid; Flavone; Phenol</td>
</tr>
<tr>
<td>Genistein&lt;sup&gt;e,f&lt;/sup&gt;</td>
<td>446-72-0</td>
<td>25 (1)</td>
<td>Pos. (1/3)&lt;sup&gt;j&lt;/sup&gt;</td>
<td>3</td>
<td>U; 407</td>
<td>Weak ER agonist and antagonist</td>
<td>Flavanoid; Isoflavone; Phenol</td>
</tr>
<tr>
<td>17β-Estradiol&lt;sup&gt;e,g,h&lt;/sup&gt;</td>
<td>50-28-2</td>
<td>Neg. (8/8)</td>
<td>7</td>
<td>IM; IUL; FRS</td>
<td>Strong ER agonist; AR agonist and antagonist</td>
<td>Steroid, phenolic; Estrene</td>
<td></td>
</tr>
<tr>
<td>Kaempferol&lt;sup&gt;e,g&lt;/sup&gt;</td>
<td>520-18-3</td>
<td>Neg. (3/3)</td>
<td>3</td>
<td></td>
<td></td>
<td>ER agonist</td>
<td>Flavanoid; Flavone; Phenol</td>
</tr>
<tr>
<td>Bisphenol A&lt;sup&gt;e,g&lt;/sup&gt;</td>
<td>80-05-7</td>
<td>Neg. (2/2)</td>
<td>2</td>
<td>U; F-PA; J(1G,F,A)</td>
<td></td>
<td>ER agonist</td>
<td>Diphenylalkane; Bisphenol; Phenol</td>
</tr>
<tr>
<td>Daidzein&lt;sup&gt;e,g&lt;/sup&gt;</td>
<td>486-66-8</td>
<td>Neg. (2/2)</td>
<td>2</td>
<td></td>
<td></td>
<td>Weak ER agonist</td>
<td>Flavanoid; Isoflavone; Phenol</td>
</tr>
</tbody>
</table>
### Table 4-2: ICCVAM Recommended Substances for Validation of In Vitro ER TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Median EC$_{50}$ Value and Range Across All MCRG Studies (µM)</th>
<th>Qualitative Response for ER Antagonism Across All MCRG Studies</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Completed/ Anticipated In Vivo Testing</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>p,p'</em>-DDE e,g</td>
<td>72-55-9</td>
<td>Neg. (2/2)</td>
<td>2</td>
<td>H; 407; M-PA; 1M; J(1G,F,A)</td>
<td>Weak AR agonist and antagonist</td>
<td>Organochlorine; Diphenylalkene</td>
<td></td>
</tr>
<tr>
<td>Kepone e,g (Chlordecone)</td>
<td>143-50-0</td>
<td>Neg. (2/2)</td>
<td>2</td>
<td></td>
<td></td>
<td>Binds to ER and AR; Chlorinated bridged cycloalkane</td>
<td></td>
</tr>
<tr>
<td><em>p,p'</em>-Methoxychlor e,g</td>
<td>72-43-5</td>
<td>Neg. (2/2)</td>
<td>2</td>
<td>U; F &amp; M-PA; IUL; 1M; FRS; 2G(avian)</td>
<td>Weak ER agonist; AR antagonist</td>
<td>Organochlorine; Chlorinated hydrocarbon</td>
<td></td>
</tr>
<tr>
<td>Atrazine e,g</td>
<td>1912-24-9</td>
<td>Neg. (1/1)</td>
<td>1</td>
<td>IM</td>
<td></td>
<td>Aromatic amine; Triazine; Arylamine</td>
<td></td>
</tr>
<tr>
<td>Butylbenzyl phthalate e,g</td>
<td>85-68-7</td>
<td>Neg. (1/1)</td>
<td>1</td>
<td>IUL</td>
<td>ER agonist</td>
<td>Phthalate</td>
<td></td>
</tr>
<tr>
<td>Di-&lt;i&gt;n&lt;/i&gt;-butyl phthalate e,g</td>
<td>84-74-2</td>
<td>Neg. (1/1)</td>
<td>1</td>
<td>U; M-PA; 1G; J(U,H,1G,F,A)</td>
<td>ER agonist</td>
<td>Phthalate</td>
<td></td>
</tr>
</tbody>
</table>

**ANTICIPATED RESPONSES (No IC$_{50}$ or Qualitative Antagonism Data Available)**

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Qualitative Response</th>
<th>No. of MCRG Assays</th>
<th>Completed/ Anticipated In Vivo Testing</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clomiphene citrate</td>
<td>50-41-9</td>
<td>Pos.</td>
<td></td>
<td></td>
<td>Binds to the ER; Selective estrogen receptor modulator</td>
<td>Chlorinated triphenylethylene; Benzylidene; Stilbene</td>
</tr>
<tr>
<td><em>o,p'</em>-DDT</td>
<td>789-02-6</td>
<td>Pos.</td>
<td></td>
<td>U; J(1G,F,A)</td>
<td>Weak AR antagonist; Weak ER agonist; Pos. for ER antagonism in yeast assay</td>
<td>Organochlorine; Diphenylalkene</td>
</tr>
<tr>
<td>Dibenzo[&lt;i&gt;a&lt;/i&gt;,&lt;i&gt;h&lt;/i&gt;]-anthracene</td>
<td>53-70-3</td>
<td>Pos.</td>
<td></td>
<td></td>
<td>Poly cyclic aromatic hydrocarbon; Anthracene</td>
<td></td>
</tr>
<tr>
<td>Substance</td>
<td>CASRN</td>
<td>Median EC$_{50}$ Value and Range Across All MCRG Studies ($\mu$M)</td>
<td>Qualitative Response for ER Antagonism Across All MCRG Studies</td>
<td>No. of MCRG Assays in Which Tested</td>
<td>Completed/Anticipated In Vivo Testing</td>
<td>Comments</td>
</tr>
<tr>
<td>------------------------</td>
<td>--------</td>
<td>----------------------------------------------------------------</td>
<td>-------------------------------------------------</td>
<td>-----------------------------------</td>
<td>-------------------------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>50-76-0</td>
<td>Neg.</td>
<td>RNA synthesis inhibitor</td>
<td>U; 407; F-PA</td>
<td>Strong ER agonist</td>
<td>Steroid, nonphenolic</td>
</tr>
<tr>
<td>4-Androstenedione</td>
<td>63-05-8</td>
<td>Neg.</td>
<td>Strong AR agonist</td>
<td></td>
<td></td>
<td>Steroid, nonphenolic</td>
</tr>
<tr>
<td>Bisphenol B</td>
<td>77-40-7</td>
<td>Neg.</td>
<td>ER agonist</td>
<td></td>
<td></td>
<td>Diphenylalkane; Bisphenol; Phenol</td>
</tr>
<tr>
<td>2-sec-Butylphenol</td>
<td>89-72-5</td>
<td>Neg.</td>
<td>Binds weakly to the AR</td>
<td></td>
<td></td>
<td>Steroid, nonphenolic</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>50-22-6</td>
<td>Neg.</td>
<td>AR agonist</td>
<td></td>
<td></td>
<td>Steroid, nonphenolic</td>
</tr>
<tr>
<td>4-Cumylphenol</td>
<td>599-64-4</td>
<td>Neg.</td>
<td>Weak ER agonist</td>
<td></td>
<td></td>
<td>Steroid, nonphenolic</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>50-02-2</td>
<td>Neg.</td>
<td>AR agonist</td>
<td></td>
<td></td>
<td>Steroid, nonphenolic</td>
</tr>
<tr>
<td>Diethylhexyl phthalate</td>
<td>117-81-7</td>
<td>Neg.</td>
<td>J(U,H,1G,F,A)</td>
<td></td>
<td></td>
<td>Phthalate</td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>56-53-1</td>
<td>Neg.</td>
<td>IUL</td>
<td></td>
<td>Strong ER agonist</td>
<td>Stilbene; Benzyldene; Diphenylalkene</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>521-18-6</td>
<td>Neg.</td>
<td>H</td>
<td></td>
<td>Weak ER agonist; Strong AR agonist</td>
<td>Steroid, nonphenolic</td>
</tr>
<tr>
<td>17α-Estradiol</td>
<td>57-91-0</td>
<td>Neg.</td>
<td>ER agonist</td>
<td></td>
<td>Strong ER agonist; AR agonist</td>
<td>Steroid, phenolic; Estrene</td>
</tr>
<tr>
<td>Estrone</td>
<td>53-16-7</td>
<td>Neg.</td>
<td>Strong ER agonist</td>
<td></td>
<td></td>
<td>Steroid, phenolic; Estrene</td>
</tr>
<tr>
<td>17α-Ethinyl estradiol</td>
<td>57-63-6</td>
<td>Neg.</td>
<td>Strong ER agonist</td>
<td></td>
<td></td>
<td>Steroid, phenolic</td>
</tr>
</tbody>
</table>
### Table 4-2: ICCVAM Recommended Substances for Validation of *In Vitro* ER TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Median EC&lt;sub&gt;50&lt;/sub&gt; Value and Range Across All MCRG Studies&lt;sup&gt;b&lt;/sup&gt; (µM)</th>
<th>Qualitative Response for ER Antagonism Across All MCRG Studies&lt;sup&gt;c&lt;/sup&gt;</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Completed/Anticipated <em>In Vivo</em> Testing&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl paraben</td>
<td>120-47-8</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Binds weakly to ER</td>
<td>Paraben; Organic acid</td>
</tr>
<tr>
<td>Fadrozole</td>
<td>102676-47-1</td>
<td>Neg.</td>
<td>F-PA; IM; FRS</td>
<td></td>
<td></td>
<td>Aromatase inhibitor</td>
<td>Imidazole; Nitrile</td>
</tr>
<tr>
<td>Fenarimol</td>
<td>60168-88-9</td>
<td>Neg.</td>
<td>F-PA</td>
<td></td>
<td></td>
<td>Aromatase inhibitor</td>
<td>Heterocycle; Pyrimidine</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>206-44-0</td>
<td>Neg.</td>
<td>AR antagonist; Neg. for ER antagonism in yeast assay</td>
<td></td>
<td></td>
<td>Polycyclic aromatic hydrocarbon; Fluorene</td>
<td></td>
</tr>
<tr>
<td>meso-Hexestrol</td>
<td>84-16-2</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Strong ER agonist</td>
<td>Diphenylalkane; Bisphenol; Phenol</td>
</tr>
<tr>
<td>Hydroxyflutamide</td>
<td>52806-53-8</td>
<td>Neg.</td>
<td>AR agonist and antagonist</td>
<td></td>
<td></td>
<td>Amide; Anilide; Nitrobenzene</td>
<td></td>
</tr>
<tr>
<td>Methyl testosterone</td>
<td>58-18-4</td>
<td>Neg.</td>
<td>H; 407; M-PA; IUL; FRS</td>
<td></td>
<td></td>
<td>ER and AR agonist</td>
<td>Steroid, nonphenolic; Androstene</td>
</tr>
<tr>
<td>Morin</td>
<td>480-16-0</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Binds weakly to ER</td>
<td>Flavanoid; Flavone; Phenol</td>
</tr>
<tr>
<td><em>p</em>-n-Nonylphenol&lt;sup&gt;f&lt;/sup&gt;</td>
<td>104-40-5</td>
<td>Neg.</td>
<td>U; 407; J(U,H,1G,F,A)</td>
<td></td>
<td></td>
<td>ER agonist; AR antagonist</td>
<td>Alkylphenol; Phenol</td>
</tr>
<tr>
<td>Norethynodrel</td>
<td>68-23-5</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Binds to ER</td>
<td>Steroid, nonphenolic; Norpregnene</td>
</tr>
<tr>
<td>4-tert-Octylphenol</td>
<td>140-66-9</td>
<td>Neg.</td>
<td>J(U,H,1G,F,A)</td>
<td></td>
<td></td>
<td>ER agonist; Neg. for ER antagonism in yeast assay</td>
<td>Alkylphenol; Phenol</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>57-30-7</td>
<td>Neg.</td>
<td>F&amp;M-PA; IM</td>
<td></td>
<td></td>
<td>Enhances thyroid hormone excretion</td>
<td>Heterocycle; Pyrimidine</td>
</tr>
<tr>
<td>Substance</td>
<td>CASRN</td>
<td>Median EC\textsubscript{50} Value and Range Across All MCRG Studies\textsuperscript{b} (µM)</td>
<td>Qualitative Response for ER Antagonism Across All MCRG Studies\textsuperscript{c}</td>
<td>No. of MCRG Assays in Which Tested</td>
<td>Completed/Anticipated In Vivo Testing\textsuperscript{d}</td>
<td>Comments</td>
<td>Chemical Class</td>
</tr>
<tr>
<td>----------------------------</td>
<td>--------</td>
<td>---------------------------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------</td>
<td>----------------------------------</td>
<td>---------------------------------</td>
<td>------------------------------------------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Phenolphthalin</td>
<td>81-90-3</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Triphenylmethane; Diphenylalkane carboxylic acid</td>
</tr>
<tr>
<td>Progesterone</td>
<td>57-83-0</td>
<td>Neg.</td>
<td></td>
<td>IM</td>
<td></td>
<td></td>
<td>Steroid, nonphenolic; Pregnenedione</td>
</tr>
<tr>
<td>Propylthiouracil</td>
<td>51-52-5</td>
<td>Neg.</td>
<td>407; F&amp;M-PA; IM; IUL; 2G</td>
<td>Inhibits T3/T4 synthesis</td>
<td></td>
<td></td>
<td>Pyrimidine; Uracil</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>26628-22-8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cytotoxic; Organic salt; Azide</td>
</tr>
<tr>
<td>Testosterone</td>
<td>58-22-0</td>
<td>Neg.</td>
<td></td>
<td>IM</td>
<td></td>
<td></td>
<td>Strong AR agonist; Steroid, nonphenolic</td>
</tr>
<tr>
<td>12-O-Tetradecanoyl-phorbol-13-acetate</td>
<td>16561-29-8</td>
<td></td>
<td></td>
<td>Activates ligand dependent cell division</td>
<td></td>
<td></td>
<td>Phorbol ester; Terpene</td>
</tr>
<tr>
<td>2,4,5-Trichlorophenoxyacetic acid</td>
<td>93-76-5</td>
<td>Neg.</td>
<td></td>
<td>Weak ER agonist</td>
<td></td>
<td></td>
<td>Organochlorine; Chlorinated aromatic hydrocarbon</td>
</tr>
<tr>
<td>Vinclozolin</td>
<td>50471-44-8</td>
<td></td>
<td></td>
<td>H; M-PA; IM; IUL; 1G; FRS</td>
<td>AR antagonist</td>
<td></td>
<td>Organochlorine; Cyclic imide; Carbamate</td>
</tr>
<tr>
<td>Ammonium perchlorate</td>
<td>7790-98-9</td>
<td></td>
<td></td>
<td></td>
<td>IUL</td>
<td>Thyroid disruptor</td>
<td>Organic acid; Organic salt</td>
</tr>
<tr>
<td>Anastrazole</td>
<td>120511-73-1</td>
<td></td>
<td></td>
<td>IM</td>
<td></td>
<td>Aromatase inhibitor</td>
<td>Nitrile; Triazole</td>
</tr>
<tr>
<td>Apomorphine</td>
<td>58-00-4</td>
<td>Neg.</td>
<td></td>
<td>IM</td>
<td></td>
<td>Dopamine D1/D2 receptor agonist</td>
<td>Heterocycle; Quinoline</td>
</tr>
<tr>
<td>Bicalutamide</td>
<td>90357-06-5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AR antagonist</td>
<td>Anilide; Nitrile; Sulfone</td>
</tr>
</tbody>
</table>
Table 4-2: ICCVAM Recommended Substances for Validation of In Vitro ER TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems\(^a\) (continued)

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Median EC(_{50}) Value and Range Across All MCRG Studies(^b) (µM)</th>
<th>Qualitative Response for ER Antagonism Across All MCRG Studies(^c)</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Completed/Anticipated In Vivo Testing(^d)</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGS 18320B</td>
<td>112808-99-8</td>
<td>Neg.</td>
<td></td>
<td>407</td>
<td>Aromatase inhibitor</td>
<td>Nitrile; Imidazole</td>
<td></td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>66-81-9</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>Protein synthesis inhibitor</td>
<td>Piperidine; Glutaramide</td>
<td></td>
</tr>
<tr>
<td>Cyproterone acetate</td>
<td>427-51-0</td>
<td>Neg.</td>
<td></td>
<td>IM</td>
<td>AR agonist and antagonist</td>
<td>Nitrile; Diphenyl ether; Organochlorine</td>
<td></td>
</tr>
<tr>
<td>Finasteride</td>
<td>98319-26-7</td>
<td>Neg.</td>
<td></td>
<td>H; M-PA; IM</td>
<td>5α-reductase inhibitor</td>
<td>Steroid, nonphenolic; Androstene</td>
<td></td>
</tr>
<tr>
<td>Fluoxymestrone</td>
<td>76-43-7</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>Weak AR agonist</td>
<td>Steroid, nonphenolic</td>
<td></td>
</tr>
<tr>
<td>Flutamide</td>
<td>13311-84-7</td>
<td>Neg.</td>
<td></td>
<td>H; 407; M-PA; IM; FRS</td>
<td>AR agonist</td>
<td>Amide; Anilide; Nitrobenzene</td>
<td></td>
</tr>
<tr>
<td>Haloperidol</td>
<td>52-86-8</td>
<td>Neg.</td>
<td></td>
<td>IM</td>
<td>Dopamine D2 receptor antagonist</td>
<td>Butyrophenone; Ketone; Piperazine</td>
<td></td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>65277-42-1</td>
<td>Neg.</td>
<td></td>
<td>F&amp;M-PA; IM</td>
<td>Weak AR agonist</td>
<td>Imidazole; Piperazine</td>
<td></td>
</tr>
<tr>
<td>Linuron</td>
<td>330-55-2</td>
<td>Neg.</td>
<td></td>
<td>H; M-PA</td>
<td>Weak AR agonist and antagonist</td>
<td>Urea</td>
<td></td>
</tr>
<tr>
<td>Medroxyprogesterone acetate</td>
<td>71-58-9</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>Weak AR agonist</td>
<td>Steroid, nonphenolic; Polycyclic hydrocarbon</td>
<td></td>
</tr>
<tr>
<td>Methyltrienolone</td>
<td>965-93-5</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>AR agonist</td>
<td>Steroid, nonphenolic; Estrene</td>
<td></td>
</tr>
<tr>
<td>Mifepristone</td>
<td>84371-65-3</td>
<td>Neg.</td>
<td></td>
<td>IM</td>
<td>AR agonist and antagonist</td>
<td>Steroid, nonphenolic; Estrene</td>
<td></td>
</tr>
<tr>
<td>Nilutamide</td>
<td>63612-50-0</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>AR antagonist</td>
<td>Heterocycle; Imidazole</td>
<td></td>
</tr>
</tbody>
</table>
### Table 4-2: ICCVAM Recommended Substances for Validation of *In Vitro* ER TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems (continued)

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Median EC$_{50}$ Value and Range Across All MCRG Studies$^b$ (µM)</th>
<th>Qualitative Response for ER Antagonism Across All MCRG Studies$^c$</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Completed/Anticipated In Vivo Testing$^d$</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxazepam</td>
<td>604-75-1</td>
<td>Neg.</td>
<td>IM</td>
<td></td>
<td></td>
<td>Enhances thyroid hormone excretion</td>
<td>Benzodiazepine</td>
</tr>
<tr>
<td>Pimozide</td>
<td>2062-78-4</td>
<td>Neg.</td>
<td>F&amp;M-PA</td>
<td></td>
<td></td>
<td>Dopamine receptor antagonist</td>
<td>Piperidine; Benzimidazole</td>
</tr>
<tr>
<td>Procymidone</td>
<td>32809-16-8</td>
<td>Neg.</td>
<td>H</td>
<td>AR antagonist</td>
<td></td>
<td></td>
<td>Organochlorine; Cyclic imide</td>
</tr>
<tr>
<td>Reserpine</td>
<td>50-55-5</td>
<td>Neg.</td>
<td>IM</td>
<td>Depletes dopamine</td>
<td></td>
<td></td>
<td>Heterocycle; Yohimban</td>
</tr>
<tr>
<td>Spironolactone</td>
<td>52-01-7</td>
<td>Neg.</td>
<td>AR agonist and antagonist</td>
<td>407</td>
<td></td>
<td></td>
<td>Steroid, nonphenolic; Pregnenolactone</td>
</tr>
<tr>
<td>L-Thyroxine</td>
<td>51-48-9</td>
<td>Neg.</td>
<td></td>
<td>Thyroid hormone</td>
<td></td>
<td></td>
<td>Aromatic amino acid</td>
</tr>
<tr>
<td>17β-Trenbolone</td>
<td>10161-33-8</td>
<td>Neg.</td>
<td>H</td>
<td>Binds strongly to the AR</td>
<td></td>
<td></td>
<td>Steroid, nonphenolic; Estrene</td>
</tr>
</tbody>
</table>

*Abbreviations:* AR = Androgen receptor; CASRN = Chemical Abstracts Service Registry Number; D1 and D2 = Two major families of dopamine receptors; DDE = 1,1-Dichloro-bis[4-chlorophenyl]ethylene; DDT = Dichlorodiphenyltrichloroethane; ER = Estrogen receptor; HDT = Highest dose tested; Neg. = Negative; Pos. = Positive; RBA = Relative binding affinity; T3 = Triiodothyronine; T4 = Thyroxine; TA = Transcriptional activation.

$^a$ Substances in bold type are those that, at a minimum, are recommended for inclusion in future validation studies. Empty cells indicate that no relevant data were identified.

$^b$ An IC$_{50}$ is the concentration of the test substance that inhibits 50% of the response of 17β-estradiol in a particular test system. Median IC$_{50}$ values are derived from *in vitro* mammalian cell reporter gene studies that were either published in the peer-reviewed scientific literature or submitted to NICEATM, and then reviewed and summarized in the NICEATM Background Review Document (BRD) titled “Current Status of Test Methods for Detecting Endocrine Disruptors: *In Vitro* Estrogen Receptor Transcriptional Activation Assays-August 2002” (available on the ICCVAM website at [http://iccvam.niehs.nih.gov/methods/endocrine.htm](http://iccvam.niehs.nih.gov/methods/endocrine.htm)). Substances for which quantitative ER antagonism data are available are ranked according to their relative potency in ER mammalian cell reporter gene antagonism assays from most potent to least potent. Numbers in parentheses to the right of the IC$_{50}$ value refer to the number of studies for which an IC$_{50}$ value was reported in the BRD. An italicized IC$_{50}$ value indicates the value was estimated from a graphical presentation of data. The range of values reported for a substance is listed below the median value.
Table 4-2: ICCVAM Recommended Substances for Validation of In Vitro ER TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems

<table>
<thead>
<tr>
<th>Substance</th>
<th>Numbers in parentheses</th>
<th>Classification</th>
<th>Notes</th>
</tr>
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<tbody>
<tr>
<td>A</td>
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<tr>
<td>B</td>
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<td>F</td>
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</table>

* Numbers in parentheses refer to the number of studies in which the substance was reported positive or negative compared to the number of studies in which it was tested. A substance is classified as “positive” for ER antagonism if it was positive in more than 50% of reported studies. A substance is classified as “presumed positive” for ER antagonism if it was positive in 50% or less of reported studies, or if it was reported positive in the single study conducted. Substances reported negative in their respective studies are classified as “presumed negative” instead of “negative” for ER antagonism, since they had not been tested in multiple studies at or above the limit dose of 1 mM recommended in Section 4.1.3. Substances without data are classified “presumed positive” or “presumed negative” based on available information, including their known mechanism of action or their responses in ER binding assays, AR binding assays, or AR TA assays.

d Several in vivo test methods are undergoing further development or validation by OECD, EPA, and the JME (J). Substances indicated are proposed for testing by OECD in the Uterotrophic assay (U), the Hershberger assay (H), or the 407 protocol (407); for testing by EPA in the female pubertal assay (F-PA), the male pubertal assay (M-PA), the intact male assay (IM), a one-generation assay (1G), a two-generation assay (2G), or a fish reproductive screen (FRS); for testing by JME in the U, H, and 1G assays, or various fish (F) and avian (A) assays. Due to the lack of CASRN for the JME studies, some of the indicated substances might not be the same substance included in this list. The in utero through lactation assay (IU) has been recommended, but EPA has not made a decision on its further development or validation.

e Information for this substance regarding its qualitative response in mammalian cell reporter gene assays, and the number of mammalian cell reporter gene assays in which it was tested was derived from data presented in Appendix D of the NICEATM ER TA BRD cited in footnote b. This BRD contains ER TA data from the published literature through January 25, 2002.

f IC₅₀ data were abstracted from publications reviewed for the NICEATM ER TA BRD; however, these data were not included in the BRD.

A No IC₅₀ data available for this substance.

h 17β-Estradiol is not considered a test substance for validation purposes, since it is the recommended reference estrogen for in vitro ER binding and TA assays (refer to Section 4.2 for more information).

i Two forms of p-nonylphenol are available for testing. One form consists of a mixture of various branched isomers (CASRN 84852-15-3), while the other contains only one isomer consisting of a linear alkyl chain (CASRN 104-40-5). ICCVAM recommends the linear form, which has a uniform chemical structure, for validation studies.

j The classification for this substance is “presumed positive” for ER antagonism since the substance was positive in 50% or less of reported studies, or was reported positive in the single study conducted.
In Vitro AR Binding Assays

In Vitro ER Transcriptional Activation Assays
5.0 **IN VITRO ANDROGEN RECEPTOR BINDING ASSAYS**

5.1 Minimum Procedural Standards

More than 11 different *in vitro* assays have been used to evaluate the AR binding ability of various substances (NIEHS 2002c). Of the 11 AR binding assays evaluated in the BRD, six used cytosolic proteins, one used nuclear protein, one used recombinant protein, and three used intact cells. No validation studies have been conducted to assess the performance and reliability of these test methods and very few substances have been tested multiple times using either the same test method or different test methods. Although there was insufficient information available to thoroughly assess the comparative performance of these 11 *in vitro* AR binding assays, the Expert Panel recommended that future validation efforts be directed to test methods using a recombinant receptor protein (see Appendix A). To assist in the development, standardization, and validation of *in vitro* AR binding assays, NICEATM and the EDWG developed proposed minimum procedural standards for consideration by the Expert Panel (NIEHS 2002c). These minimum procedural standards focused on test methods that used a radiolabeled reference androgen to detect substances that could bind to the AR. The purpose of minimum procedural standards is to specify information essential for maximizing test method intra- and inter-laboratory reproducibility while minimizing the likelihood of erroneous results. Also, adherence to such standards will enhance any assessment of the comparative performance of *in vitro* AR binding assays. The minimum procedural standards provided here have been revised to incorporate recommendations and comments of the Expert Panel, the EDWG, and the public. Except as noted, all *in vitro* AR binding assays should incorporate these minimum procedural standards in their protocols, and scientific justification should be provided for any deviations.

5.1.1 Animal Studies

All studies requiring animal tissues should have animal use procedures approved by an IACUC or its equivalent.

*Rationale:* An IACUC review will help ensure that animals needed as sources of tissue for isolation of the AR will be used in a humane manner. The review will also ensure consideration of alternative test methods that do not require animal tissues and appropriate justification if animal tissues are used.

5.1.2 Reference Androgen

The displacement of a radiolabeled reference androgen from the AR in a competitive binding study is used to identify substances that bind to the AR. Methyltrienolone (R1881) (CASRN 965-93-5) is recommended as the reference androgen in all AR binding assays. The RBA, a measure of relative activity, of a test substance is equal to the IC$_{50}$ of the unlabeled reference androgen divided by the IC$_{50}$ of the test substance, multiplied by 100. The IC$_{50}$ is the (calculated) concentration that inhibits the binding of the radiolabeled reference androgen to the AR by 50%, and is determined by simultaneously incubating the AR with a saturating amount of the radiolabeled androgen and a range of concentrations of the unlabeled androgen or the unlabeled reference androgen. The concentration range used for the unlabeled androgen should be 1 nM to 1 µM. IC$_{50}$ and RBA values should be calculated and presented for all *in vitro* AR binding assays.

*Rationale:* 5α-Dihydrotestosterone (DHT) has been frequently used as the reference androgen in AR binding studies, especially when recombinant proteins are used as the
source of the AR. However, since DHT is metabolized by animal tissue cytosolic preparations, R1881 is the reference androgen of choice for such binding assays. Since DHT is metabolized by many cell lines, R1881 is the reference androgen of choice for in vitro AR TA assays (see Section 6.0). Thus, to allow for a more direct comparison of the relative performance of in vitro AR binding and TA assays, R1881 is recommended as the most suitable reference androgen for AR binding assays.

### 5.1.3 Dissociation Constant of the Reference Androgen

Prior to conducting studies to evaluate the AR-binding ability of test substances, the dissociation constant ($K_d$) of the reference androgen and the total number of receptors in the AR preparation ($B_{max}$, which is expressed as fmol/mg protein) should be determined using a saturation binding experiment. To determine the $K_d$ and $B_{max}$, the AR should be exposed to the radiolabeled reference androgen at seven to ten concentrations, spaced across a three to four log interval. The ligand binding array of Raffelsberger and Wittliff (1997)¹ has the advantage of determining simultaneously in each study the $K_d$ of the radiolabeled reference androgen, the $B_{max}$ at different concentrations of the AR (if desired, but not required), and the IC₅₀ values of the unlabeled reference androgen and the test substance. Thus, the Expert Panel recommended this method for determining the $K_d$ of the reference androgen.

¹ The ligand binding array differs from the conventional binding assay in that the competitive binding assay is conducted using a range of concentrations of both the radiolabeled reference androgen and the test substance that generates an array of isotherms that permits the simultaneous calculation of $K_d$ and $B_{max}$ for the radiolabeled reference estrogen and the IC₅₀ values of the unlabeled reference estrogen and the test substance.

**Rationale:** The purpose of determining $B_{max}$ is to demonstrate that a finite number of receptors are saturated with the reference androgen, which ensures that the test system is optimized with respect to receptor and ligand concentrations. The purpose of determining the $K_d$ is to identify the appropriate concentration of the radiolabeled reference androgen to be used in competitive binding studies. Furthermore, the ability to obtain $K_d$ and $B_{max}$ values that are within the accepted limits for a specific test method (i.e., reference androgen and AR protein) is a critical measure of the robustness of the procedure.

### 5.1.4 Preparation of Test Substances and Volume of Administered Solvent

Test substances should be dissolved in a solvent that is miscible with an aqueous solution. Water, ethanol (95 to 100%), or DMSO is the preferred solvent. Preference should be given to the solvent that allows testing of the test substance at the maximal concentration possible, but without exceeding the limit dose (see Section 5.1.5). However, in testing situations where more than one solvent could be used, preference should be given to water, followed by ethanol (95 to 100%), and then DMSO. Other solvents may be used if it can be demonstrated that they do not interact or otherwise interfere with the test system. The volume of the solvent included in the reaction mixture generally has ranged from 0.1 to 1% of the total volume of the reactants. For any solvent, it should be demonstrated that the maximum volume used does not interfere with the test system. This can be accomplished by comparing the $K_d$ obtained for the radiolabeled reference androgen in the presence of the highest volume of the solvent with the $K_d$ of the reference androgen in the absence of the solvent. The stability of the dissolved test substance should be determined prior to testing. In the absence of stability
information, the stock solution should be prepared fresh prior to use.

**Rationale:** Selection of water, ethanol (95 to 100%), or DMSO as solvents is based on historical usage. Members of the Expert Panel stated that water or ethanol (95 to 100%) is preferred to DMSO because some substances, when dissolved in DMSO, appear to bind with lower affinity to the receptor. For this reason, most investigators have not used DMSO when dissolved in DMSO, and because of possible differences in receptor protein sensitivity, the maximal concentration of a solvent that does not interfere with performance should be determined for each test method.

### 5.1.5 Concentration Range of Test Substances

In the absence of solubility constraints, the maximum test substance concentration (i.e., the limit dose) should be 1 mM. Seven test substance concentrations spaced at log intervals up to the limit dose (i.e., 1 nM, 10 nM, 100 nM, 1 µM, 10 µM, 100 µM, 1 mM) should be tested.

**Rationale:** Most test method guidelines include a limit dose to ensure that all substances are tested over the same dose range while avoiding excessive amounts of a test substance that can perturb the test system through physicochemical mechanisms. An established limit dose also helps to minimize the effort and cost of screening and testing. Based on the range of published IC$_{50}$ values for AR binding (NIEHS 2002c), a limit dose of 1 mM, unless precluded by solubility constraints, was deemed suitable by the Expert Panel, the EDWG and ICCVAM for assessing the ability of test substances to bind to the AR.

The seven recommended test substance concentrations, spaced at log intervals, should be sufficient to determine an IC$_{50}$ value with sufficient accuracy because, currently, the experimental results will be used in a semiquantitative manner only (i.e., RBA values should not be used to rank substances regarding possible in vivo potency). If a lower maximum concentration is tested because of solubility constraints, the number of concentrations tested should remain the same by adding intermediate concentrations within the adjusted range.

### 5.1.6 Negative, Solvent and Positive Controls

Controls are required for the development of a saturation binding curve to determine the $B_{\text{max}}$ and $K_{d}$, and in subsequent competitive binding studies to evaluate the AR binding ability of test substances (see NIEHS 2002c, *Appendix B1*). For the saturation binding curve, a control set of tubes containing the AR and the radiolabeled reference androgen is required to determine total (maximum) binding of the radiolabeled reference androgen to the AR. A set of tubes containing unlabeled reference androgen at a concentration that will saturate the AR, the radiolabeled reference androgen, and the AR is required to measure non-specific binding. A set of tubes containing the radiolabeled androgen alone is required to determine the total radioactivity of the reference androgen added to each tube. In addition, a set of negative control tubes containing the AR, the radiolabeled reference androgen, and a negative control substance (e.g., a substance such as corticosterone that does not bind to the AR) is included to demonstrate the specificity of the interaction between the AR and the reference androgen.

For a competitive binding assay, a set of solvent control tubes containing the AR, the radiolabeled reference androgen, and the solvent used to dissolve the test substance is required to determine total (maximum)
binding of the radiolabeled reference androgen to the AR. The solvent control should be added at the highest volume used to administer the test substance to the reaction mixture. A set of tubes to measure nonspecific binding and those containing a negative control substance, as described above, are also included in each study. In addition to the unlabeled reference androgen, another positive control substance (e.g., hydroxyflutamide) with a binding affinity that is between two and three orders of magnitude lower than the reference androgen should be included in each study, and its IC\textsubscript{50} and RBA values reported.

**Rationale:** In *in vitro* competitive AR binding assays, the binding of a test substance to the AR is demonstrated by its ability to reduce the amount of radiolabeled reference androgen bound to the receptor at the end of the incubation period. Thus, the control response in each study is the total (maximum) binding of radiolabeled reference androgen to the AR that occurs in the absence of the test substance. The inclusion of the various sets of control and negative substance control tubes are to ensure that the saturation binding and the competitive binding studies are performed properly. The inclusion in each study of an additional positive control substance with an RBA of two to three orders of magnitude lower than the reference androgen provides another quality control (QC) measure by which to judge the sensitivity and acceptability of a test method for detecting substances that bind weakly to the receptor, and by which to evaluate the intralaboratory reproducibility of the test method. The usefulness of an additional positive control androgen with an RBA value that is two to three times lower than that of the reference androgen in each study should be evaluated during the validation process.

5.1.7 Within-Test Replicates
All concentration levels of the various controls, the reference androgen, and the test substance should be tested in triplicate.

**Rationale:** The purpose of triplicate assay tubes for each concentration of the various controls, the reference androgen, and the test substance is to ensure robust data and the ability to evaluate interreplicate variability. The most appropriate number of replicate tubes, however, should be evaluated after sufficient data has been collected using an optimized test method protocol.

5.1.8 Data Analysis
The first step in determining the IC\textsubscript{50} value for the test substance is to determine the B\textsubscript{max} and K\textsubscript{d} values of the radiolabeled reference androgen in the AR preparation. These parameters are obtained from a saturation binding experiment which is usually analyzed using a non-linear regression model (see Section 5.1.3). Several different software programs (e.g., Compete\textsuperscript{®} and OneSite\textsuperscript{®} [Lundon Software, Inc., Cleveland Heights, Ohio], GraphPad Prism\textsuperscript{®} [GraphPad Software, Inc., San Diego, California], and LIGAND [Munson and Rodbard 1980]) have been used to compute the K\textsubscript{d} and B\textsubscript{max} values of the radiolabeled reference androgen in a particular AR preparation. Once these parameters are known, the IC\textsubscript{50} values of the unlabeled reference androgen and the test substance can be determined using either a conventional competitive binding assay or a ligand binding array (Raffelsberger and Wittliff 1997). The experimental design differs between the two methodologies and, thus, the most appropriate methods for data analyses will differ also. Although stating that the more frequently used competitive binding assay is acceptable, the Expert Panel recommended the ligand binding array for future validation studies. The IC\textsubscript{50} values for the unlabeled reference androgen
and the test substance are used to calculate the RBA value of the test substance.

The statistical methods used to calculate the $B_{\text{max}}$, $K_d$, and $IC_{50}$ values should be justified. This includes a formal assessment of the nature of the statistical characteristics of the data (distribution, variance patterns, specific nonlinear models, etc.) and of how the models fit the data. Confidence limits should be calculated and provided for these values. In addition, the corresponding historical mean and confidence intervals for the $K_d$ value for the radiolabeled reference androgen, the $B_{\text{max}}$ for the AR preparation, and the $IC_{50}$ values for the unlabeled reference androgen and the additional positive control (if used) should be calculated and presented. For those test substances that significantly reduce the extent of binding of the radiolabeled reference substance (as determined using an appropriate statistical test) but without achieving an $IC_{50}$, it might be useful to determine whether inhibition is via a competitive or noncompetitive mechanism. In the former case, the test substance binds to the AR at the same amino acid sequence (cognate sequence) as the natural or synthetic ligand, whereas, in the latter case, the test substance binds to an amino acid sequence different from the binding domain and acts allosterically to prevent receptor binding.

**Rationale:** The different statistical methods for calculating the $K_d$, $B_{\text{max}}$, and $IC_{50}$ values or methods for determining a statistically significant decrease in AR binding of the radiolabeled reference androgen that does not achieve a 50% reduction have not been formally evaluated for their appropriateness. Data generated from a prevalidation study are needed for this purpose.

### 5.1.9 Good Laboratory Practice Compliance

Studies should be performed in compliance with GLP guidelines (EPA 2001, 2002; FDA 2002; OECD 1998).

**Rationale:** Conducting studies in compliance with GLP guidelines increases confidence in the quality and reliability of test data. Furthermore, if data using these test methods are to be submitted to the EPA in response to Federal testing requirements, then compliance with appropriate GLP guidelines will be required.

### 5.1.10 Study Acceptance Criteria

- The $IC_{50}$ value for the unlabeled reference androgen should be approximately equal to the molar concentration of the radiolabeled reference androgen plus the $K_d$ value.
- The $K_d$ and $IC_{50}$ values for the reference androgen should be within the 95% confidence limits for historical data.
- The ratio of total binding in the absence of a competitor to the amount of the radiolabeled reference androgen added per assay tube should not be greater than 10%.
- The $IC_{50}$ and RBA values for the concurrent additional positive control (if used) should be within the 95% confidence limits for historical data.
- The solvent control, at the concentration used, should not alter the performance of the assay.
- The limit dose should be 1 mM, unless precluded by solubility constraints.
- The study should comply with GLP guidelines.

**Rationale:** Established study acceptance criteria are required to ensure that each study is conducted appropriately.
5.1.11 Interpretation of Results
A substance is classified as positive for binding to the AR if an IC$_{50}$ value can be calculated. In general, the test substance should induce a sigmoid-shaped dose response curve over at least a few log concentrations. If a precipitous decrease in binding of the radiolabeled reference androgen to the AR occurs over a narrow concentration range (i.e., over a one log increment), the response might reflect precipitation of the AR rather than competitive binding by the test substance. If a substance does not bind to the AR after testing to the limit dose or to the maximum concentration possible based on its solubility (while not exceeding the limit dose), the test substance is classified as “negative” for binding to the AR under the conditions of the test. Test substances that induce a statistically significant reduction, but less than 50%, in binding of the radiolabeled reference androgen to the AR, are classified as “equivocal”.

Rationale: Until information becomes available about the biological relevance of studies in which the test substance induces a significant but less than 50% reduction in binding of the radiolabeled reference androgen to the AR, such responses should be noted and the substances classified as equivocal. The inability of a substance to decrease binding by at least 50% might be due to its relative insolubility, or its nonspecific binding to proteins other than the AR.

5.1.12 Repeat Studies
Generally, in a validation study, repeat studies would be conducted in order to evaluate intralaboratory repeatability and reproducibility. In contrast, in screening studies, repeat studies are not needed except to clarify equivocal results. If a study is repeated, the use of test substance concentrations more closely distributed in the range of interest might facilitate a more accurate analysis of the dose-response relationship for the test substance.

Rationale: Repeat studies are used in a validation study to demonstrate the intralaboratory repeatability and reproducibility of a test method. However, for a screening study, if the acceptance criteria are met and a clear negative or positive response is obtained, a repeat study to verify the original result usually is not considered necessary. In studies where an accurate IC$_{50}$ value cannot be calculated or where an equivocal response is obtained, a repeat study using adjusted dose levels might be needed to ensure a reliable conclusion.

5.1.13 Study Report
At a minimum, the study report should include the following information.

Reference Androgen
- name, CASRN, purity, and supplier of the reference androgen (radiolabeled and unlabeled), and specific activity of the radiolabeled reference androgen
- concentrations and volumes used

Additional Positive Control (if used)
- name, CASRN, purity, and supplier or source
- concentrations and volumes used

Negative Binding Control Substance
- name, CASRN, purity, and supplier or source
- concentrations and volumes used

Test Substance
- name, chemical structure (if known), and CASRN (if known), and supplier or source
- physical nature (solid or liquid) and purity, if known (every attempt should be made to determine the purity)
physicochemical properties relevant to the study (e.g., solubility, pH, stability, volatility)
concentrations and volumes used

Solvent
name, CASRN, purity, and supplier or source
justification for choice of solvent
information on the solubility of the test substance in all solvents in which it was tested
information to demonstrate that the solvent, at the maximum volume used, does not interact or otherwise interfere with the assay

Androgen Receptor
type and source of AR and the supplier
if the AR is isolated from animal tissues, information on species, strain, age, and gender of the animals used, the surgical procedure used to remove the tissue, and the method used to isolate the AR
if a recombinant AR protein is used, information on the cloning procedure used, the methods used to express the protein, and the procedures used for isolation of the protein
protein concentration of AR preparation
method used to measure protein concentration
method for storage of AR, if applicable

Study Conditions
$K_d$ of the reference androgen and $B_{\text{max}}$ of the AR
rationale for the concentration of the radiolabeled reference androgen in the binding assay
protein concentration of AR used in the assay
name(s) and concentration(s) of protease inhibitor(s) included in the animal tissue isolation buffer, if used

composition of buffers used
concentration range of the test substance, with justification
volume of the solvent used to dissolve the test substance and the volume added to the reaction mixture
incubation volume, duration, and temperature
description of the solvent control
type and composition of metabolic activation system, if used
description of the method used to separate AR-bound and -unbound radiolabeled reference androgen
method used to analyze concentration of receptor-ligand complexes
statistical method used to determine $K_d$, $B_{\text{max}}$, and $IC_{50}$ values
any other statistical method(s) used to assess the ability of the test substance to inhibit the binding of the radiolabeled reference androgen

Results
observations for and extent of any test substance precipitation
the IC data for each replicate at each concentration of the test substance, along with confidence levels or other measure of intradose repeatability
graphically presented dose-response curves for the unlabeled reference androgen, the positive control, and the test substance
$IC_{50}$ values and confidence limits for the unlabeled reference androgen, the additional positive control, if used, and the test substance
calculated RBA values for the additional positive control and the test substance

Discussion of Results
reproducibility of the $K_d$ of the reference androgen and $B_{\text{max}}$ of the AR, compared to historical data
• historical IC\textsubscript{50} values for the unlabeled reference androgen, including ranges, means, standard deviations, confidence intervals
• reproducibility of the IC\textsubscript{50} values of the unlabeled reference androgen, compared to historical data
• historical IC\textsubscript{50} and RBA values for the additional positive control substance (if used) with ranges, means, standard deviations, and confidence intervals
• reproducibility of the IC\textsubscript{50} and RBA values for the additional positive control substance, if used, compared to historical data
• the test substance dose-response relationship for inhibition of binding of the radiolabeled reference androgen to the AR

Conclusion
• classification of the test substance with regard to in vitro AR binding activity

Rationale: Minimum reporting standards are needed to ensure that a study report contains the level of information and detail that would be required if the study results are reviewed by the applicable regulatory agency, or for independent replication of the study, if deemed necessary.

5.2 Recommended Substances for Validation of In Vitro Androgen Receptor Binding Assays\textsuperscript{2}

To facilitate validation of in vitro AR binding assays, ICCVAM has compiled a list of 78 recommended substances for use in future validation studies. The 78 substances are presented in Table 5-1, with a summary of available quantitative in vitro AR binding data for each substance. Section 2.0 provides a detailed account of how these substances were selected. RBA data are available for 33 (42\%) of these 78 recommended substances. Although methyltrienolone is included in the list of recommended substances, it was not included in the count of substances for validation as it is the recommended reference standard against which all test substances are compared. Quantitative in vitro AR binding data are provided for substances that induced a positive response in at least one study. This includes the median RBA value and the range of RBA values where more than one positive study had been conducted, and the number of studies and assays in which each substance was tested. In situations where only one positive study was reported, the RBA value obtained in that study is reported. The substances with RBA data are listed first, sorted by potency from strongest to weakest, based on the median or single RBA value of each substance across all positive studies. The median or single RBA values range from 126 to 0.00009, extending over eight orders of magnitude. Positive and “presumed positive” substances have been grouped into six RBA categories in log decrements: \(>10\), <10 to 1, <1 to 0.1, <0.1 to 0.01, <0.01 to 0.001, and <0.001. Presumed positive substances induced a positive response in 50\% or fewer of the AR binding studies in which they were tested. Substances were classified as negative if they did not induce at least a 50\% reduction in binding of the radiolabeled reference androgen to the AR in multiple studies when tested up to the limit dose as defined in this document (i.e., 1 mM). Substances reported as negative for AR binding were classified as “presumed negative” if they had not been tested to the limit dose in multiple studies (i.e., reproducibility for a

\textsuperscript{2}Inclusion of a substance in this list does not mean that EPA, NICEATM, ICCVAM, or the Expert Panel has or will make a determination that any use of the substance will pose a significant risk. Further, these substances should not be interpreted to be “endocrine disruptors”; the substances listed are simply compounds that have been or may prove to be useful in developing, standardizing, or validating screening and testing methods.
negative response had not been demonstrated at test substance concentration up to 1 mM). Using these criteria, no substances could be classified as negative for AR binding. The presumed negative substances are listed below the sixth RBA category (<0.001) and include the maximum HDT used among studies, if available, in addition to the number of studies and assays in which the substance was tested. No effort was made to assess the validity and quality of each negative or positive response reported for each substance in each study. Following the presumed negative substances are those that have not been tested for AR binding activity. These substances have been assigned a presumed positive or negative response in in vitro AR binding assays based on the substances’ anticipated or known mechanism of action and their response in in vitro AR TA assays. Presumed positive substances are listed first, followed by presumed negative substances that have been selected for the minimal list of substances (see below and Section 2.4.4). Both categories are sorted alphabetically by substance name. The other substances that are presumed negative are sorted alphabetically at the end of the list.

Substances have been classified as presumed positive even when reported as positive for AR binding in less than 50% of the studies conducted. This classification is because erroneous positive studies are probably less likely than erroneous negative studies due to the nature of AR binding assays and the protocols generally used. For example, in many negative studies, the HDT was below the IC50 value obtained in positive studies reported for that substance. The classification of a substance as positive (and its ranking), presumed positive, or presumed negative in this list is based sometimes on the results of a single study and, therefore, the accuracy of the classification is questionable. However, it is anticipated that testing these presumed positive and negative substances will provide critical information on the comparative sensitivity and reproducibility of different in vitro AR binding assays, when such methods are standardized and conducted using the recommended minimum procedural standards.

The quantitative and qualitative data provided with this substance list summarize information obtained from peer-reviewed scientific reports. Because the positive data were obtained from studies using different in vitro AR binding assays, they show a great deal of variability and, thus, the reported values should not be used as definitive target values to be obtained during the validation process. The data summary presented in Table 5-1 is provided to inform interested investigators of the historical quantitative values obtained for these substances in in vitro AR binding studies.

As described in Section 2.4.4, a subset of 44 substances has been identified that, at a minimum, should be used in any validation of in vitro AR binding assays. These 44 substances are in bold type in Table 5-1. Of these substances, 75% (33) are classified as positive (17) or presumed positive (16) for AR binding, and 25% (11) are classified as presumed negative.
## Table 5-1: ICCVAM Recommended Substances for Validation of *In Vitro* AR Binding Assays

<table>
<thead>
<tr>
<th>RBA Range (Classification)</th>
<th>Substance</th>
<th>CASRN</th>
<th>Median RBA Value and RBA Range</th>
<th>No. Positive Responses/No. Times Tested</th>
<th>No. AR Binding Assays in Which Tested</th>
<th>Completed/Anticipated <em>In Vivo</em> Testing</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥10 (Positive)</td>
<td>Methyltrienolone (<em>R1881</em>)</td>
<td>965-93-5</td>
<td>126 (6.7 - 290)</td>
<td>7/7</td>
<td>4</td>
<td>AR agonist; Recommended reference androgen</td>
<td>Steroid, nonphenolic; Estrene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17β-Trenbolone</td>
<td>10161-33-8</td>
<td>108.9</td>
<td>1/1</td>
<td>1</td>
<td>H</td>
<td>Steroid, nonphenolic; Estrene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5α-Dihydrotestosterone</td>
<td>521-18-6</td>
<td>93 (6.8 - 233)</td>
<td>13/13</td>
<td>6</td>
<td>H</td>
<td>Strong AR agonist; Weak ER agonist</td>
<td>Steroid, nonphenolic</td>
</tr>
<tr>
<td></td>
<td>Methyl testosterone</td>
<td>58-18-4</td>
<td>38 (35.9 - 40)</td>
<td>2/2</td>
<td>2</td>
<td>H; 407; M-PA; IUL; FRS</td>
<td>AR and ER agonist</td>
<td>Steroid, nonphenolic; Androstene</td>
</tr>
<tr>
<td></td>
<td>Spironolactone</td>
<td>52-01-7</td>
<td>33.9 (0.76 - 67)</td>
<td>2/2</td>
<td>1</td>
<td></td>
<td>AR agonist and antagonist</td>
<td>Steroid, nonphenolic; Pregnen lactone</td>
</tr>
<tr>
<td></td>
<td>Testosterone</td>
<td>58-22-0</td>
<td>31.3 (0.45 - 125)</td>
<td>13/13</td>
<td>8</td>
<td>IM</td>
<td>Strong AR agonist</td>
<td>Steroid, nonphenolic</td>
</tr>
<tr>
<td></td>
<td>Medroxyprogesterone acetate</td>
<td>71-58-9</td>
<td>11.6 (1.33 - 48.61)</td>
<td>5/5</td>
<td>4</td>
<td></td>
<td>Weak AR agonist</td>
<td>Steroid, nonphenolic; Polycyclic hydrocarbon</td>
</tr>
<tr>
<td>&lt;10 to 1 (Positive)</td>
<td>Bicalutamide</td>
<td>90357-06-5</td>
<td>4.08 (1.8 - 6.36)</td>
<td>2/2</td>
<td>2</td>
<td></td>
<td>AR antagonist</td>
<td>Anilide; Nitrile; Sulfone</td>
</tr>
<tr>
<td></td>
<td>Cyproterone acetate</td>
<td>427-51-0</td>
<td>2.8 (0.588 - 12.4)</td>
<td>11/12</td>
<td>5</td>
<td>IM</td>
<td>AR agonist and antagonist</td>
<td>Nitrile; Diphenyl ether; Organochlorine</td>
</tr>
<tr>
<td></td>
<td>Progesterone</td>
<td>57-83-0</td>
<td>2 (0.000056 - 6.6)</td>
<td>9/10</td>
<td>7</td>
<td>IM</td>
<td></td>
<td>Steroid, nonphenolic; Pregnenediene</td>
</tr>
<tr>
<td></td>
<td>Mifepristone</td>
<td>84371-65-3</td>
<td>2.2</td>
<td>1/1</td>
<td>1</td>
<td>IM</td>
<td>AR agonist and antagonist</td>
<td>Steroid, nonphenolic; Estrene</td>
</tr>
<tr>
<td></td>
<td>17β-Estradiol</td>
<td>50-28-2</td>
<td>1.7 (0.00112 - 8.5)</td>
<td>13/13</td>
<td>7</td>
<td>IM; IUL; FRS</td>
<td>AR agonist and antagonist; Strong ER agonist</td>
<td>Steroid, phenolic; Estrene</td>
</tr>
</tbody>
</table>
### Table 5-1: ICCVAM Recommended Substances for Validation of In Vitro AR Binding Assays

<table>
<thead>
<tr>
<th>RBA Range</th>
<th>Substance</th>
<th>CASRN</th>
<th>Median RBA Value and RBA Range</th>
<th>No. Positive Responses/No. Times Tested</th>
<th>No. AR Binding Assays in Which Tested</th>
<th>Completed/Anticipated In Vivo Testing</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10 to 1 (Positive)</td>
<td>Nilutamide e</td>
<td>63612-50-0</td>
<td>1.24 (0.2 - 1.7)</td>
<td>4/4</td>
<td>2</td>
<td>AR antagonist</td>
<td>Heterocycle; Imidazole</td>
<td></td>
</tr>
<tr>
<td>&lt;1 to 0.1 (Positive)</td>
<td>4-Androstenedione e</td>
<td>63-05-8</td>
<td>1.03 (0.056 - 2)</td>
<td>2/2</td>
<td>2</td>
<td>Strong AR agonist</td>
<td>Steroid, nonphenolic</td>
<td></td>
</tr>
<tr>
<td>&lt;1 to 0.1 (Positive)</td>
<td>17α-Ethyl estradiol e,h</td>
<td>57-63-6</td>
<td>0.29</td>
<td>1/1</td>
<td>1</td>
<td>U; 407; F-PA</td>
<td>Strong ER agonist</td>
<td>Steroid, phenolic</td>
</tr>
<tr>
<td>&lt;1 to 0.1 (Positive)</td>
<td>Hydroxyflutamide e</td>
<td>52806-53-8</td>
<td>0.585 (0.00143 - 1.75)</td>
<td>10/10</td>
<td>4</td>
<td>AR agonist and antagonist</td>
<td>Amide; Anilide; Nitrobenzene</td>
<td></td>
</tr>
<tr>
<td>&lt;1 to 0.1 (Positive)</td>
<td>Fluoxymestrone e,h</td>
<td>76-43-7</td>
<td>0.3</td>
<td>1/1</td>
<td>1</td>
<td>Weak AR agonist</td>
<td>Steroid, nonphenolic</td>
<td></td>
</tr>
<tr>
<td>&lt;1 to 0.1 (Positive)</td>
<td>Estrone e,h,j</td>
<td>53-16-7</td>
<td>0.1</td>
<td>1/1</td>
<td>1</td>
<td>AR agonist; Strong ER agonist</td>
<td>Steroid, phenolic; Estrene</td>
<td></td>
</tr>
<tr>
<td>&lt;0.1 to 0.01 (Positive)</td>
<td>Flutamide e</td>
<td>13311-84-7</td>
<td>0.02 (0.0065 - 0.079)</td>
<td>3/5</td>
<td>5</td>
<td>H; 407; M-PA; IM; FRS</td>
<td>AR antagonist</td>
<td>Amide; Anilide; Nitrobenzene</td>
</tr>
<tr>
<td>&lt;0.1 to 0.01 (Positive)</td>
<td>Vinclozolin e</td>
<td>50471-44-8</td>
<td>0.018 (0.000068 - 0.035)</td>
<td>2/4</td>
<td>3</td>
<td>H; M-PA; IM; IUL; 1G; FRS</td>
<td>AR antagonist</td>
<td>Organochlorine; Cyclic imide; Carbamate</td>
</tr>
<tr>
<td>&lt;0.1 to 0.01 (Positive)</td>
<td>p,p’-DDE e</td>
<td>72-55-9</td>
<td>0.016 (0.0058 - 0.02)</td>
<td>3/3</td>
<td>2</td>
<td>H; 407; M-PA; IM; J(1G,F,A)</td>
<td>Weak AR agonist and antagonist</td>
<td>Organochlorine; Diphenylalkene</td>
</tr>
<tr>
<td>&lt;0.1 to 0.01 (Positive)</td>
<td>Diethylstilbestrol e</td>
<td>56-53-1</td>
<td>0.01 (0.0065 - 0.036)</td>
<td>3/4</td>
<td>3</td>
<td>IUL</td>
<td>Strong ER agonist</td>
<td>Stilbene; Benzylidene; Diphenylalkene</td>
</tr>
<tr>
<td>&lt;0.01 to 0.001 (Positive)</td>
<td>Linuron e</td>
<td>330-55-2</td>
<td>0.0055 (0.0005 - 0.025)</td>
<td>4/4</td>
<td>4</td>
<td>H; M-PA</td>
<td>Weak AR agonist and antagonist</td>
<td>Urea</td>
</tr>
<tr>
<td>&lt;0.01 to 0.001 (Positive)</td>
<td>Atrazine e,h,j</td>
<td>1912-24-9</td>
<td>0.0018</td>
<td>1/1</td>
<td>1</td>
<td>IM</td>
<td>Aromatic amine; Triazine; Arylamine</td>
<td></td>
</tr>
</tbody>
</table>
Table 5-1: ICCVAM Recommended Substances for Validation of *In Vitro* AR Binding Assays *(continued)*

<table>
<thead>
<tr>
<th>RBA Range^h (Classification)</th>
<th>Substance</th>
<th>CASRN</th>
<th>Median RBA Value and RBA Range^e</th>
<th>No. Positive Responses/No. Times Tested</th>
<th>No. AR Binding Assays in Which Tested</th>
<th>Completed/Anticipated <em>In Vivo</em> Testing^d</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.01 to 0.001 (Positive)</td>
<td><em>o,p'-DDT^e</em></td>
<td>789-02-6</td>
<td>0.00105 (0.00058 - 0.00786)</td>
<td>3/3</td>
<td>2</td>
<td>U; J(1G,F,A)</td>
<td>Weak AR and ER antagonist; Weak ER agonist</td>
<td>Organochlorine; Diphenylalkene</td>
</tr>
<tr>
<td></td>
<td><em>4-tert-Octylphenol^k,k</em></td>
<td>140-66-9</td>
<td>0.0001</td>
<td>1/1</td>
<td>1</td>
<td>J(U,H,1G,F,A)</td>
<td>ER agonist</td>
<td>Alkylphenol; Phenol</td>
</tr>
<tr>
<td>&lt;0.001 (Presumed Positive)</td>
<td>Kepone (Chlordecone)^e</td>
<td>143-50-0</td>
<td>0.00072 (0.00063 - 0.008)</td>
<td>2/2</td>
<td>2</td>
<td></td>
<td>Binds to AR and ER</td>
<td>Organochlorine; Chlorinated bridged cycloalkane</td>
</tr>
<tr>
<td></td>
<td><em>p,p'-Methoxychlor^e</em></td>
<td>72-43-5</td>
<td>0.00053 (0.000068 - 0.001)</td>
<td>2/2</td>
<td>2</td>
<td>U; F&amp;M-PA; IUL; IM; FRS; 2G(avian)</td>
<td>AR antagonist; Weak ER agonist</td>
<td>Organochlorine; Chlorinated hydrocarbon</td>
</tr>
<tr>
<td></td>
<td>Corticosterone^e</td>
<td>50-22-6</td>
<td>0.000068</td>
<td>1/1</td>
<td>1</td>
<td></td>
<td></td>
<td>Steroid, nonphenolic</td>
</tr>
<tr>
<td></td>
<td>Procymidone^e</td>
<td>32809-16-8</td>
<td>0.000068</td>
<td>1/1</td>
<td>1</td>
<td>H</td>
<td>AR antagonist</td>
<td>Organochlorine; Cyclic imide</td>
</tr>
<tr>
<td></td>
<td>Bisphenol A^k</td>
<td>80-05-7</td>
<td>0.000094</td>
<td>1/1</td>
<td>1</td>
<td>U; F-PA; J(1G,F,A)</td>
<td>Weak ER agonist</td>
<td>Diphenylalkane; Bisphenol; Phenol</td>
</tr>
<tr>
<td>No RBA Value (Presumed Negative)</td>
<td>Diethylhexylphthalate^k</td>
<td>117-81-7</td>
<td>HDT - 10 µM</td>
<td>0/1</td>
<td>1</td>
<td>J(U,H,1G,F,A)</td>
<td></td>
<td>Phthalate</td>
</tr>
<tr>
<td></td>
<td>Dexamethasone^e,j</td>
<td>50-02-2</td>
<td>HDT - 10 µM</td>
<td>0/2</td>
<td>2</td>
<td></td>
<td>AR agonist</td>
<td>Steroid, nonphenolic</td>
</tr>
</tbody>
</table>

**ANTICIPATED RESPONSES**

<table>
<thead>
<tr>
<th>RBA Data Not Available (Presumed Positive)</th>
<th>Substance</th>
<th>CASRN</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluoranthene</td>
<td>206-44-0</td>
<td>Pos.</td>
<td>AR antagonist</td>
<td>Polycyclic aromatic hydrocarbon; Fluorene</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>65277-42-1</td>
<td>Pos.</td>
<td>F&amp;M-PA; IM</td>
<td>Weak AR agonist</td>
<td>Imidazole; Piperazine</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Note: *RBA = Relative Binding Affinity; ANTICIPATED RESPONSES = Data Not Available (Presumed Positive)
Table 5-1: ICCVAM Recommended Substances for Validation of *In Vitro* AR Binding Assays<sup>a</sup> (continued)

<table>
<thead>
<tr>
<th>RBA Range&lt;sup&gt;b&lt;/sup&gt; (Classification)</th>
<th>Substance</th>
<th>CASRN</th>
<th>Median RBA Value and RBA Range&lt;sup&gt;c&lt;/sup&gt;</th>
<th>No. Positive Responses/No. Times Tested</th>
<th>No. AR Binding Assays in Which Tested</th>
<th>Completed/Anticipated <em>In Vivo</em> Testing&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBA Data Not Available (Presumed Positive)</td>
<td><em>p</em>-n-Nonylphenol&lt;sup&gt;m&lt;/sup&gt;</td>
<td>104-40-5</td>
<td>Pos.</td>
<td></td>
<td></td>
<td>U; 407; J(U,H,I,G,F,A)</td>
<td>AR antagonist</td>
<td>Alkylphenol; Phenol</td>
</tr>
<tr>
<td>RBA Data Not Available (Presumed Negative)</td>
<td>Actinomycin D</td>
<td>50-76-0</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>RNA synthesis inhibitor</td>
<td>Phenoxazine; Lactone; Peptide</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Di-n-butyl phthalate</td>
<td>84-74-2</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>U; M-PA; 1G; J(U,H,I,G,F,A)</td>
<td>ER agonist</td>
<td>Phthalate</td>
</tr>
<tr>
<td></td>
<td>Fadrozole</td>
<td>102676-47-1</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>F-PA; IM; FRS</td>
<td>Aromatase inhibitor</td>
<td>Imidazole; Nitrile</td>
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<td></td>
<td>Finasteride</td>
<td>98319-26-7</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>H; M-PA; IM</td>
<td>5α-reductase inhibitor</td>
<td>Steroid, nonphenolic; Androstene</td>
</tr>
<tr>
<td></td>
<td>4-Hydroxytamoxifen</td>
<td>68047-06-3</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>ER antagonist</td>
<td>Triphenylethylene; Benzyldione;</td>
<td></td>
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<td></td>
<td>Phenobarbital</td>
<td>57-30-7</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>F&amp;M-PA; IM</td>
<td>Enhances thyroid hormone excretion</td>
<td>Heterocycle; Pyrimidine</td>
</tr>
<tr>
<td></td>
<td>Sodium azide</td>
<td>26628-22-8</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>Cytotoxic</td>
<td>Organic salt; Azide</td>
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</tr>
<tr>
<td></td>
<td>12-O-Tetradecanoylphorbol-13acetate</td>
<td>16561-29-8</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>Activates ligand independent cell</td>
<td>Phorbol ester; Terpene</td>
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<tr>
<td></td>
<td>2,4,5-Trichlorophenoxyacetic acid</td>
<td>93-76-5</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>Weak ER agonist</td>
<td>Organochlorine; Chlorinated aromatic hydrocarbon</td>
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<tr>
<td></td>
<td>Ammonium perchlorate</td>
<td>7790-98-9</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>IUL</td>
<td>Thyroid disruptor</td>
<td>Organic acid; Organic salt</td>
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</tbody>
</table>
## Table 5-1: ICCVAM Recommended Substances for Validation of *In Vitro* AR Binding Assays *(continued)*

<table>
<thead>
<tr>
<th>RBA Range (Classification)</th>
<th>Substance</th>
<th>CASRN</th>
<th>Median RBA Value and RBA Range</th>
<th>No. Positive Responses/No. Times Tested</th>
<th>No. AR Binding Assays in Which Tested</th>
<th>Completed/Anticipated <em>In Vivo</em> Testing</th>
<th>Chem. Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBA Data Not Available (Presumed Negative)</td>
<td>Anastrazole</td>
<td>120511-73-1</td>
<td>Neg.</td>
<td>IM</td>
<td>Aromatase inhibitor</td>
<td>Nitrile; Triazole</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Apigenin</td>
<td>520-36-5</td>
<td>Neg.</td>
<td>IUL</td>
<td>ER agonist</td>
<td>Flavanoid; Flavone; Phenol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Apomorphine</td>
<td>58-00-4</td>
<td>Neg.</td>
<td>IM</td>
<td>Dopamine D1/D2 receptor agonist</td>
<td>Heterocycle; Quinoline</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bisphenol B</td>
<td>77-40-7</td>
<td>Neg.</td>
<td>IM</td>
<td>ER agonist</td>
<td>Diphenylalkane; Bisphenol; Phenol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Butylbenzyl phthalate</td>
<td>85-68-7</td>
<td>Neg.</td>
<td>IUL</td>
<td>ER agonist</td>
<td>Phthalate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-sec-Butylphenol</td>
<td>89-72-5</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>Phenol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CGS 18320B</td>
<td>112808-99-8</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>407</td>
<td>Aromatase inhibitor</td>
</tr>
<tr>
<td></td>
<td>Clomiphene citrate</td>
<td>50-41-9</td>
<td>Neg.</td>
<td></td>
<td>Binds to the ER</td>
<td>Chlorinated triphenylethylene; Benzylidene; Stilbene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Coumestrol</td>
<td>479-13-0</td>
<td>Neg.</td>
<td>IM</td>
<td>ER agonist</td>
<td>Coumestan; Benzopyranoone; Coumarin; Ketone</td>
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<tr>
<td></td>
<td>4-Cumylphenol</td>
<td>599-64-4</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>Weak ER agonist</td>
<td>Phenol</td>
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<tr>
<td></td>
<td>Cycloheximide</td>
<td>66-81-9</td>
<td>Neg.</td>
<td></td>
<td>Protein synthesis inhibitor</td>
<td>Piperidine; Glutaramide</td>
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</tr>
<tr>
<td></td>
<td>Daidzein</td>
<td>486-66-8</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>Weak ER agonist</td>
<td>Flavanoid; Isoflavone; Phenol</td>
</tr>
<tr>
<td></td>
<td>Dibenzo[a,h]-anthracene</td>
<td>53-70-3</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Polycyclic aromatic hydrocarbon; Anthracene</td>
</tr>
</tbody>
</table>
### Table 5-1: ICCVAM Recommended Substances for Validation of *In Vitro* AR Binding Assays*(continued)*

<table>
<thead>
<tr>
<th>RBA Range (Classification)</th>
<th>Substance</th>
<th>CASRN</th>
<th>Median RBA Value and RBA Range</th>
<th>No. Positive Responses/No. Times Tested</th>
<th>No. AR Binding Assays in Which Tested</th>
<th>Completed/Anticipated <em>In Vivo</em> Testing</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBA Data Not Available (Presumed Negative)</td>
<td>17α-Estradiol</td>
<td>57-91-0</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>ER agonist</td>
<td>Steroid, phenolic; Estrene</td>
</tr>
<tr>
<td></td>
<td>Ethyl paraben</td>
<td>120-47-8</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Binds weakly to ER</td>
<td>Paraben; Organic acid</td>
</tr>
<tr>
<td></td>
<td>Fenarimol</td>
<td>60168-88-9</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>F-PA</td>
<td>Aromatase inhibitor</td>
</tr>
<tr>
<td></td>
<td>Flavone</td>
<td>525-82-6</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>M-PA; IM</td>
<td>Weak ER antagonist</td>
</tr>
<tr>
<td></td>
<td>Genistein</td>
<td>446-72-0</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>U; 407</td>
<td>Weak ER agonist and antagonist</td>
</tr>
<tr>
<td></td>
<td>Haloperidol</td>
<td>52-86-8</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>IM</td>
<td>Dopamine D2 receptor antagonist</td>
</tr>
<tr>
<td></td>
<td>meso-Hexestrol</td>
<td>84-16-2</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Strong ER agonist</td>
</tr>
<tr>
<td></td>
<td>ICI 182,780</td>
<td>129453-61-8</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>IM</td>
<td>ER antagonist</td>
</tr>
<tr>
<td></td>
<td>Kaempferol</td>
<td>520-18-3</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ER agonist</td>
</tr>
<tr>
<td></td>
<td>Morin</td>
<td>480-16-0</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Binds weakly to the ER</td>
<td>Flavanoid; Flavone; Phenol</td>
</tr>
<tr>
<td></td>
<td>Norethynodrel</td>
<td>68-23-5</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Binds to the ER</td>
<td>Steroid, nonphenolic; Norpregnene</td>
</tr>
<tr>
<td></td>
<td>Oxazepam</td>
<td>604-75-1</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>IM</td>
<td>Enhances thyroid hormone excretion</td>
</tr>
<tr>
<td></td>
<td>Phenolphthalin</td>
<td>81-90-3</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Triphenylmethane; Diphenylalkane</td>
</tr>
</tbody>
</table>

*Notes:*
- RBA: Relative Binding Activity
- ER: Estrogen Receptor
- U: Ambigous results
- M-PA: Moderate affinity to the ER
- IM:  Incomplete results
- **Bold**: Indicates recommended substances for validation of *In Vitro* AR binding assays.

*Abbreviations:*
- NOR: Norpregnene
- E: Estrogen
- ER: Estrogen Receptor
- M-PA: Moderate affinity to the ER
- IM: Incomplete results
- U: Ambiguous results
### Table 5-1: ICCVAM Recommended Substances for Validation of *In Vitro* AR Binding Assays

<table>
<thead>
<tr>
<th>RBA Range</th>
<th>Substance</th>
<th>CASRN</th>
<th>Median RBA Value and RBA Range</th>
<th>No. Positive Responses/No. Times Tested</th>
<th>No. AR Binding Assays in Which Tested</th>
<th>Completed/Anticipated <em>In Vivo</em> Testing</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. AR Binding Assays</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pimozide</td>
<td>2062-78-4</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>F&amp;M-PA</td>
<td>Dopamine receptor antagonist</td>
</tr>
<tr>
<td></td>
<td>Propylthiouracil</td>
<td>51-52-5</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>407; F&amp;M-PA; IM; IUL; 2G</td>
<td>Inhibits T3/T4 synthesis</td>
</tr>
<tr>
<td></td>
<td>Reserpine</td>
<td>50-55-5</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>IM</td>
<td>Depletes dopamine</td>
</tr>
<tr>
<td></td>
<td>Tamoxifen</td>
<td>10540-29-1</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>IM</td>
<td>ER antagonist</td>
</tr>
<tr>
<td></td>
<td>L-Thyroxine</td>
<td>51-48-9</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>407</td>
<td>Thyroid hormone</td>
</tr>
<tr>
<td></td>
<td>Zearalenone</td>
<td>17924-92-4</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>IM</td>
<td>ER agonist</td>
</tr>
</tbody>
</table>

**Abbreviations:** AR = Androgen receptor; CASRN = Chemical Abstracts Service Registry Number; D1 and D2 = Two major families of dopamine receptors; DDE = 1,1-Dichloro-bis[4-chlorophenyl]ethylene; DDT = Dichlorodiphenyltrichloroethane; ER = Estrogen receptor; HDT = Highest dose tested; Neg. = Negative; Pos. = Positive; RBA = Relative binding affinity; T3 = Triiodothyronine; T4 = Thyroxine.

* Substances in bold type are those that, at a minimum, are recommended for inclusion in future validation studies. Empty cells indicate that no relevant data were identified.

* Substances for which RBA data are available are sorted into six RBA categories in log decrements: $\geq10$, $<10-1$, $<1-0.1$, $<0.1-0.01$, $<0.01-0.001$, and $<0.001$. A substance is classified as “presumed positive” for AR binding if it was positive in more than 50% of reported studies. A substance is classified as “presumed positive” for AR binding if it was positive in 50% or less of reported studies, if it was reported positive in the single study conducted, or if its median RBA from reported studies was less than 0.001. The two substances that did not produce an IC$_{50}$ value in an AR binding assay are classified as “presumed negative” instead of “negative” for AR binding since they had not been tested in multiple studies at or above the limit dose of 1 mM recommended in *Section 5.1.5*. Substances without RBA data are classified as “presumed positive” or “presumed negative” based on available information, including their known mechanism of action or their responses in AR transcriptional activation (TA) assays, ER binding assays, or ER TA assays.

* Substances for which RBA data are available are ranked according to their relative potency in AR binding assays from most potent to least potent. Substances for which no relevant RBA data are available have been assigned an anticipated positive (Pos.) or negative (Neg.) response for AR binding based on available information, including their known mechanism of action or their responses in AR TA assays, ER binding assays, or ER TA assays.

* The RBA for a test substance is calculated as $[\text{IC}_{50} \text{(reference androgen)}/\text{IC}_{50} \text{(test substance)}] \times 100$, where IC$_{50}$ is the inhibitory concentration of the test substance that displaces 50% of the radiolabeled reference androgen from the receptor. Median RBA values are derived from *in vitro* AR binding data published in the peer-reviewed scientific literature, which were then reviewed and summarized in the NIEHS Background Review Document (BRD) titled “Current Status of Test Methods for Detecting Endocrine Disruptors: *In Vitro* Androgen Receptor Binding Assays—July 2002” (available on the ICCVAM website at [http://iccvam.niehs.nih.gov/methods/endocrine.htm](http://iccvam.niehs.nih.gov/methods/endocrine.htm)). Substances for which RBA data are available are ranked according to their relative potency in AR binding assays from most potent to least potent. Substances for which no relevant RBA data are available have been assigned an anticipated positive (Pos.) or negative (Neg.) response for AR binding based on available information, including their known mechanism of action or their responses in AR TA assays, ER binding assays, or ER TA assays.
Table 5-1: ICCVAM Recommended Substances for Validation of In Vitro AR Binding Assays

Several in vivo test methods are undergoing further development or validation by OECD, EPA, and the JME (J). Substances indicated are proposed for testing by OECD in the Uterotrophic assay (U), the Hershberger assay (H), or the 407 protocol (407); for testing by EPA in the female pubertal assay (F-PA), the male pubertal assay (M-PA), the intact male assay (IM), a one-generation assay (1G), a two-generation assay (2G), or a fish reproductive screen (FRS); for testing by JME in the U, H, and 1G assays, or various fish (F) and avian (A) assays. Due to the lack of CASRN for the JME studies, some of the indicated substances might not be the same substance included in this list. The in utero through lactation assay (IUL) has been recommended, but EPA has not made a decision on its further development or validation.

Information regarding the median RBA value, the corresponding RBA range, the number of AR binding test methods used, and the number of positive responses per number of studies conducted was derived from data presented in Appendix D of the NICEATM AR Binding BRD cited in footnote c. This document contains AR binding data from the published literature through September 30, 2001. The median RBA values and the counts for number of assays and number of positive responses do not include the LNCaP assay described in the BRD, because the AR in this assay contains a point mutation in the ligand binding domain that alters the binding properties of the receptor. The LNCaP assay uses a cell line derived from the lymph node of a patient with metastatic prostatic adenocarcinoma.

The median RBA values and RBA ranges for 5α-dihydrotestosterone, testosterone, and methyltrienolone were determined from studies in which these substances were not used as the reference androgen. Additionally, the counts (No. Positive Responses/No. Times Tested, and No. AR Binding Assays in Which Tested) for 5α-dihydrotestosterone, testosterone, and methyltrienolone exclude studies in which these substances were used as the reference androgen.

R1881 is the recommended reference androgen for in vitro AR binding and TA assays and, thus, is not considered a test substance for validation purposes (refer to Section 5.2 for more information).

17β-Trenbolone, mifepristone, 17α-ethinyl estradiol, fluoxymestrone, estrone, atrazine and 4-tert-octylphenol are classified “presumed positive” because only a single positive study was reported for these substances.

Data for estrone are from the LNCaP assay, the only assay in which this substance was tested. LNCaP data are not included for any other substance in this table.

Atrazine has been associated with delayed pubertal development in male rats (Stoker et al. 2000), but it is thought to act through a mechanism other than binding to the AR (Sanderson et al. 2001; Stoker et al. 2000). To be consistent with the classification scheme used for other substances where only a single positive study was reported, atrazine is classified “presumed positive”.

Information for this substance was abstracted from a publication that was published or reviewed after the literature search was completed for the NICEATM AR Binding BRD (i.e., Paris et al. 2002 and Parks et al. 2000 in Section 7.0).

The HDT for dexamethasone was 10 µM in one study and 0.3 µM in one study.

Two forms of p-nonylphenol are available for testing. One form consists of a mixture of various branched isomers (CASRN 84852-15-3), while the other contains only one isomer consisting of a linear alkyl chain (CASRN 104-40-5). ICCVAM recommends the linear form, which has a uniform chemical structure, for validation studies.
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6.0 IN VITRO ANDROGEN RECEPTOR TRANSCRIPTIONAL ACTIVATION ASSAYS

6.1 Minimum Procedural Standards

More than 18 different in vitro assays have been used to evaluate the ability of substances to act as AR TA agonists or antagonists (NIEHS 2002d). Of the 18 in vitro AR TA assays considered in the AR TA BRD, 15 used mammalian cell lines, 1 used yeast cells, 1 used a fish cell line, and 1 measured cell proliferation. The Expert Panel recommended that assays using yeast and those measuring cell proliferation not be considered for future validation efforts. Yeast-based assays were not recommended due to the poor transport of many substances across the yeast cell wall, while assays based on cell proliferation were not recommended because cell proliferation can be mediated through pathways other than those involving transcriptional activation of androgen responsive genes. No validation studies have been conducted to assess the performance and reliability of these test methods, and the few substances tested multiple times within and across assays preclude an assessment of comparative test method performance. Although the Expert Panel concluded that no specific in vitro AR TA test method could be recommended currently as a priority for validation, assays using cells (e.g., MDA-MB-453) with an endogenous AR that has been transduced with an adenovirus carrying a Luc reporter gene were thought to be the most effective and reliable (see Appendix A). To assist in the development, standardization, and validation of in vitro AR TA assays, NICEATM and the EDWG developed proposed minimum procedural standards for consideration by the Expert Panel (NIEHS 2002d). The purpose of minimum procedural standards is to specify information essential for maximizing test method intra- and interlaboratory reproducibility while minimizing the likelihood of erroneous results. Such standards also enhance any assessment of the comparative performance of different AR TA assays. The minimum procedural standards provided here have been revised to incorporate recommendations and comments of the Expert Panel, the EDWG, and the public. Except where noted, all in vitro AR TA assays should incorporate these minimal procedural standards in their protocols, and scientific justification should be provided for deviations.

6.1.1 Reference Androgen and TA Response

6.1.1.1 Agonism Assays

The purpose of the reference androgen in AR TA agonism assays is to demonstrate the adequacy of the test method for detecting AR agonists (i.e., the reference androgen serves as a positive control). The recommended reference androgen is methyltrienolone (R1881, CASRN 965-93-5). The TA-inducing ability of the reference androgen should be demonstrated by generating a full dose-response curve in each study. The concentration of R1881 used in most in vitro TA agonism assays ranges from 1 pM to 1 μM.

Rationale: Due to the possible metabolism of natural androgens in some cell lines, R1881, which is not metabolized, is the recommended reference androgen. Test acceptance criteria for the positive control should be established based on historical data for the maximum induction and on the calculated concentration of the reference androgen that induces a half-maximal response (i.e., the effective concentration [EC$_{50}$] value).

6.1.1.2 Antagonism assays

In AR TA antagonism assays, test substances are evaluated for their ability to reduce the level of TA induced by a reference androgen.
The concentration of the reference androgen selected for antagonism assays should be within the upper linear region of the dose-response curve; 70 to 80% of maximal induction is recommended. The recommended reference androgen for these assays is R1881.

Rationale: Due to the possible metabolism of natural androgens in some cell lines, R1881, which is not metabolized, is the recommended reference androgen. The ability to detect a weak antagonist depends on the magnitude of the TA response induced by the reference androgen. Using a reference androgen concentration that elicits a response within the upper linear portion of the dose response curve will maximize the sensitivity of the test method.

6.1.2 Preparation of Test Substances and Volume of Administered Solvent
Test substances should be dissolved in a solvent that is miscible with the cell medium. Water, ethanol (95 to 100%), or DMSO is the preferred solvent. Preference should be given to the solvent that allows testing of the test substance at the maximal concentration possible without exceeding the limit dose (see Section 6.1.3). However, in testing situations where more than one solvent could be used, preference should be given to water, followed by ethanol (95 to 100%), and then DMSO. Other solvents may be used if it can be demonstrated that they are not cytotoxic and otherwise do not interact with the test system. The volume of the solvent included in the reaction mixture generally has ranged from 0.1 to 1% of the total volume. For any solvent, it should be demonstrated that the maximum volume used does not interfere with the test system. This can be accomplished by comparing the maximum fold induction and the mean EC$_{50}$ value for the reference androgen in the presence and absence of the solvent at the highest volume to be used in the TA studies. The stability of the dissolved test substance should be determined prior to testing. In the absence of stability information, the stock solution should be prepared fresh prior to use.

Rationale: Selection of water, ethanol (95 to 100%), or DMSO as suitable solvents is based on historical usage. Members of the Expert Panel stated that water or ethanol (95 to 100%) is preferred to DMSO because some substances, when dissolved in DMSO, might result in reduced activity (see Section 6.1.4). For this reason, most investigators have limited the final concentration of DMSO to less than 0.1%. Because of differences in the sensitivities of various cell lines, the maximal concentration of a solvent that does not interfere with performance should be determined for each test method.

6.1.3 Concentration Range of the Test Substances
In the absence of solubility or cytotoxicity constraints, the maximum test substance concentration (i.e., the limit dose) for agonism or antagonism assays should be 1 mM. Seven test substance concentrations spaced at log intervals up to the limit dose (i.e., 1 nM, 10 nM, 100 nM, 1 μM, 10 μM, 100 μM, 1 mM) should be tested. An evaluation of cell cytotoxicity should be included in each study, and only those dose levels not associated with toxicity greater than 10% of the concurrent solvent control should be considered in the analysis of the data.

Rationale: Most test method guidelines include a limit dose to ensure that all substances are tested over the same dose range while avoiding excessive amounts of a test substance that can perturb the test system through physicochemical mechanisms. An established limit dose also minimizes the effort and cost of screening and testing. Based on the range of published EC$_{50}$ values for AR agonists and IC$_{50}$ values for AR antagonists (NIEHS
2002d), a limit dose of 1 mM was deemed suitable by the Expert Panel, the EDWG, and ICCVAM for assessing the ability of a test substance to act as either an AR agonist or an antagonist.

The seven recommended test substance concentrations, spaced at log intervals, should be sufficient for a screening test because, currently, the study results will be used in a semi-quantitative manner only. If a lower maximum concentration is tested because of solubility or cytotoxicity constraints, the number of concentrations tested should remain the same by adding intermediate concentrations within the adjusted range. The purpose of the cytotoxicity assay is to ensure that only responses at nontoxic doses are considered.

6.1.4 Solvent and Positive Controls
6.1.4.1 Solvent controls

**Agonism Assays**

In each study, a set of concurrent solvent control cultures should be included. The solvent control consists of the solvent in which the reference androgen and the test substance are dissolved plus the cell line containing the AR, but without the reference androgen. The solvent for the reference androgen and test substance should be present at the highest volume that they are used to add these substances to the test system. As indicated in Section 6.1.2, the solvent at the concentration used must not be cytotoxic or otherwise interact with the test system.

**Rationale:** The concurrent solvent control in TA agonism assays provides a measure of the extent of TA in the absence of the reference androgen, other positive controls (if used), or the test substance, and is the baseline against which the extent of TA induced by these substances is compared.

**Antagonism Assays**

A concurrent set of solvent control cultures should be included in each study. The solvent control consists of the solvent in which the reference androgen and the test substance are dissolved, the cell line containing the AR, and the test method specific concentration of the reference androgen (based on achieving 70 to 80% of the maximum TA of the reference androgen). The solvent for the reference androgen and test substance should be present at the highest volume that they are used to add these substances to the test system. As indicated in Section 6.1.2, the solvent at the concentration used must not be cytotoxic or otherwise interact with the test system.

**Rationale:** The extent of TA in the presence of the reference androgen is the baseline against which the antagonism of a test substance is measured.

6.1.4.3 Positive control

**Agonism Assays**

In addition to the standard potent reference androgen, it might be useful to include in each study a positive control androgen with a maximal TA response two to three orders of magnitude lower than the reference androgen. Due to the paucity of quantitative data for AR TA agonism assays, a specific substance cannot be recommended at this time as an additional positive control.

**Rationale:** The inclusion in each study of a second positive control, in addition to the reference androgen, would provide another QC measure by which to judge the sensitivity and acceptability of a study for detecting a weak agonist, and by which to evaluate the historical intralaboratory reproducibility of the test method. The necessity for inclusion of an additional positive control androgen in each study should be evaluated during the validation process.
Antagonism Assays
A known AR antagonist (e.g., hydroxyflutamide) should be included as a positive antagonist control in each antagonism study. The concentration of the reference antagonist that is used should be one that reduces the ability of the reference androgen to induce TA in the test system by 70 to 90%. The positive antagonist control should also be tested in the absence of the reference androgen to determine whether it alone can induce TA.

Rationale: The purpose of the positive antagonist control is to demonstrate the sensitivity and reproducibility of the in vitro AR TA antagonism assay. A range of doses of a positive control antagonist that inhibits the ability of the reference androgen to induce TA will allow for historical confidence intervals to be calculated, which can be used as a QC measure to ensure the adequacy of each study. Hydroxyflutamide is suggested as the candidate AR antagonist as this substance historically has been shown to be negative as an agonist but positive as an antagonist at concentrations lower than 10μM. Other substances that might be used as a positive control antagonist should produce a similar response.

6.1.5 Within-Test Replicates
All concentration levels of the controls, the reference androgen, and the test substance should be tested in triplicate.

Rationale: The purpose of triplicate tubes for each concentration and volume of the various controls, the reference androgen, and the test substance is to ensure robust data and the ability to evaluate interreplicate variability. The most appropriate number of replicate tubes, however, should be evaluated after sufficient data has been collected using an optimized test method protocol.

6.1.6 Data Analysis
No standardized statistical methods for analyzing data obtained from in vitro AR TA assays have been developed. For agonism assays, an EC$_{50}$ is calculated for the concentration of the test substance and the positive control(s) that results in 50% of the maximal TA response. TA induction may also be reported as fold increase above the concurrent solvent control response. For antagonism assays, the TA response induced by a test substance in the presence of the reference androgen is compared to the response induced by the reference androgen alone and an IC$_{50}$ is calculated (i.e., the test substance concentration that reduces the reference androgen response by 50%). Approaches for data analysis have varied from a visual inspection of the data to more formal statistical approaches involving either one- or two-way analysis of variance (ANOVA) (with main effects being treatment and replicates), using a general linear model based on means and variances for the fold induction above the concurrent solvent control level. The EC$_{50}$ (agonism assays) or IC$_{50}$ (antagonism assays) values have been calculated using various curve-fitting programs. One curve-fitting approach is based on a logistic dose-response model where the asymptotic minimum and maximum response, the dose that is halfway between the minimum and maximum, and the slope of the line tangent to the logistic curve at this midpoint are determined (Gaido et al. 1997). Asymptotic standard errors of the parameter estimates are employed to perform two-sided Student’s t tests. However, when EC$_{50}$ or IC$_{50}$ values cannot be calculated, an appropriate trend analysis could be used to evaluate for a significant dose-response relationship for agonism or antagonism. Then, an appropriate pair-wise test could be used to evaluate for a significant effect at the different test substance concentrations. In addition, the corresponding historical mean and confidence intervals for the EC$_{50}$ or IC$_{50}$ values for the reference androgen/
positive controls in agonism and antagonism studies, respectively, should be calculated and presented.

**Rationale:** Various statistical and non-statistical approaches have been used to analyze the results of AR TA agonism and antagonism assays. Statistical methods are more informative than nonstatistical methods. However, before deciding on which statistical approaches to use, an understanding of the underlying variability in the data should be obtained, and suitable diagnostics will need to be performed to ensure that all underlying assumptions regarding the statistical procedure are valid.

### 6.1.7 Good Laboratory Practice Compliance

Studies should be performed in compliance with GLP guidelines (EPA 2001, 2002; FDA 2002; OECD 1998).

**Rationale:** Conducting studies in compliance with GLP guidelines increases confidence in the quality and reliability of test data. Furthermore, if data using these test methods are to be submitted to the EPA in response to Federal testing requirements, then compliance with appropriate GLP guidelines will be required.

### 6.1.8 Study Acceptance Criteria

- The limit dose should be 1 mM, unless precluded by solubility or cytotoxicity constraints.
- The response (fold-increase, EC\textsubscript{50} or IC\textsubscript{50} values) for the reference androgen and the positive control should be within the appropriate historical acceptance range.
- The study should comply with GLP guidelines.

**Rationale:** Established study acceptance criteria are required to ensure that the study is conducted appropriately.

### 6.1.9 Interpretation of Results

A substance is classified as an AR agonist if the response (e.g., luciferase activity) elicited by the substance is increased significantly above the concurrent solvent control level, as determined by an appropriate statistical test. A substance is classified as an AR antagonist if the substance causes a significant decrease in the ability of the reference androgen to induce TA, as determined by an appropriate statistical test. However, interpretation of the results should not rely solely on statistics but also on scientific judgment and should incorporate consideration of the nature and shape of the dose-response relationship and, if needed, the reproducibility of the response in independent experiments. If a substance does not induce TA or inhibit the ability of the reference androgen to induce TA after testing to the limit dose or to the maximum concentration possible based on its solubility or cytotoxicity, the tests substance is classified as negative for agonism and antagonism, respectively, under conditions of the test.

**Rationale:** Criteria that incorporate appropriate statistical methods and sound scientific judgment for classifying a substance as an AR agonist or antagonist are essential for ensuring the credibility of the results.

### 6.1.10 Repeat Studies

Generally, in a validation study, repeat studies would be conducted to evaluate intralaboratory repeatability and reproducibility. In contrast, in screening studies, repeat studies are not conducted, except to clarify equivocal results. If a study is repeated, the use of test substance concentrations more closely distributed in the range of interest might facilitate a more accurate analysis of the dose-response relationship for the test substance.

**Rationale:** Repeat studies are used in a validation study to demonstrate the intralaboratory
repeatability and reproducibility of a test method. However, for a screening study, if the acceptance criteria are met and a clear negative or positive response is obtained, a repeat study to verify the original result usually is not considered necessary. In studies where an accurate EC\textsubscript{50} or IC\textsubscript{50} value cannot be calculated or where an equivocal response is obtained, a repeat study using adjusted dose levels might be needed to ensure a reliable conclusion.

6.1.11 Study Report
At a minimum, the study report should include the following information:

**Reference Androgen**
- name, CASRN, purity, and supplier or source of the reference androgen
- concentrations and volumes used

**Additional Positive Control (if used)**
- name, CASRN, purity, and supplier or source
- concentrations and volumes used

**Test Substance**
- name, chemical structure (if known), CASRN (if known), and supplier or source
- physical nature (solid or liquid) and purity, if known (every attempt should be made to determine the purity)
- physicochemical properties relevant to the study (e.g., solubility, pH, stability, volatility)
- concentrations and volumes used

**Solvent**
- name, CASRN, purity, and supplier or source
- justification for choice of solvent
- information on the solubility of the test substance in all solvents in which it was tested
- information to demonstrate that the solvent, at the maximum volume used, is not cytotoxic and otherwise does not interfere with the study

**Androgen Receptor**
- type and source of AR and the supplier
- isolation procedure or method for making constructs
- nomenclature and components of the expression construct
- complete DNA sequence of AR incorporated into expression construct

**Reporter Plasmid**
- type of reporter gene
- type and structure of response elements
- name, identification and source of original plasmid used to make construct
- sequence of the inserts in each plasmid
- description and methodology used to make the transfected plasmid
- nomenclature and genetic components comprising the reporter construct

**Cell Line**
- source and nomenclature of the cell line and protocol for its maintenance before and after transfection
- source of plasticware used to culture cells and source of other materials used in the study
- passage number of cell line used for transfection and passage number of cell line used in the study
- growth parameters of the cell line before initiation of the study
- method used to transiently transfect the reporter construct into the cells
- method used to monitor transient transfection efficiency between cell preparations
- methods for establishment and propagation of a stably transfected cell line and what is
required for growth of the cell line (e.g., charcoal-stripped serum)
• method used to monitor the stability of a stably transfected cell line used for testing
• rationale, based on data, for deciding on the number of passages a cell line can undergo without a decrease in activity
• details regarding the selection requirements needed for maintaining stable cell lines

Study Conditions
• rationale for the concentration of the reference androgen used
• composition of media and buffers used
• concentration range of the test substance, with justification
• volume of the solvent used to dissolve the test substance and the volume added to the reaction mixture
• incubation volume, duration, and temperature
• description of the solvent control
• level of carbon dioxide in the incubator when growing cells and throughout study
• type and composition of metabolic activation system, if used
• concentration ranges of positive controls
• method used to lyse cells after incubation
• method used to measure TA based on reporter activity
• statistical methods used to determine the response and EC_{50} value for agonism studies or IC_{50} value for antagonism studies

Results
• observations for and extent of any precipitation of test substance
• extent of cytotoxicity at each dose level
• reporter response for each replicate at each dose for all test substances, along with confidence levels or other measure of intradose repeatability
• graphically presented dose-response curves for the reference androgen (agonism studies), the positive control(s), and the test substance
• calculated EC_{50} value for agonism studies or IC_{50} value for antagonism studies and confidence limits for the reference androgen (agonism studies), positive control(s), and test substance
• in agonism studies, the fold increase above the concurrent solvent control in TA for each concentration of the reference androgen, the additional positive control (if used), and the test substance
• for antagonism studies, the percent decrease in TA for each concentration of the positive control and the test substance

Discussion of Results
• in each agonism study, reproducibility of fold increases in activity and in the EC_{50} value for the reference androgen control, including ranges, means, standard deviations, and confidence intervals compared to historical data
• in agonism studies, historical EC_{50} values for the positive control androgen with ranges, means, standard deviations, and confidence intervals
• in antagonism studies, reproducibility of fold decreases in activity for the reference androgen and the IC_{50} values for the reference antagonist, including ranges, means, and standard deviations, compared to historical data

Conclusion
• classification of test substance with regard to in vitro AR TA agonist or antagonist activity

Rationale: Minimum reporting standards are needed to ensure that a study report contains the level of information and detail that would be required if the study results are reviewed by the applicable regulatory agency, or for
independent replication of the study, if deemed necessary.

6.2 Recommended Substances for Validation of In Vitro Androgen Receptor Transcriptional Activation Assays

To facilitate validation of in vitro AR TA assays, ICCVAM has compiled a list of 78 recommended substances for use in future validation studies. Separate lists are provided of the available quantitative and qualitative data and anticipated responses of each of the 78 substances in in vitro AR TA agonism (Table 6-1) and antagonism (Table 6-2) assays. Section 2.0 provides a detailed account of how these substances were selected. EC\(_{50}\) and IC\(_{50}\) data are available for 6 (8%) and 18 (23%) of these 78 recommended substances for agonism and antagonism, respectively. Qualitative data are available for 45 (58%) and 27 (35%) of these 78 recommended substances for agonism and antagonism, respectively. Thus, there is incomplete information regarding how all 78 of the recommended substances will respond in in vitro AR agonism and antagonism assays utilizing mammalian cell reporter gene systems. Although methyltrienolone is included in the list of recommended substances, it was not included in the count of substances for validation as it is a required component of the test system to measure antagonism and is the positive control for agonism studies. Quantitative in vitro AR TA data are provided for the substances inducing a positive response in at least one study. This includes the median EC\(_{50}\) or IC\(_{50}\) values for agonism and antagonism studies, respectively, a range of values where more than one study had been conducted, and the number of studies and test methods in which each substance was tested. In situations where only one positive study was reported, the EC\(_{50}\) or IC\(_{50}\) value obtained in that study is reported. The substances with EC\(_{50}\) or IC\(_{50}\) data are listed first, sorted by potency from strongest to weakest, based on the median EC\(_{50}\) or IC\(_{50}\) value of each substance across all positive studies. Substances that induced a positive response in 50% or fewer of the AR TA studies in which they were tested are classified in this table as “presumed positive” for AR agonism or antagonism. No effort was made to assess the validity and quality of each negative or positive study reported for each substance. Substances were classified as negative for AR TA agonism or antagonism activity if they were reported as negative in multiple studies when tested up to the limit dose as defined in this document (i.e., 1 mM). Substances were classified as “presumed negative” for AR TA activity if they had not been tested to the limit dose in multiple studies (i.e., reproducibility for a negative response had not been demonstrated at test substance concentration up to 1 mM). Using these criteria, no substances could be classified as negative for AR TA activity. Following the presumed negative substances are those without relevant in vitro AR TA data. Substances lacking either quantitative or qualitative data have been assigned a presumed positive or negative response in in vitro AR TA assays, based on the substances’ anticipated or known mechanism of action and response in in vitro AR binding assays. Presumed positive substances are listed first, followed by presumed negative substances that have been selected for the minimal list of substances (see below and Section 2.4.4). Both categories are sorted alphabetically by substance name. The remaining substances that are presumed negative are sorted alphabetically at the end of the list.

1Inclusion of a substance does not mean that EPA, NICEATM, ICCVAM, or the Expert Panel has or will make a determination that any use of the substance will pose a significant risk. Further, these substances should not be interpreted to be “endocrine disruptors”; the substances listed are simply compounds that have been or may prove to be useful in developing, standardizing, or validating screening and testing methods.
Substances have been classified as presumed positive for agonism even when less than 50% of the studies were positive. Without detailed information regarding the experimental protocol used, it is not possible to assess the quality of the data. However, with the AR TA agonism tests, false positive responses are possible if the cell line used in the study contains a glucocorticoid or progesterone receptor and the mouse mammary tumor virus hormone response element is incorporated into the reporter construct. The classification of a substance as positive (and its ranking) or negative in this list is based sometimes on the results of a single study and, therefore, the accuracy of the classification is questionable. However, it is anticipated that testing these presumed positive and negative substances will provide critical information on the comparative sensitivity and reproducibility of different in vitro AR TA assays, when such assays are standardized and conducted using the recommended minimum procedural standards.

The quantitative and qualitative data provided with this substance list summarize information obtained primarily from peer-reviewed scientific reports. Because the positive data were obtained from studies using different in vitro AR TA assays, they show a great deal of variability and, thus, the reported values should not be used as definitive target values to be obtained during the validation process. The data summaries presented in Tables 6-1 and 6-2 are provided to inform interested investigators of the historical quantitative values obtained for these substances in in vitro AR TA assays.

As described in Section 2.4.4, and mentioned above, a subset of 44 substances has been identified that, at a minimum, should be used in any validation of in vitro AR TA assays. These 44 substances are in bold type in Table 6-1 for agonism. Of these substances, 45% (20) are classified as positive (15) or presumed positive (5) for AR agonism, and 55% (24) are classified as presumed negative. The same 44 substances are in bold type in Table 6-2 for antagonism. Of these substances, 45% (20) are classified as positive (16) or presumed positive (4) for AR antagonism, and 55% (24) are classified as presumed negative.
### Table 6-1: ICCVAM Recommended Substances for Validation of *In Vitro* AR TA Agonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Median EC₅₀ Value and Range Across All MCRG Studies (µM)</th>
<th>Qualitative Response for AR Agonism Across All MCRG Studies</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Completed/Anticipated <em>In Vivo</em> Testing</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>5α-Dihydrotestosterone e</td>
<td>521-18-6</td>
<td>0.00015 (3) 0.000004 - 0.000153</td>
<td>Pos. (21/21)</td>
<td>13</td>
<td>H</td>
<td>Strong AR agonist; Weak ER agonist</td>
<td>Steroid, nonphenolic</td>
</tr>
<tr>
<td>Testosterone e</td>
<td>58-22-0</td>
<td>0.0002 (3) 0.000107 - 0.000527</td>
<td>Pos. (11/11)</td>
<td>10</td>
<td>IM</td>
<td>Strong AR agonist</td>
<td>Steroid, nonphenolic</td>
</tr>
<tr>
<td>Methyl testosterone e</td>
<td>58-18-4</td>
<td>0.00081 (2) 0.0000274 - 0.000135</td>
<td>Pos. (2/2)</td>
<td>2</td>
<td>H; 407; M-PA; IUL; FRS</td>
<td>AR and ER agonist</td>
<td>Steroid, nonphenolic; Androstene</td>
</tr>
<tr>
<td>4-Androstenedione e</td>
<td>63-05-8</td>
<td>0.0015 (2) 0.000645 - 0.00242</td>
<td>Pos. (3/3)</td>
<td>3</td>
<td>AR agonist and antagonist</td>
<td>Steroid, nonphenolic</td>
<td>Aragonist</td>
</tr>
<tr>
<td>Mifepristone e</td>
<td>84371-65-3</td>
<td>0.014 (1)</td>
<td>Pos. (3/4)</td>
<td>4</td>
<td>IM</td>
<td>AR agonist and antagonist</td>
<td>Steroid, nonphenolic; Estrene</td>
</tr>
<tr>
<td>Estrone e</td>
<td>53-16-7</td>
<td>0.055 (1)</td>
<td>Pos. (2/2)</td>
<td>2</td>
<td>AR agonist; Strong ER agonist</td>
<td>Steroid, phenolic; Estrene</td>
<td></td>
</tr>
</tbody>
</table>

**QUALITATIVE DATA ONLY**

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Qualitative Response for AR Agonism Across All MCRG Studies</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Completed/Anticipated <em>In Vivo</em> Testing</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-Estradiol e,g</td>
<td>50-28-2</td>
<td>Pos. (10/11)</td>
<td>9</td>
<td>IM; IUL; FRS</td>
<td>AR agonist and antagonist; Strong ER agonist</td>
<td>Steroid, phenolic; Estrene</td>
</tr>
<tr>
<td>Cyproterone acetate e,g</td>
<td>427-51-0</td>
<td>Pos. (8/8)</td>
<td>7</td>
<td>IM</td>
<td>AR agonist and antagonist</td>
<td>Nitrile; Diphenyl ether; Organochlorine</td>
</tr>
<tr>
<td><em>Methyltrienolone (R1881)</em> e,g</td>
<td>965-93-5</td>
<td>Pos. (8/8)</td>
<td>5</td>
<td>AR agonist; <em>Recommended reference androgen</em></td>
<td>Steroid, nonphenolic; Estrene</td>
<td></td>
</tr>
<tr>
<td>Progesterone e,g</td>
<td>57-83-0</td>
<td>Pos. (7/9)</td>
<td>7</td>
<td>IM</td>
<td>AR agonist and antagonist</td>
<td>Steroid, nonphenolic; Pregnenedione</td>
</tr>
<tr>
<td>Hydroxyflutamide e,g</td>
<td>52806-53-8</td>
<td>Pos. (5/6)</td>
<td>4</td>
<td>AR agonist and antagonist</td>
<td>Steroid, nonphenolic; Nitrobenzene</td>
<td></td>
</tr>
</tbody>
</table>
Table 6-1: ICCVAM Recommended Substances for Validation of *In Vitro* AR TA Agonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems\(^a\) (continued)

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Median EC(_{50}) Value and Range Across All MCRG Studies(^b) (µM)</th>
<th>Qualitative Response for AR Agonism Across All MCRG Studies(^c)</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Completed/ Anticipated In Vivo Testing(^d)</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medroxyprogesterone acetate(^e,g)</td>
<td>71-58-9</td>
<td>Pos. (4/4)</td>
<td>Weak AR agonist</td>
<td>AR agonist</td>
<td>Weak AR agonist</td>
<td>Steroid, nonphenolic; Polycyclic hydrocarbon</td>
<td></td>
</tr>
<tr>
<td>Dexamethasone(^e,g)</td>
<td>50-02-2</td>
<td>Pos. (3/4)</td>
<td>AR agonist</td>
<td>AR agonist</td>
<td>AR agonist</td>
<td>Steroid, nonphenolic</td>
<td></td>
</tr>
<tr>
<td>Bicalutamide(^e,g)</td>
<td>90357-06-5</td>
<td>Pos. (2/2)</td>
<td>AR antagonist</td>
<td>AR agonist and antagonist</td>
<td>AR agonist and antagonist</td>
<td>Steroid, nonphenolic; Pregnen lactone</td>
<td></td>
</tr>
<tr>
<td>Spironolactone(^e,g)</td>
<td>52-01-7</td>
<td>Pos. (2/2)</td>
<td>Weak AR agonist and antagonist</td>
<td></td>
<td>Weak AR agonist and antagonist</td>
<td>Organochlorine; Diphenylalkene</td>
<td></td>
</tr>
<tr>
<td>p,p'-DDE(^e,g)</td>
<td>72-55-9</td>
<td>Pos. (2/3)</td>
<td>Weak AR agonist and antagonist</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluoxymestrone(^e,g)</td>
<td>76-43-7</td>
<td>Pos. (1/1)</td>
<td>Weak AR agonist</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linuron(^e,g)</td>
<td>330-55-2</td>
<td>Pos. (1/1)</td>
<td>Weak AR agonist and antagonist</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nilutamide(^e,g)</td>
<td>63612-50-0</td>
<td>Pos. (1/1)</td>
<td>AR antagonist</td>
<td></td>
<td></td>
<td>Heterocycle; Imidazole</td>
<td></td>
</tr>
<tr>
<td>Dibenzo[a,h]-anthracene(^e,g)</td>
<td>53-70-3</td>
<td>Pos. (1/1)</td>
<td>AR antagonist</td>
<td></td>
<td></td>
<td>Polycyclic aromatic hydrocarbon; Anthracene</td>
<td></td>
</tr>
<tr>
<td>Flutamide(^e,g)</td>
<td>13311-84-7</td>
<td>Neg. (5/5)</td>
<td>AR antagonist</td>
<td></td>
<td></td>
<td>Amide; Anilide; Nitrobenzene</td>
<td></td>
</tr>
<tr>
<td>Diethylstilbestrol(^e,g)</td>
<td>56-53-1</td>
<td>Neg. (2/2)</td>
<td>Strong ER agonist</td>
<td></td>
<td></td>
<td>Stilbene; Benzylidene; Diphenylalkene</td>
<td></td>
</tr>
<tr>
<td>Kepone(^e,g) (Chlordecone)</td>
<td>143-50-0</td>
<td>Neg. (2/2)</td>
<td>Binds to AR and ER</td>
<td></td>
<td></td>
<td>Organochlorine; Chlorinated bridged cycloalkane</td>
<td></td>
</tr>
</tbody>
</table>
Table 6-1: ICCVAM Recommended Substances for Validation of *In Vitro* AR TA Agonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems\(^a\) (continued)

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Median EC(_{50}) Value and Range Across All MCRG Studies(^b) (µM)</th>
<th>Qualitative Response for AR Agonism Across All MCRG Studies(^c)</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Completed/Anticipated <em>In Vivo</em> Testing(^d)</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrazine (^e,g)</td>
<td>1912-24-9</td>
<td>Neg. (1/1)</td>
<td></td>
<td>1</td>
<td>M-PA; IUL</td>
<td>Weak ER agonist</td>
<td>Aromatic amine; Triazine; Arylamine</td>
</tr>
<tr>
<td>Bisphenol A (^e,g)</td>
<td>80-05-7</td>
<td>Neg. (1/1)</td>
<td></td>
<td>1</td>
<td>U; F-PA; J(1G,F,A)</td>
<td>Weak ER agonist</td>
<td>Diphenylalkane; Bisphenol; Phenol</td>
</tr>
<tr>
<td>Corticosterone (^e,g)</td>
<td>50-22-6</td>
<td>Neg. (1/1)</td>
<td></td>
<td>1</td>
<td>Binds weakly to AR</td>
<td></td>
<td>Steroid, nonphenolic</td>
</tr>
<tr>
<td>(o,p')-DDT (^e,g)</td>
<td>789-02-6</td>
<td>Neg. (1/1)</td>
<td></td>
<td>1</td>
<td>U; J(1G,F,A)</td>
<td>Weak AR and ER antagonist; Weak ER agonist</td>
<td>Organochlorine; Diphenylalkane</td>
</tr>
<tr>
<td>Di-n-butyl phthalate (^e,g)</td>
<td>84-74-2</td>
<td>Neg. (1/1)</td>
<td></td>
<td>1</td>
<td>U; M-PA; 1G; J(U,H,1G,F,A)</td>
<td>ER agonist</td>
<td>Phthalate</td>
</tr>
<tr>
<td>Diethylhexyl phthalate (^e,g)</td>
<td>117-81-7</td>
<td>Neg. (1/1)</td>
<td></td>
<td>1</td>
<td>J(U,H,1G,F,A)</td>
<td></td>
<td>Phthalate</td>
</tr>
<tr>
<td>17α-Ethynyl estradiol (^e,g)</td>
<td>57-63-6</td>
<td>Neg. (1/1)</td>
<td></td>
<td>1</td>
<td>U; 407; F-PA</td>
<td>Strong ER agonist</td>
<td>Steroid, phenolic</td>
</tr>
<tr>
<td>Fluoranthene (^e,h)</td>
<td>206-44-0</td>
<td>Neg. (1/1)</td>
<td></td>
<td>1</td>
<td>AR antagonist</td>
<td>Polycyclic aromatic hydrocarbon; Fluorene</td>
<td></td>
</tr>
<tr>
<td>4-Hydroxytamoxifen (^e,g)</td>
<td>68047-06-3</td>
<td>Neg. (1/1)</td>
<td></td>
<td>1</td>
<td>ER antagonist</td>
<td>Triphenylethylene; Benzylidene; Stilbene; Phenol</td>
<td></td>
</tr>
<tr>
<td>(p,p')-Methoxychlor (^e,g)</td>
<td>72-43-5</td>
<td>Neg. (1/1)</td>
<td></td>
<td>1</td>
<td>U; F&amp;M-PA; IUL; IM; 2G(avian); FRS</td>
<td>AR antagonist; Weak ER agonist</td>
<td>Organochlorine; Chlorinated hydrocarbon</td>
</tr>
<tr>
<td>(p-n)-Nonylphenol (^e,g,j)</td>
<td>104-40-5</td>
<td>Neg. (1/1)</td>
<td></td>
<td>1</td>
<td>U; 407; J(U,H,1G,F,A)</td>
<td>AR and ER antagonist; ER agonist</td>
<td>Alkylphenol; Phenol</td>
</tr>
<tr>
<td>4-tert-Octylphenol (^e,g)</td>
<td>140-66-9</td>
<td>Neg. (1/1)</td>
<td></td>
<td>1</td>
<td>J(U,H,1G,F,B)</td>
<td>ER agonist</td>
<td>Alkylphenol; Phenol</td>
</tr>
</tbody>
</table>
Table 6-1: ICCVAM Recommended Substances for Validation of In Vitro AR TA Agonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems\(^a\) (continued)

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Median EC(_{50}) Value and Range Across All MCRG Studies(^b) (µM)</th>
<th>Qualitative Response for AR Agonism Across All MCRG Studies(^c)</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Completed/Anticipated In Vivo Testing(^d)</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenobarbital(^e,g)</td>
<td>57-30-7</td>
<td>Neg. (1/1)</td>
<td></td>
<td>1</td>
<td>F&amp;M-PA; IM</td>
<td>Enhances thyroid hormone excretion</td>
<td>Heterocycle; Pyrimidine</td>
</tr>
<tr>
<td>Procymidone(^e,g)</td>
<td>32809-16-8</td>
<td>Neg. (1/1)</td>
<td></td>
<td>1</td>
<td>H</td>
<td>AR antagonist</td>
<td>Organochlorine; Cyclic imide</td>
</tr>
<tr>
<td>Vinclozolin(^e,g)</td>
<td>50471-44-8</td>
<td>Neg. (1/1)</td>
<td></td>
<td>1</td>
<td>H; M-PA; IM; IUL; 1G; FRS</td>
<td>AR antagonist</td>
<td>Organochlorine; Cyclic imide; Carbamate</td>
</tr>
<tr>
<td>Bisphenol B(^e,g)</td>
<td>77-40-7</td>
<td>Neg. (1/1)</td>
<td></td>
<td>1</td>
<td>IUL</td>
<td>ER agonist</td>
<td>Diphenylalkane; Bisphenol; Phenol</td>
</tr>
<tr>
<td>Butylbenzyl phthalate(^e,g)</td>
<td>85-68-7</td>
<td>Neg. (1/1)</td>
<td></td>
<td>1</td>
<td>IUL</td>
<td>ER agonist</td>
<td>Phthalate</td>
</tr>
<tr>
<td>Coumestrol(^e,g)</td>
<td>479-13-0</td>
<td>Neg. (1/1)</td>
<td></td>
<td>1</td>
<td>IM</td>
<td>ER agonist</td>
<td>Coumestan; Benzopyranone; Coumarin; Ketone</td>
</tr>
<tr>
<td>4-Cumylphenol(^e,g)</td>
<td>599-64-4</td>
<td>Neg. (1/1)</td>
<td></td>
<td>1</td>
<td></td>
<td>Weak ER agonist</td>
<td>Phenol</td>
</tr>
<tr>
<td>17α-Estradiol(^e,g)</td>
<td>57-91-0</td>
<td>Neg. (1/1)</td>
<td></td>
<td>1</td>
<td></td>
<td>ER agonist</td>
<td>Steroid, phenolic; Estrene</td>
</tr>
<tr>
<td>Tamoxifen(^e,g)</td>
<td>10540-29-1</td>
<td>Neg. (1/1)</td>
<td></td>
<td>1</td>
<td></td>
<td>ER antagonist</td>
<td>Triphenylethylene; Benzylidene; Stilbene</td>
</tr>
<tr>
<td>Zearalenone(^e,g)</td>
<td>17924-92-4</td>
<td>Neg. (1/1)</td>
<td></td>
<td>1</td>
<td></td>
<td>ER agonist</td>
<td>Resorcylic acid lactone; Phenol</td>
</tr>
</tbody>
</table>

ANTICIPATED RESPONSES (No EC\(_{50}\) or Qualitative Agonism Data Available)

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Qualitative Response</th>
<th>No. of MCRG Assays</th>
<th>Completed/Anticipated In Vivo Testing</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketoconazole</td>
<td>65277-42-1</td>
<td>Pos.</td>
<td>1</td>
<td>Pos. for AR agonism in yeast assay</td>
<td>Imidazole; Piperazine</td>
<td></td>
</tr>
</tbody>
</table>
### Table 6-1: ICCVAM Recommended Substances for Validation of In Vitro AR TA Agonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems * (continued)

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Median EC&lt;sub&gt;50&lt;/sub&gt; Value and Range Across All MCRG Studies a (µM)</th>
<th>Qualitative Response for AR Agonism Across All MCRG Studies b</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Completed/Anticipated In Vivo Testing c</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-Trenbolone</td>
<td>10161-33-8</td>
<td>Pos.</td>
<td></td>
<td>H</td>
<td>Binds strongly to AR</td>
<td>Steroid, nonphenolic; Estrene</td>
<td></td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>50-76-0</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>RNA synthesis inhibitor</td>
<td>Phenoxazone; Lactone; Peptide</td>
<td></td>
</tr>
<tr>
<td>Fadrozole</td>
<td>102676-47-1</td>
<td>Neg.</td>
<td></td>
<td>F-PA; IM; FRS</td>
<td>Aromatase inhibitor</td>
<td>Imidazole; Nitrile</td>
<td></td>
</tr>
<tr>
<td>Finasteride</td>
<td>98319-26-7</td>
<td>Neg.</td>
<td></td>
<td>H; M-PA; IM</td>
<td>5α-reductase inhibitor; Neg. for AR agonism in yeast assay</td>
<td>Steroid, nonphenolic; Androstene</td>
<td></td>
</tr>
<tr>
<td>Sodium azide</td>
<td>26628-22-8</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>Cytotoxic</td>
<td>Organic salt; Azide</td>
<td></td>
</tr>
<tr>
<td>12-O-Tetradecanoyl-phorbol-13-acetate</td>
<td>16561-29-8</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>Activates ligand independent cell division</td>
<td>Phorbol ester; Terpene</td>
<td></td>
</tr>
<tr>
<td>2,4,5-Trichlorophenoxy-acetic acid</td>
<td>93-76-5</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>Weak ER agonist</td>
<td>Organochlorine; Chlorinated aromatic hydrocarbon</td>
<td></td>
</tr>
<tr>
<td>Ammonium perchlorate</td>
<td>7790-98-9</td>
<td>Neg.</td>
<td></td>
<td>IUL</td>
<td>Thyroid disruptor</td>
<td>Organic acid; Organic salt</td>
<td></td>
</tr>
<tr>
<td>Anastrazole</td>
<td>120511-73-1</td>
<td>Neg.</td>
<td></td>
<td>IM</td>
<td>Aromatase inhibitor; Neg. for AR agonism in yeast assay</td>
<td>Nitrile; Triazole</td>
<td></td>
</tr>
<tr>
<td>Apigenin</td>
<td>520-36-5</td>
<td>Neg.</td>
<td></td>
<td>IUL</td>
<td>ER agonist</td>
<td>Flavanoid; Flavone; Phenol</td>
<td></td>
</tr>
<tr>
<td>Apomorphine</td>
<td>58-00-4</td>
<td>Neg.</td>
<td></td>
<td>IM</td>
<td>Dopamine D1/D2 receptor agonist</td>
<td>Heterocycle; Quinoline</td>
<td></td>
</tr>
</tbody>
</table>
Table 6-1: ICCVAM Recommended Substances for Validation of *In Vitro* AR TA Agonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Median EC_{50} Value and Range Across All MCRG Studies^b (µM)</th>
<th>Qualitative Response for AR Agonism Across All MCRG Studies^c</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Completed/Anticipated In Vivo Testing^d</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-sec-Butylphenol</td>
<td>89-72-5</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Phenol</td>
</tr>
<tr>
<td>CGS 18320B</td>
<td>112808-99-8</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Aromatase inhibitor</td>
<td>Nitrile; Imidazole</td>
</tr>
<tr>
<td>Clomiphene citrate</td>
<td>50-41-9</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Binds to the ER</td>
<td>Chlorinated triphenylethylene; Benzyldiene; Stilbene</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>66-81-9</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Protein synthesis inhibitor</td>
<td>Piperidine; Glutaramide</td>
</tr>
<tr>
<td>Daidzein</td>
<td>486-66-8</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Weak ER agonist</td>
<td>Flavanoid; Isoflavone; Phenol</td>
</tr>
<tr>
<td>Ethyl paraben</td>
<td>120-47-8</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Binds weakly to ER</td>
<td>Paraben; Organic acid</td>
</tr>
<tr>
<td>Fenarimol</td>
<td>60168-88-9</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Aromatase inhibitor</td>
<td>Heterocycle; Pyrimidine</td>
</tr>
<tr>
<td>Flavone</td>
<td>525-82-6</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>M-PA; IM</td>
<td>Flavanoid; Flavone</td>
</tr>
<tr>
<td>Genistein</td>
<td>446-72-0</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Weak ER agonist and antagonist</td>
<td>Flavanoid; Isoflavone; Phenol</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>52-86-8</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Dopamine D2 receptor antagonist</td>
<td>Butyrophenone; Ketone; Piperazine</td>
</tr>
<tr>
<td>meso-Hexestrol</td>
<td>84-16-2</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Strong ER agonist</td>
<td>Diphenylalkane; Bisphenol; Phenol</td>
</tr>
<tr>
<td>ICI 182,780</td>
<td>129453-61-8</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>ER antagonist; Neg. for AR agonism in yeast assay</td>
<td>Steroid, phenolic</td>
</tr>
</tbody>
</table>

^a (continued)
### Table 6-1: ICCVAM Recommended Substances for Validation of *In Vitro* AR TA Agonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Median EC$_{50}$ Value and Range Across All MCRG Studies $^b$ ($\mu$M)</th>
<th>Qualitative Response for AR Agonism Across All MCRG Studies $^c$</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Completed/Anticipated <em>In Vivo</em> Testing $^d$</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaempferol</td>
<td>520-18-3</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>ER agonist</td>
<td>Flavanoid; Flavone; Phenol</td>
<td></td>
</tr>
<tr>
<td>Morin</td>
<td>480-16-0</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>Binds weakly to ER</td>
<td>Flavanoid; Flavone; Phenol</td>
<td></td>
</tr>
<tr>
<td>Norethynodrel</td>
<td>68-23-5</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>Binds to ER</td>
<td>Steroid, nonphenolic; Norpregnene</td>
<td></td>
</tr>
<tr>
<td>Oxazepam</td>
<td>604-75-1</td>
<td>Neg.</td>
<td>IM</td>
<td></td>
<td></td>
<td>Enhances thyroid hormone excretion</td>
<td>Benzodiazepine</td>
</tr>
<tr>
<td>Phenolphthalin</td>
<td>81-90-3</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Triphenylmethane; Diphenylalkane carboxylic acid</td>
</tr>
<tr>
<td>Pimozide</td>
<td>2062-78-4</td>
<td>Neg.</td>
<td>F&amp;M-PA</td>
<td></td>
<td></td>
<td>Dopamine receptor antagonist</td>
<td>Piperidine; Benzimidazole</td>
</tr>
<tr>
<td>Propylthiouracil</td>
<td>51-52-5</td>
<td>Neg.</td>
<td>407; F&amp;M-PA; IM; IUL; 2G</td>
<td></td>
<td></td>
<td>Inhibits T3/T4 synthesis</td>
<td>Pyrimidine; Uracil</td>
</tr>
<tr>
<td>Reserpine</td>
<td>50-55-5</td>
<td>Neg.</td>
<td>IM</td>
<td></td>
<td></td>
<td>Depletes dopamine</td>
<td>Heterocycle; Yohimbine</td>
</tr>
<tr>
<td>L-Thyroxine</td>
<td>51-48-9</td>
<td>Neg.</td>
<td>407</td>
<td></td>
<td></td>
<td>Thyroid hormone</td>
<td>Aromatic amino acid</td>
</tr>
</tbody>
</table>

*Abbreviations: AR = Androgen receptor; CASRN = Chemical Abstracts Service Registry Number; D1 and D2 = Two major families of dopamine receptors; DDE = 1,1-Dichloro-bis[4-chlorophenyl]ethylene; DDT = Dichlorodiphenyltrichloroethane; ER = Estrogen receptor; HDT = Highest dose tested; Neg. = Negative; Pos. = Positive; RBA = Relative binding affinity; T3 = Triiodothyronine; T4 = Thyroxine; TA = Transcriptional activation.*

*Substances in bold type are those that, at a minimum, are recommended for inclusion in future validation studies. Empty cells indicate that no relevant data were identified.*

*An EC$_{50}$ is the effective concentration of the test substance that elicits 50% of the maximum response in a particular test system. Median EC$_{50}$ values are derived from *in vitro* mammalian cell reporter gene studies that were either published in the peer-reviewed scientific literature or submitted to NICEATM.*
Table 6-1: ICCVAM Recommended Substances for Validation of In Vitro AR TA Agonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems

<table>
<thead>
<tr>
<th>Substance</th>
<th>Median EC$_{50}$ Value</th>
<th>Qualitative Response</th>
<th>Number of Mammalian Cell Reporter Gene Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1881</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Note: R1881 is the recommended reference androgen for in vitro AR binding and TA assays and, thus, is not considered a test substance for validation purposes (refer to Section 6.2 for more information).*

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*Numbers in parentheses refer to the number of studies in which the substance was reported positive or negative compared to the number of studies in which it was tested. A substance is classified as “positive” for AR agonism if it was positive in more than 50% of reported studies. A substance is classified as “presumed positive” for AR agonism if it was positive in 50% or less of reported studies, or if it was reported positive in the single study conducted. Substances reported negative in their respective studies are classified as “presumed negative” instead of “negative” for AR agonism, since they had not been tested in multiple studies at or above the limit dose of 1 mM recommended in Section 6.1.3. Substances without data are classified “presumed positive” or “presumed negative” based on available information, including their known mechanism of action or their responses in AR binding assays, ER binding assays, or ER TA assays.*

*Several in vivo test methods are undergoing further development or validation by OECD, EPA, and the JME (J). Substances indicated are proposed for testing by OECD in the Uterotrophic assay (U), the Hershberger assay (H), or the 407 protocol (407); for testing by EPA in the female pubertal assay (F-PA), the male pubertal assay (M-PA), the intact male assay (IM), a one-generation assay (1G), a two-generation assay (2G), or a fish reproductive screen (FRS); and for testing by JME in the U, H, and 1G assays, or various fish (F) and avian (A) assays. Due to the lack of CASRN for the JME studies, some of the indicated substances might not be the same substance included in this list. The in utero through lactation assay (IUL) has been recommended, but EPA has not made a decision on its further development or validation.*

*Information for this substance regarding its median EC$_{50}$ value (if available), its qualitative response in mammalian cell reporter gene assays, and the number of mammalian cell reporter gene assays in which it was tested was derived from data presented in Appendix D of the NICEATM AR TA BRD cited in footnote b. This BRD contains AR transcriptional activation data from the published literature through January 25, 2002.*

*No EC$_{50}$ data available for this substance.*

*Information for this substance was abstracted from a publication that was published or reviewed after the literature search was completed for the NICEATM AR TA BRD (i.e., Vinggaard et al. 2000 in Section 7.0).*

*Two forms of p-nonylphenol are available for testing. One form consists of a mixture of various branched isomers (CASRN 84852-15-3), while the other contains only one isomer consisting of a linear alkyl chain (CASRN 104-40-5). ICCVAM recommends the linear form, which has a uniform chemical structure, for validation studies.*

*The classification for this substance is “presumed positive” for AR agonism since the substance was positive in 50% or less of reported studies, or was reported positive in the single study conducted.*
### Table 6-2: ICCVAM Recommended Substances for Validation of *In Vitro* AR TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Median EC$_{50}$ Value and Range Across All MCRG Studies (µM)</th>
<th>Qualitative Response for AR Antagonism Across All MCRG Studies</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Completed/Anticipated In Vivo Testing</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mifepristone</td>
<td>84371-65-3</td>
<td>0.05 (1)</td>
<td>Pos. (2/2)</td>
<td>2</td>
<td>IM</td>
<td>AR agonist and antagonist</td>
<td>Steroid, nonphenolic; Estrene</td>
</tr>
<tr>
<td>Cyproterone acetate</td>
<td>427-51-0</td>
<td>0.1 (5) 0.01 - 45</td>
<td>Pos. (6/6)</td>
<td>5</td>
<td>IM</td>
<td>AR agonist and antagonist</td>
<td>Nitrile; Diphenyl ether; Organochlorine</td>
</tr>
<tr>
<td>Hydroxyflutamide</td>
<td>52806-53-8</td>
<td>0.1 (6) 0.01 - 45</td>
<td>Pos. (11/11)</td>
<td>7</td>
<td>IM</td>
<td>AR agonist and antagonist</td>
<td>Amide; Anilide; Nitrobenzene</td>
</tr>
<tr>
<td>Vinlozolin</td>
<td>50471-44-8</td>
<td>0.28 (2) 0.05 - 0.5</td>
<td>Pos. (3/3)</td>
<td>3</td>
<td>IM</td>
<td>AR antagonist</td>
<td>Organochlorine; Cyclic imide; Carbamate</td>
</tr>
<tr>
<td>Spironolactone</td>
<td>52-01-7</td>
<td>0.3 (2) 0.09 - 0.5</td>
<td>Pos. (2/3)</td>
<td>3</td>
<td>IM</td>
<td>AR agonist and antagonist</td>
<td>Steroid, nonphenolic; pregnenedione</td>
</tr>
<tr>
<td>Nilutamide</td>
<td>63612-50-0</td>
<td>0.3 (3) 0.15 - 10</td>
<td>Pos. (3/3)</td>
<td>2</td>
<td>IM</td>
<td>AR antagonist</td>
<td>Heterocycle; Imidazole</td>
</tr>
<tr>
<td>Progesterone</td>
<td>57-83-0</td>
<td>0.3 (2) 0.1 - 0.5</td>
<td>Pos. (3/3)</td>
<td>3</td>
<td>IM</td>
<td>AR agonist and antagonist</td>
<td>Steroid, nonphenolic; pregnenedione</td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>56-53-1</td>
<td>0.36 (1)</td>
<td>Pos. (2/2)</td>
<td>2</td>
<td>IUL</td>
<td>Strong ER agonist</td>
<td>Stilbene; Benzylidine; Diphenylalkene</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>50-28-2</td>
<td>0.5 (3) 0.05 - 1</td>
<td>Pos. (5/5)</td>
<td>4</td>
<td>IM; IUL; FRS</td>
<td>AR agonist and antagonist</td>
<td>Steroid, phenolic; Estrene</td>
</tr>
<tr>
<td>Bicalutamide</td>
<td>90357-06-5</td>
<td>0.63 (4) 0.5 - 18</td>
<td>Pos. (5/5)</td>
<td>4</td>
<td>IM; IUL; FRS</td>
<td>Strong ER agonist</td>
<td>Steroid, phenolic; Estrene</td>
</tr>
<tr>
<td>Bisphenol A</td>
<td>80-05-7</td>
<td>1 (1)</td>
<td>Pos. (1/2)</td>
<td>2</td>
<td>U; F-PA; J(1G,F,B)</td>
<td>Weak ER agonist</td>
<td>Diphenylalkane; Bisphenol; Phenol</td>
</tr>
<tr>
<td>p,p'-DDE</td>
<td>72-55-9</td>
<td>3 (4) 0.75 - 15.2</td>
<td>Pos. (7/7)</td>
<td>5</td>
<td>IM</td>
<td>Weak AR agonist and antagonist</td>
<td>Organochlorine; Diphenylalkene</td>
</tr>
</tbody>
</table>
### Table 6-2: ICCVAM Recommended Substances for Validation of In Vitro AR TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Median EC_{50} Value and Range Across All MCRG Studies</th>
<th>Qualitative Response for AR Antagonism Across All MCRG Studies</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Completed/ Anticipated In Vivo Testing</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-tert-Octylphenol</td>
<td>140-66-9</td>
<td>3 (1)</td>
<td>Pos. (1/1)</td>
<td>1</td>
<td>J(U,H,1G,F,B)</td>
<td>ER agonist</td>
<td>Alkylphenol; Phenol</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>206-44-0</td>
<td>4.6 (1)</td>
<td>Pos. (1/1)</td>
<td>1</td>
<td>AR antagonist</td>
<td>Polycyclic aromatic hydrocarbon; Fluorene</td>
<td></td>
</tr>
<tr>
<td>Linuron</td>
<td>330-55-2</td>
<td>5 (3) 5 - 10</td>
<td>Pos. (3/3)</td>
<td>2</td>
<td>H; M-PA</td>
<td>Weak AR agonist and antagonist</td>
<td>Urea</td>
</tr>
<tr>
<td>Kepone</td>
<td>143-50-0</td>
<td>6.9 (1)</td>
<td>Pos. (1/2)</td>
<td>2</td>
<td>Binds to AR and ER</td>
<td>Organochlorine; Chlorinated bridged cycloalkane</td>
<td></td>
</tr>
<tr>
<td>Procymidone</td>
<td>32809-16-8</td>
<td>7.5 (2) 5 - 10</td>
<td>Pos. (2/2)</td>
<td>2</td>
<td>H</td>
<td>AR antagonist</td>
<td>Organochlorine; Cyclic imide</td>
</tr>
<tr>
<td>Fenarimol</td>
<td>60168-88-9</td>
<td>15</td>
<td>Pos. (1/1)</td>
<td>1</td>
<td>F-PA</td>
<td>Aromatase inhibitor</td>
<td>Heterocycle; Pyrimidine</td>
</tr>
<tr>
<td>Flutamide</td>
<td>13311-84-7</td>
<td>Pos. (3/3)</td>
<td>3</td>
<td>H; 407; M-PA; IM; FRS</td>
<td>AR agonist</td>
<td>Amide; Anilide; Nitrobenzene</td>
<td></td>
</tr>
<tr>
<td>o,p'-DDT</td>
<td>789-02-6</td>
<td>Pos. (2/2)</td>
<td>2</td>
<td>U; J(1G,F,B)</td>
<td>Weak AR and ER antagonist; Weak ER agonist</td>
<td>Organochlorine; Diphenylalkane</td>
<td></td>
</tr>
<tr>
<td>p,p'-Methoxychlor</td>
<td>72-43-5</td>
<td>Pos. (2/2)</td>
<td>1</td>
<td>U; F&amp;M-PA; IUL; IM; FRS; 2G(avian)</td>
<td>AR antagonist; Weak ER agonist</td>
<td>Organochlorine; Chlorinated hydrocarbon</td>
<td></td>
</tr>
<tr>
<td>Atrazine</td>
<td>1912-24-9</td>
<td>Neg. (1/1)</td>
<td>1</td>
<td>M-PA; IUL</td>
<td></td>
<td>Aromatic amine; Triazine; Arylamine</td>
<td></td>
</tr>
<tr>
<td>Fluoxymestrone</td>
<td>76-43-7</td>
<td>Neg. (1/1)</td>
<td>1</td>
<td></td>
<td>Weak AR agonist</td>
<td>Steroid, nonphenolic</td>
<td></td>
</tr>
<tr>
<td>Medroxyprogesterone acetate</td>
<td>71-58-9</td>
<td>Neg. (1/1)</td>
<td>1</td>
<td></td>
<td>Weak AR agonist</td>
<td>Steroid, nonphenolic; Polycyclic hydrocarbon</td>
<td></td>
</tr>
</tbody>
</table>
### Table 6-2: ICCVAM Recommended Substances for Validation of *In Vitro* AR TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems\(^a\) (continued)

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Median EC(_{50}) Value and Range Across All MCRG Studies(^b) (µM)</th>
<th>Qualitative Response for AR Antagonism Across All MCRG Studies(^c)</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Completed/Anticipated <em>In Vivo</em> Testing(^d)</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Methyltrienolone</em> (R1881)(^e,g,h)</td>
<td>965-93-5</td>
<td>Neg. (1/1)</td>
<td>1</td>
<td></td>
<td>AR agonist; <em>Recommended reference androgen</em></td>
<td>Steroid, nonphenolic; Estrene</td>
<td></td>
</tr>
<tr>
<td><em>Testosterone</em>(^e,g)</td>
<td>58-22-0</td>
<td>Neg. (1/1)</td>
<td>1</td>
<td>IM</td>
<td>Strong AR agonist</td>
<td>Steroid, nonphenolic</td>
<td></td>
</tr>
<tr>
<td>Butylbenzyl phthalate(^e,g)</td>
<td>85-68-7</td>
<td>Neg. (1/1)</td>
<td>1</td>
<td>IUL</td>
<td>ER agonist</td>
<td>Phthalate</td>
<td></td>
</tr>
</tbody>
</table>

**ANTICIPATED RESPONSES (No IC\(_{50}\) or Qualitative Antagonism Data Available)**

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Qualitative Response</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Actinomycin D</em></td>
<td>50-76-0</td>
<td>Neg.</td>
<td></td>
<td>RNA synthesis inhibitor</td>
<td>Phenoxazone; Lactone; Peptide</td>
</tr>
<tr>
<td><em>4-Androstenedione</em></td>
<td>63-05-8</td>
<td>Neg.</td>
<td></td>
<td>Strong AR agonist</td>
<td>Steroid, nonphenolic</td>
</tr>
<tr>
<td><em>Corticosterone</em></td>
<td>50-22-6</td>
<td>Neg.</td>
<td></td>
<td>Binds weakly to AR</td>
<td>Steroid, nonphenolic</td>
</tr>
<tr>
<td><em>Dexamethasone</em></td>
<td>50-02-2</td>
<td>Neg.</td>
<td></td>
<td>AR agonist</td>
<td>Steroid, nonphenolic</td>
</tr>
<tr>
<td><em>Di-n-butyl phthalate</em></td>
<td>84-74-2</td>
<td>Neg.</td>
<td>U; M-PA; IG; J(U,H,1G,F,B)</td>
<td>ER agonist</td>
<td>Phthalate</td>
</tr>
<tr>
<td><em>Diethylhexyl phthalate</em></td>
<td>117-81-7</td>
<td>Neg.</td>
<td>J(U,H,1G,F,B)</td>
<td></td>
<td>Phthalate</td>
</tr>
<tr>
<td><em>5α-Dihydrotestosterone</em></td>
<td>521-18-6</td>
<td>Neg.</td>
<td>H</td>
<td>Weak ER agonist; Strong AR agonist</td>
<td>Steroid, nonphenolic</td>
</tr>
<tr>
<td><em>Estrone</em></td>
<td>53-16-7</td>
<td>Neg.</td>
<td></td>
<td>Strong ER agonist; R agonist</td>
<td>Steroid, phenolic; Estrene</td>
</tr>
<tr>
<td><em>17α-Ethinyl estradiol</em></td>
<td>57-63-6</td>
<td>Neg.</td>
<td>U; 407; F-PA</td>
<td>Strong ER agonist</td>
<td>Steroid, phenolic</td>
</tr>
<tr>
<td>Substance</td>
<td>CASRN</td>
<td>Median EC&lt;sub&gt;50&lt;/sub&gt; Value and Range Across All MCRG Studies&lt;sup&gt;b&lt;/sup&gt; (µM)</td>
<td>Qualitative Response for AR Antagonism Across All MCRG Studies&lt;sup&gt;c&lt;/sup&gt;</td>
<td>No. of MCRG Assays in Which Tested</td>
<td>Completed/Anticipated &lt;em&gt;In Vivo&lt;/em&gt; Testing&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>------------------------</td>
<td>------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------</td>
<td>------------------------------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Fadrozole</td>
<td>102676-47-1</td>
<td>Neg.</td>
<td>F-PA; IM; FRS</td>
<td></td>
<td>Aromatase inhibitor</td>
</tr>
<tr>
<td>Finasteride</td>
<td>98319-26-7</td>
<td>Neg.</td>
<td>H; M-PA; IM</td>
<td>5α-reductase inhibitor</td>
<td></td>
</tr>
<tr>
<td>4-Hydroxytamoxifen</td>
<td>68047-06-3</td>
<td>Neg.</td>
<td></td>
<td>ER antagonist</td>
<td></td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>65277-42-1</td>
<td>Neg.</td>
<td>F&amp;M-PA; IM</td>
<td></td>
<td>Weak AR agonist; Neg. for AR antagonism in yeast assay</td>
</tr>
<tr>
<td>Methyl testosterone</td>
<td>58-18-4</td>
<td>Neg.</td>
<td>H; 407; M-PA; IUL; FRS</td>
<td>AR and ER agonist</td>
<td></td>
</tr>
<tr>
<td>&lt;sup&gt;p&lt;/sup&gt;-n-Nonylphenol&lt;sup&gt;f&lt;/sup&gt;</td>
<td>104-40-5</td>
<td>Neg.</td>
<td>U; 407; J(U,H,1G,F,B)</td>
<td>ER agonist and antagonist; Neg. for AR antagonism in yeast assay</td>
<td></td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>57-30-7</td>
<td>Neg.</td>
<td>F&amp;M-PA; IM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium azide</td>
<td>26628-22-8</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>Cytotoxic</td>
</tr>
<tr>
<td>12-O-Tetradecanoylphorbol-13-acetate</td>
<td>16561-29-8</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>Activates ligand independent cell division</td>
</tr>
<tr>
<td>17β-Trenbolone</td>
<td>10161-33-8</td>
<td>Neg.</td>
<td>H</td>
<td>Binds strongly to the AR</td>
<td></td>
</tr>
<tr>
<td>2,4,5-Trichlorophenoxyacetic acid</td>
<td>93-76-5</td>
<td>Neg.</td>
<td></td>
<td></td>
<td>Weak ER agonist</td>
</tr>
</tbody>
</table>
### Table 6-2: ICCVAM Recommended Substances for Validation of In Vitro AR TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems\(^a\) (continued)

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Median EC(_{50}) Value and Range Across All MCRG Studies(^b) (µM)</th>
<th>Qualitative Response for AR Antagonism Across All MCRG Studies(^c)</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Completed/ Anticipated In Vivo Testing(^d)</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium perchlorate</td>
<td>7790-98-9</td>
<td>Neg.</td>
<td></td>
<td>IUL</td>
<td>Thyroid disruptor</td>
<td>Organic acid; Organic salt</td>
<td></td>
</tr>
<tr>
<td>Anastrazole</td>
<td>120511-73-1</td>
<td>Neg.</td>
<td></td>
<td>IM</td>
<td>Aromatase inhibitor; Neg. for AR antagonism in yeast assay</td>
<td>Nitrile; Triazole</td>
<td></td>
</tr>
<tr>
<td>Apigenin</td>
<td>520-36-5</td>
<td>Neg.</td>
<td></td>
<td>IUL</td>
<td>ER agonist</td>
<td>Flavanoid; Flavone; Phenol</td>
<td></td>
</tr>
<tr>
<td>Apomorphine</td>
<td>58-00-4</td>
<td>Neg.</td>
<td></td>
<td>IM</td>
<td>Dopamine D1/D2 receptor agonist</td>
<td>Heterocycle; Quinoline</td>
<td></td>
</tr>
<tr>
<td>Bisphenol B</td>
<td>77-40-7</td>
<td>Neg.</td>
<td></td>
<td>ER agonist</td>
<td></td>
<td>Diphenylalkane; Bisphenol; Phenol</td>
<td></td>
</tr>
<tr>
<td>2-sec-Butylphenol</td>
<td>89-72-5</td>
<td>Neg.</td>
<td></td>
<td>ER agonist</td>
<td></td>
<td>Phenol</td>
<td></td>
</tr>
<tr>
<td>CGS 18320B</td>
<td>112808-99-8</td>
<td>Neg.</td>
<td></td>
<td>407</td>
<td>Aromatase inhibitor</td>
<td>Nitrile; Imidazole</td>
<td></td>
</tr>
<tr>
<td>Clomiphene citrate</td>
<td>50-41-9</td>
<td>Neg.</td>
<td></td>
<td>Binds to ER</td>
<td></td>
<td>Chlorinated triphenylethylene; Benzylidene; Stilbene</td>
<td></td>
</tr>
<tr>
<td>Coumestrol</td>
<td>479-13-0</td>
<td>Neg.</td>
<td></td>
<td>IM</td>
<td>ER agonist</td>
<td>Coumestan; Benzopyranone; Coumarin; Ketone</td>
<td></td>
</tr>
<tr>
<td>4-Cumylphenol</td>
<td>599-64-4</td>
<td>Neg.</td>
<td></td>
<td>Weak ER agonist</td>
<td></td>
<td>Phenol</td>
<td></td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>66-81-9</td>
<td>Neg.</td>
<td></td>
<td>Protein synthesis inhibitor</td>
<td></td>
<td>Piperidine; Glutaramide</td>
<td></td>
</tr>
<tr>
<td>Daidzein</td>
<td>486-66-8</td>
<td>Neg.</td>
<td></td>
<td>Weak ER agonist</td>
<td></td>
<td>Flavanoid; Isoflavone; Phenol</td>
<td></td>
</tr>
</tbody>
</table>
### Table 6-2: ICCVAM Recommended Substances for Validation of *In Vitro* AR TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Median EC$_{50}$ Value and Range Across All MCRG Studies$^b$ (µM)</th>
<th>Qualitative Response for AR Antagonism Across All MCRG Studies$^c$</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Completed/Anticipated In Vivo Testing$^d$</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dibenzo[a,h]anthracene</td>
<td>53-70-3</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Polycyclic aromatic hydrocarbon; Anthracene</td>
<td></td>
</tr>
<tr>
<td>17α-Estradiol</td>
<td>57-91-0</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>ER agonist</td>
<td>Steroid, phenolic; Estrene</td>
</tr>
<tr>
<td>Ethyl paraben</td>
<td>120-47-8</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Binds weakly to ER</td>
<td>Paraben; Organic acid</td>
</tr>
<tr>
<td>Flavone</td>
<td>525-82-6</td>
<td>Neg.</td>
<td>M-PA; IM</td>
<td></td>
<td></td>
<td>Weak ER antagonist</td>
<td>Flavanoid; Flavone</td>
</tr>
<tr>
<td>Genistein</td>
<td>446-72-0</td>
<td>Neg.</td>
<td>U; 407</td>
<td></td>
<td></td>
<td>Weak ER agonist and antagonist</td>
<td>Flavanoid; Isoflavone; Phenol</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>52-86-8</td>
<td>Neg.</td>
<td>IM</td>
<td></td>
<td></td>
<td>Dopamine D2 receptor antagonist</td>
<td>Butyrophenone; Ketone; Piperazine</td>
</tr>
<tr>
<td><em>meso</em>-Hexestrol</td>
<td>84-16-2</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Strong ER agonist</td>
<td>Diphenylalkane; Bisphenol; Phenol</td>
</tr>
<tr>
<td>ICI 182,780</td>
<td>129453-61-8</td>
<td>Neg.</td>
<td>IM</td>
<td></td>
<td></td>
<td>ER antagonist; Neg. for AR antagonism in yeast assay</td>
<td>Steroid, phenolic</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>520-18-3</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>ER agonist</td>
<td>Flavanoid; Flavone; Phenol</td>
</tr>
<tr>
<td>Morin</td>
<td>480-16-0</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Binds weakly to ER</td>
<td>Flavanoid; Flavone; Phenol</td>
</tr>
<tr>
<td>Norethynodrel</td>
<td>68-23-5</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Binds to ER</td>
<td>Steroid, nonphenolic; Norpregnene</td>
</tr>
<tr>
<td>Oxazepam</td>
<td>604-75-1</td>
<td>Neg.</td>
<td>IM</td>
<td></td>
<td></td>
<td>Enhances thyroid hormone excretion</td>
<td>Benzodiazepine</td>
</tr>
</tbody>
</table>

$^a$ (continued)
### Table 6-2: ICCVAM Recommended Substances for Validation of *In Vitro* AR TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems<sup>a</sup> (continued)

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Median EC&lt;sub&gt;50&lt;/sub&gt; Value and Range Across All MCRG Studies&lt;sup&gt;b&lt;/sup&gt; (µM)</th>
<th>Qualitative Response for AR Antagonism Across All MCRG Studies&lt;sup&gt;c&lt;/sup&gt;</th>
<th>No. of MCRG Assays in Which Tested</th>
<th>Completed/Anticipated <em>In Vivo</em> Testing&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Comments</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolphthalin</td>
<td>81-90-3</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Triphenylmethane; Diphenylalkane carboxylic acid</td>
</tr>
<tr>
<td>Pimozide</td>
<td>2062-78-4</td>
<td>Neg.</td>
<td>F&amp;M-PA</td>
<td>407; F&amp;M-PA; IM; IUL; 2G</td>
<td></td>
<td>Dopamine receptor antagonist</td>
<td>Piperidine; Benzimidazole</td>
</tr>
<tr>
<td>Propylthiouracil</td>
<td>51-52-5</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
<td>Inhibits T3/T4 synthesis</td>
<td>Pyrimidine; Uracil</td>
</tr>
<tr>
<td>Reserpine</td>
<td>50-55-5</td>
<td>Neg.</td>
<td>IM</td>
<td>Depletes dopamine</td>
<td></td>
<td></td>
<td>Heterocycle; Yohimban</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>10540-29-1</td>
<td>Neg.</td>
<td></td>
<td>ER antagonist</td>
<td></td>
<td></td>
<td>Triphenylethylene; Benzylidene; Stilbene</td>
</tr>
<tr>
<td>L-Thyroxine</td>
<td>51-48-9</td>
<td>Neg.</td>
<td></td>
<td>407</td>
<td></td>
<td>Thyroid hormone</td>
<td>Aromatic amino acid</td>
</tr>
<tr>
<td>Zearalenone</td>
<td>17924-92-4</td>
<td>Neg.</td>
<td></td>
<td>ER agonist</td>
<td></td>
<td></td>
<td>Resorcylic acid lactone; Phenol</td>
</tr>
</tbody>
</table>

**Abbreviations**: AR = Androgen receptor; CASRN = Chemical Abstracts Service Registry Number; D1 and D2 = Two major families of dopamine receptors; DDE = 1,1-Dichloro-bis[4-chlorophenyl]ethylene; DDT = Dichlorodiphenyltrichloroethane; ER = Estrogen receptor; HDT = Highest dose tested; Neg. = Negative; Pos. = Positive; RBA = Relative binding affinity; T3 = Triiodothyronine; T4 = Thyroxine; TA = Transcriptional activation.

<sup>a</sup> Substances in bold type are those that, at a minimum, are recommended for inclusion in future validation studies. Empty cells indicate that no relevant data were identified.

<sup>b</sup> An IC<sub>50</sub> is the concentration of the test substance that inhibits 50% of the response of the reference androgen in a particular test system. Median IC<sub>50</sub> values are derived from *in vitro* mammalian cell reporter gene studies that were either published in the peer-reviewed scientific literature or submitted to NICEATM, and then reviewed and summarized in the NICEATM Background Review Document (BRD) titled “Current Status of Test Methods for Detecting Endocrine Disruptors: *In Vitro* Androgen Receptor Transcriptional Activation Assays-July 2002” (available on the ICCVAM website at [http://iccvam.niehs.nih.gov/methods/endocrine.htm](http://iccvam.niehs.nih.gov/methods/endocrine.htm)). Substances for which quantitative AR antagonism data are available are ranked according to their relative potency in AR mammalian cell reporter gene antagonism assays from most potent to least potent. Numbers in parentheses to the right of the IC<sub>50</sub> value refer to the number of studies for which an IC<sub>50</sub> value was reported in the BRD. An italicized IC<sub>50</sub> value indicates the value was estimated from a graphical presentation of data. The range of values reported for a substance is listed below the median value.
Table 6-2: ICCVAM Recommended Substances for Validation of *In Vitro* AR TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems<sup>a</sup> (continued)

<sup>c</sup> Numbers in parentheses refer to the number of studies in which the substance was reported positive or negative compared to the number of studies in which it was tested. A substance is classified as “positive” for AR antagonism if it was positive in more than 50% of reported studies. A substance is classified as “presumed positive” for AR antagonism if it was positive in 50% or less of reported studies, or if it was reported positive in the single study conducted. Substances reported negative in their respective studies are classified as “presumed negative” instead of “negative” for AR antagonism, since they had not been tested in multiple studies at or above the limit dose of 1 mM recommended in *Section 6.1.3*. Substances without data are classified “presumed positive” or “presumed negative” based on available information, including their known mechanism of action or their responses in AR binding assays, ER binding assays, or ER TA assays.

<sup>d</sup> Several *in vivo* test methods are undergoing further development or validation by OECD, EPA, and the JME (J). Substances indicated are proposed for testing by OECD in the Uterotrophic assay (U), the Hershberger assay (H), or the 407 protocol (407); for testing by EPA in the female pubertal assay (F-PA), the male pubertal assay (M-PA), the intact male assay (IM), a one-generation assay (1G), a two-generation assay (2G), or a fish reproductive screen (FRS); for testing by JME in the U, H, and 1G assays, or various fish (F) and avian (A) assays. Due to the lack of CASRN for the JME studies, some of the indicated substances might not be the same substance included in this list. The *in utero* through lactation assay (IUL) has been recommended, but EPA has not made a decision on its further development or validation.

<sup>e</sup> Information for this substance regarding its median IC<sub>50</sub> value (if available), its qualitative response in mammalian cell reporter gene assays, and the number of mammalian cell reporter gene assays in which it was tested was derived from data presented in Appendix D of the NICEATM AR TA BRD cited in footnote b. This BRD contains AR TA data from the published literature through January 25, 2002.

<sup>f</sup> Information for this substance was abstracted from a publication that was published or reviewed after the literature search was completed for the NICEATM AR TA BRD (i.e., Andersen et al. 2002 and Paris et al. 2002 in *Section 7.0*).

<sup>g</sup> No IC<sub>50</sub> data available for this substance.

<sup>h</sup> R1881 is the recommended reference androgen for *in vitro* AR binding and TA assays and, thus, is not considered a test substance for validation purposes (refer to *Section 6.2* for more information).

<sup>i</sup> Two forms of *p*-nonylphenol are available for testing. One form consists of a mixture of various branched isomers (CASRN 84852-15-3), while the other contains only one isomer consisting of a linear alkyl chain (CASRN 104-40-5). ICCVAM recommends the linear form, which has a uniform chemical structure, for validation studies.

<sup>j</sup> The classification for this substance is “presumed positive” for AR antagonism since the substance was positive in 50% or less of reported studies, or was reported positive in the single study conducted.
7.0 REFERENCES


Expert Panel Evaluation of the Validation Status of *In Vitro* Test Methods for Detecting Endocrine Disruptors:

Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays

Expert Panel Final Report

September 2002

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)

National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

National Institute of Environmental Health Sciences
National Institutes of Health
U.S. Public Health Service
Department of Health and Human Services
# Table of Contents

Table of Contents ........................................................................................................... A-3
ED Expert Panel Roster ................................................................................................... A-4
Executive Summary ......................................................................................................... A-5

I. **In Vitro** Estrogen Receptor (ER) Binding Assays ............................................... A-17
   1.0 Recommendations and Priority for Validation Studies ...................................... A-17
   2.0 Minimum Procedural Standards for **In Vitro** ER Binding Assays ................ A-18
   3.0 Recommendations for **In Vitro** ER Binding Test Method Protocols for Validation Studies .......................................................... A-23
   4.0 Recommended List of Substances to be Used for Validation of **In Vitro** ER Binding Assays .......................................................... A-24
   References ................................................................................................................ A-25
   Appendix 1: Ligand Titration Assay ................................................................. A-26
   Appendix 2: Ligand Competition Array .......................................................... A-29

II. **In Vitro** Estrogen Receptor (ER) Transcriptional Activation (TA) Assays .... A-34
   1.0 Recommendations and Priority for Validation Studies .................................. A-34
   2.0 Minimum Procedural Standards for **In Vitro** ER TA Assays ....................... A-36
   3.0 Recommendations for **In Vitro** ER TA Test Method Protocols for Validation Studies .......................................................... A-41
   4.0 Recommended List of Substances to be Used for Validation of **In Vitro** ER TA Assays .......................................................... A-43
   Table 1: Substances in Common Between **In Vitro** ER Binding Assays and **In Vitro** ER TA Agonism and Antagonism Assays .................................................... A-47
   Additional Panel Recommendations ............................................................... A-48

III. **In Vitro** Androgen Receptor (AR) Binding Assays ........................................ A-50
   1.0 Recommendations and Priority for Validation Studies .................................. A-50
   2.0 Minimum Procedural Standards for **In Vitro** AR Binding Assays ............ A-54
   3.0 Recommendations for **In Vitro** AR Binding Test Method Protocols for Validation Studies .......................................................... A-58
   4.0 Recommended List of Substances to be Used for Validation of **In Vitro** AR Binding Assays .......................................................... A-60

IV. **In Vitro** Androgen Receptor (AR) Transcriptional Activation (TA) Assays .... A-62
   1.0 Recommendations and Priority for Validation Studies .................................. A-62
   2.0 Minimum Procedural Standards for **In Vitro** AR TA Assays ....................... A-75
   3.0 Recommendations for **In Vitro** AR TA Test Method Protocols for Validation Studies .......................................................... A-80
   4.0 Recommended List of Substances to be Used for Validation of **In Vitro** AR TA Assays .......................................................... A-82
   Summary ............................................................................................................... A-84
   Additional Comments on the BRD ................................................................. A-85
   References ............................................................................................................ A-88
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EXECUTIVE SUMMARY

Introduction
In response to public concern that pesticides may interfere with endocrine processes in humans and wildlife, Congress in 1996 directed the U.S. EPA, through the Food Quality Protection Act (FQPA) (Public Law 104-170), to develop a screening program for evaluating the potential of pesticides and other substances to induce hormone-related health effects. In 1998, the U.S. EPA Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) issued a report recommending that the agency evaluate the effects of these substances on both human and ecological (wildlife) health, and that a screening and testing program be implemented for identifying endocrine disruptors. In response, the U.S. EPA proposed the Endocrine Disruptor Screening Program (EDSP) (Federal Register, Vol. 63, No. 248, pp. 71541-71568, December 28, 1998, available at http://www.epa.gov/fedrgstr/EPA-TOX/1998/December/Day-28/t34298.htm). The proposed EDSP consists of a Tier 1 screening battery of tests that is designed to identify substances capable of interacting with the endocrine system, and different Tier 2 testing assays that are designed to confirm and extend the Tier 1 results. If, based on a weight of evidence evaluation of the results from the Tier 1 screening battery, the test substance is identified as a potential endocrine disruptor, Tier 2 in vivo tests are conducted to provide detailed information on concentration response relationships and specific abnormal effects that may result. The proposed Tier 1 in vitro assays include estrogen receptor (ER) and androgen receptor (AR) assays. Currently, the U.S. EPA proposes that either a binding assay or a transcriptional activation (TA) assay be used. These in vitro assays are relevant for screening purposes because they might identify substances that alter natural endocrine processes by binding with estrogen and/or androgen receptors, resulting in agonist and/or antagonist activity.

To assess the current validation status of these in vitro methods, the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), which provides operational support to the Interagency Coordinating Committee for the Validation of Alternative Methods (ICCVAM), prepared Background Review Documents (BRD) on:

- In vitro ER binding assays
- In vitro ER TA assays
- In vitro AR binding assays
- In vitro AR TA assays

As part of the ICCVAM evaluation, the U.S. EPA asked for development of minimum performance criteria that could be used to define acceptable in vitro ER/AR binding and TA assays. It was envisioned that these criteria would be based on the performance of existing standardized in vitro ER binding assays, and would be used to assess the acceptability of future new or revised assays.

An extensive literature search for relevant publications was conducted and a formal request through the U.S. Federal Register (Vol. 66, No. 57, pp. 16278-16279, March 23, 2000) was made for data and information from completed, ongoing, or planned studies using or evaluating ER/AR binding and TA assays. BRDs, prepared for each of the four types of assays, are included:

- A review of the different protocols used for each type of assay
During development of the BRDs, ICCVAM and NICEATM determined that no validation studies have been completed on the assays being considered. With agreement from the U.S. EPA, NICEATM and ICCVAM decided to proceed with an expert panel evaluation of the current status of ER/AR binding and TA assays and the development of recommendations for their future validation.

An Expert Panel meeting, sponsored by the National Institute of Environmental Health Sciences (NIEHS) and the NTP and organized by NICEATM in collaboration with ICCVAM, was held on May 21-22, 2002 at the Sheraton Imperial Hotel in Research Triangle Park, NC. The Panel was charged with assessing the current validation status of these four types of in vitro endocrine disruptor screening methods and to develop recommendations on the following:

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

In Vitro ER Binding Assays
The Panel reviewed 14 different in vitro ER binding assays in which 635 different substances had been tested in one or more of the assays. The sources of ER used in these assays included cytosol prepared from MCF-7 cells and from the uteri of mice, rats, and rabbits; intact MCF-7 cells; purified recombinant human ERα and ERβ, and fusion proteins between glutathione-S-transferase and the binding domains of the human ERα, mouse ER, chicken ER, anole ER, and rainbow trout ER.

The Panel agreed that the in vitro ER binding assays considered in the BRD still required standardization and that the available data were not adequate to assess the validation status of the test methods. The Panel recommended that test methods using recombinant ER receptors (both α and β subtypes) should be given the highest priority for further assay standardization and validation. Either human or rat receptors were considered acceptable. For screening for possible ecological effects, recombinant receptors from wildlife were considered to be potentially more relevant and should be evaluated.

There was consensus among the Panel on the adequacy of the following minimum procedural standards described in the In Vitro ER Binding BRD:

- All studies utilizing animals should be approved by the Institutional Animal Care and Use Committee (IACUC) or its equivalent.
- The dissociation constant ($K_d$) of the reference estrogen must be determined with each set of assays.
• The reference estrogen for the assays should be 17β-estradiol.
• Test substances should be prepared in water or 95-100% ethanol but dimethyl sulfoxide (DMSO) could be used, if necessary. A set of solvent/vehicle-only controls (with final solvent concentrations identical to those used in the reaction mixtures containing the test substance) must be included in each set of assays.
• The limit concentration should be 1 mM but the solubility characteristics of each test substance must be taken into consideration. In addition, possible denaturation of the receptor at high test substance concentrations, and for some substances (e.g., surfactants), at concentrations below 1 mM, need to be considered when the results of positive assays are interpreted.
• The concentration range of test substances should consist of at least seven different concentrations spaced at one order of magnitude apart from each other (e.g., 1, 10, 100 nM, 1, 10, 100 μM, 1 mM). However, if a lower maximum concentration is tested due to solubility constraints or excessive toxicity, the number of concentrations tested can be adjusted to account for the reduced concentration range.
• The control solvent/vehicle volume must be the same as that used in the reaction mixtures containing the test substances and should remain constant throughout the concentration range tested.
• A substance (e.g., tamoxifen, coumestrol, estriol) with a binding affinity of two or three orders of magnitude below that of 17β-estradiol should be used as a concurrent positive control.
• Triplicate measurements should be performed at each concentration (negative and positive controls, reference estrogen, test substance) tested.
• For data analysis, it is essential that both the B_{max} (number of binding sites or specific binding capacity) and the K_d values be computed.
• For an assay to be acceptable, the reference estrogen/positive control responses must be acceptable, based on historical data.
• If an IC_{50} cannot be obtained after testing to the limit dose or the highest dose possible, the test substance is usually classified as being “negative” for in vitro ER binding. However, when test substances induce a significant reduction in binding, but do not achieve at least a 50% reduction in the binding of the reference estrogen to the ER, such responses should be noted and the substances classified as "equivocal" until additional information becomes available about the significance of this category of dose response curves.
• The test report should include information on the test substance, the solvent used, the type and source of the ER, the test conditions, the results, a discussion of the results, and a determination as to whether the substance is positive or negative.
• Replicate studies are not mandated but questionable data needs to be confirmed by re-testing the substance.
• The assays should be performed following Good Laboratory Practice guidelines.

Additional or modified minimum procedural standards that were recommended by the Panel included:
• Classification of a test substance as “positive” for binding should be based on the use of statistical inferences pertinent to the characteristics of the assay. The state of the art for making statistical inferences with endocrine disruptor data requires more detailed research and study.
• Hexa-tritium labeled 17β-estradiol (i.e., [2,3,6,7,16,17-3H] 17β-estradiol) should be used as the ligand for all assays.
• In situations where more than one solvent could be used, preference should be given to water, then ethanol, followed by DMSO.
• The type of protein assay and the concentration of the protein used in the assays must be specified in the study report.
• Sodium molybdate and a cocktail of protease inhibitors must be added to the assay to protect the ER from degradation.
• The use of dextran-coated charcoal was recommended as the most suitable method for separating bound from free labeled 17β-estradiol.
• For data analysis, the use of a ligand titration array (Raffelsberger and Wittliff, 1997) would provide simultaneous evaluation of a laboratory’s performance and determination of the estrogen binding properties (e.g., IC₅₀, K_d and K_i values) of both the reference estrogen and the test substances.

The Panel concluded that each of the binding parameters (i.e., B_max, K_d, K_i values) of the reference estrogen and test substances should be measured in each ligand titration/binding assay. Since the binding of a test substance to the receptor is often not directly proportional to the concentration of the substance being tested, a non-linear response is often obtained. Thus, it seemed appropriate that a non-linear statistical model would be the best approach for the analysis of this data. However, the Panel concluded that a decision on this statistical approach needed further evaluation before the most appropriate statistical method could be identified. This evaluation would be facilitated by the collection of data generated by different laboratories using the same defined protocol and testing the same set of substances.

The Panel did not recommend the inclusion of a metabolic activation system in in vitro ER binding assays.

The Panel was comfortable with the “Example Protocol for the In Vitro Estrogen Receptor (ER) Competitive Binding Assay using Rat Uterine Cytosol (RUC)” included in the ER Binding BRD (Section 12, Annex - based on the U.S. EPA RUC Protocol, 2002), provided that it is amended to include the additional details presented in the discussion on minimum procedural standards in the BRD and the points discussed in the ER Binding Group report responses to Questions 1 and 2. This amended protocol can serve as a prototype for developing protocols for other ER binding assays, such as those using a purified ER protein.

The Panel endorsed the 33 substances recommended in the In Vitro ER Binding BRD for the validation of in vitro ER binding assays. While this list provided substances across the range of binding activities, the Panel recommended that the list be reviewed to ensure that it represents the diversity of chemical classes and the range of potencies that the U.S. EPA is interested in screening. The Panel recommended that the proportion of negative ER binding substances in the list should be increased to at least 25% to enable the specificity of the assay to be accurately determined.
In regard to future validation studies, the Panel concluded that both inter- and intra-laboratory reproducibility must be assessed using the same reference ER preparation and identical set of test substances.

**In Vitro ER TA Assays**

The Panel reviewed 95 different ER TA assays (63 mammalian reporter gene assays, 10 mammalian proliferation assays, 22 yeast strain reporter gene assays) in which 698 different substances had been tested in one or more of the assays. The source of the ER used in these assays included unspecified ER from human, mouse, and rat; or ERα and ERβ subtypes found endogenously or transiently/stably transfected into various cell lines. The luciferase and chloramphenicol acetyltransferase reporter genes were used in the mammalian cell line assays and the β-galactosidase reporter gene was used in the yeast strain assays.

The Panel, while indicating that an assay using a stably transfected cell line appeared to be more amenable to high throughput screening, was not convinced that such an assay was the most appropriate. Stable cell lines lose their stability over time, are limited in availability, and are difficult to isolate. To resolve this issue, the Panel recommended that a comparative study be conducted in which the response of cell lines transiently or stably transfected with the same ER receptor/reporter gene constructs be compared using a selected set of test substances. A third cell line expressing an endogenous ER and transfected with the same reporter construct should be included in this study.

There was consensus among the Panel on the adequacy of the following minimum procedural standards provided in the *In Vitro* ER TA BRD:

- The reference estrogen should be 17β-estradiol; the transcriptional activation response with this substance must be demonstrated by a full concentration response curve.
- Test substances should be prepared preferably in absolute ethanol or culture medium but DMSO could be used, if necessary. A set of solvent/vehicle-only controls (with final solvent concentrations identical to those used in the reaction mixtures containing the test substance) must be included in each set of assays.
- The solvent/vehicle volume must be the same as that used in the reaction mixtures containing the test substance, and should remain constant throughout the concentration range tested.
- A relatively active antagonist (e.g., ICI 182,780) should be used as the positive control for antagonist studies.
- The limit concentration should be 1 mM but the solubility characteristics of each test substance must be taken into consideration. (One Panel member felt that this concentration was excessive and that 0.1 mM should be adequate).
- The concentration range of test substances should consist of at least seven different concentrations spaced at one order of magnitude apart from each other (e.g., 1, 10, 100 nM, 1, 10, 100 μM, 1 mM). However, if a lower maximum concentration is tested due to solubility constraints or excessive cytotoxicity, the number of concentrations tested can be reduced to account for the altered concentration range. At least one Panel member suggested that five concentration levels would be adequate.
- Triplicate measurements should be performed at each test substance concentration level.
• Classification of a test substance as "positive" for agonist or antagonist activity in transcriptional activation assays should be based on the generation of a concentration response curve.
• Historical data should be used as part of the assay acceptance criteria (i.e., reference substances for agonism and antagonism must give appropriate responses).
• The test report should include information on the test substance, the solvent used, the ER, the reporter plasmid, the cell line, the test conditions, the results, a discussion of the results, and a determination as to whether the substance is positive or negative.
• Replicate studies are not mandated but questionable data needs to be confirmed by re-testing of the substance. However, one Panel member recommended testing each substance at least three times in different experiments.
• The assays should be performed following Good Laboratory Practice guidelines.

Additional or modified minimum procedural standards that were recommended by the Panel included:
• Concentrations to be tested for the reference positive control, 17β-estradiol, should range from 1 pM to 1 μM, and clear guidance is needed about the expected response.
• With regard to the preparation of test substances, the level of solvent that does not adversely affect assay response should be determined before testing by performing appropriate pre-validation studies using the reference estrogen.
• A relatively weak estrogenic agonist (e.g., estriol) should be included as an additional control for agonist studies.
• A measure of cellular cytotoxicity should be incorporated into the assay to help define the upper limit for test material concentrations, similar to the Maximum Tolerated Dose (MTD) approach used in in vivo studies.
• To ensure that a positive agonist response reflects a receptor-mediated activity, the test substance could be re-tested with ICI 182,780 (the candidate ER antagonist) present in the culture medium.
• For transient transfection assays, there is a need to include a constitutive reporter gene assay to control for transfection efficiency.
• A standard definition for "relative activity" must be decided upon so that a positive/ negative call for agonism and antagonism can be made.
• Suitable diagnostics must be performed on any statistical procedure to ensure that the model fits the data before it is finally chosen for analysis of the data.
• The test report should also include the complete DNA sequences of constructs and vectors, the transfection methods used, the cell passage number(s) during the study, and the CO₂ % level in the incubator.

The Panel did not recommend the inclusion of an exogenous metabolic activation system in in vitro ER TA studies at this time. However, the Panel recommended that available information on the Phase I/Phase II metabolic capabilities of the cell lines employed in validation studies, as well as available information on the metabolism of the validation chemicals be compiled. The Panel concluded that studies should be conducted to obtain information on the importance of metabolism for systems ultimately employed in screening.
The Panel concluded that the protocols provided in the *In Vitro* ER TA BRD were adequate, provided that the minimum procedural standards are included, and that a laboratory with cell or yeast culture expertise should be able to perform the assays. However, there are a number of issues relating to standardization that will have to be added to the protocols.

In terms of the list of reference substances recommended in the *In Vitro* ER TA BRD for future *in vitro* ER TA assay validation studies, the Panel recommended that selection be based on solid scientific rationale such as a combination of existing ER binding, antagonist, or agonist data. To the extent possible, all the same reference substances should be used for validation of both the *in vitro* ER binding and the *in vitro* ER TA assays. The Panel also recommended that the proportion of negative substances be increased to more effectively evaluate the specificity of these assays. During development of the final list, consideration should be given to substances selected by the U.S. EPA and the Organisation for Economic Co-operation and Development (OECD) for validating *in vivo* endocrine disruptor assays. Also, the Panel encouraged the development of a centralized repository of chemicals with verified purity for future validation studies.

The Panel recommended that a sequential testing strategy be evaluated for utility during the pre-validation of *in vitro* ER/AR binding and TA agonism/antagonism assays. In this approach, if a substance induces a positive response in any assay, then testing in any of the other binding/TA assays would not need to be conducted. In support of this strategy, the Panel concluded that further classification of the activity of a positive test substance using additional binding/TA endpoints would provide little additional information that would assist with prioritization and the design of subsequent *in vivo* studies.

**In Vitro AR Binding Assays**

The Panel reviewed 11 different *in vitro* AR binding assays in which 109 different substances had been tested in one or more of the assays. The sources of AR used in these assays included cytosol from calf uterus, rat epididymis and prostate, and MCF-7 cells; rat epididymal nuclear fraction; COS-1 cells transiently transfected with a human AR; LnCaP cells and human genital fibroblasts with an endogenous AR; and semi-purified recombinant human AR.

The Panel acknowledged the lack of an existing acceptable standardized *in vitro* AR binding assay protocol, and that the published data were inadequate for assessing the reliability and comparative performance of these assays. Based on the available data, the Panel recommended that the highest priority for future efforts is the development of a high-throughput assay using a purified, recombinant full-length AR protein. A truncated AR protein (consisting of the AR ligand binding domain) has been purified but this protein is less desirable than the full-length protein because it appears that domains other than the actual binding domain modulate the binding of substances to the AR. However, in contrast to the human ER protein, the commercial availability of the intact AR cDNA is apparently limited due to restrictions by the exclusive license of the patent. The Panel recommended that the appropriate government agencies investigate the status of patents and licenses attendant to the use of the human and rat AR and provide guidance as to how the scientific community should proceed with the development of such AR assays. An alternative approach might be to proceed with a truncated AR protein that is commercially available but that has not yet been evaluated for sensitivity and reliability.
There was consensus among the Panel on the adequacy of the following minimum procedural standards provided in the In Vitro AR Binding BRD:

- All studies utilizing animals should be approved by the IACUC or its equivalent.
- The $K_d$ of the reference androgen must be determined with each set of assays.
- Test substances should be prepared preferably in water, and then absolute ethanol but DMSO can be used, if necessary. A set of solvent/vehicle-only controls (with final solvent concentrations identical to those used in the reaction mixtures containing the test substance) must be included in each set of assays.
- The control solvent/vehicle volume must be the same as that used in the reaction mixtures containing the test substance, and should remain constant throughout the concentration range tested.
- The limit concentration should be 1 mM but the solubility characteristics of each test substance must be taken into consideration. In addition, possible denaturation of the receptor at high test substance concentrations, and for some substances (e.g., surfactants), at concentrations below 1 mM, need to be considered when the results of positive assays are interpreted.
- The concentration range of test substances must consist of at least seven different concentrations spaced at one order of magnitude apart from each other (e.g., 1, 10, 100 nM, 1, 10, 100 μM, 1 mM). However, if a lower maximum concentration is tested due to solubility constraints or excessive cytotoxicity, the number of concentrations tested can be reduced to account for the altered concentration range.
- A substance (e.g., cyproterone acetate) with a binding affinity of two or three orders of magnitude below that of the reference androgen should be used as a positive control.
- Triplicate measurements should be performed at each concentration level.
- For data analysis, it is essential to compute both the $B_{max}$ and the $K_d$ values.
- If an IC$_{50}$ cannot be obtained after testing to the limit dose or the highest dose possible, the test substance is usually classified as being “negative" for in vitro AR binding. However, when test substances induce a significant reduction in binding, but do not achieve at least a 50% reduction in the binding of the reference androgen to the AR, such responses should be noted and the substances classified as "equivocal" until additional information becomes available about the significance of this category of dose response curves.
- The test report should include information on the test substance, the solvent used, the type and source of the AR, the test conditions, the results, a discussion of the results, and a determination as to whether the substance is positive or negative.
- Replicate studies are not mandated but questionable data needs to be confirmed by re-testing of the substance.
- The assays should be performed following Good Laboratory Practice guidelines.

Additional or modified minimum procedural standards that were recommended by the Panel included:

- Classification of a test substance as “positive” for binding should be based on the use of statistical inferences pertinent to the characteristics of the assay. The state of the art for making statistical inferences with endocrine disruptor data requires more detailed research and study.
The endogenous ligand, 5α-dihydrotestosterone (DHT), was recommended as the reference androgen for recombinant protein-based assays where metabolism of DHT would not occur. Due to its high affinity, lack of metabolism, and low non-specific protein binding, R1881 was recommended as the reference androgen for most other assays. However, as R1881 binds to the progesterone receptor (PR), binding assays based on cells or tissues that contain this receptor should include triamcinolone acetonide to block its binding to the PR. Alternatively, mibolerone, which has a low affinity for PR, was considered appropriate as the reference androgen for such assays.

- An additional positive control substance with a binding affinity within two orders of magnitude of the limit of sensitivity of the assay should be included also.
- The dextran-coated charcoal procedure should be used for the separation of free and bound ligand.
- The type of protein assay and the concentration of the protein used in the assays must be specified.
- Sodium molybdate and a cocktail of protease inhibitors must be added to the assay to protect the AR from degradation.
- For data analysis, the use of a ligand titration array (Raffelsberger and Wittliff, 1997) would provide simultaneous evaluation of a laboratory’s performance and determination of the androgen binding properties (e.g., IC\textsubscript{50}, K\textsubscript{d}, and K\textsubscript{i} values) of both the reference androgen and the test substances.
- The study report should include information on the chemical and radiochemical purity of the radiolabeled androgen, as well as information on the assay used for protein determination.

The addition of an exogenous metabolic activation system was not recommended for current use by the Panel in \textit{in vitro} AR binding assays.

For the same reasons described for the \textit{in vitro} ER binding assays, the Panel concluded that the statistical approaches for the analysis of data generated using \textit{in vitro} AR binding assays required further investigation. The Panel recommended that prevalidation studies be conducted to evaluate an \textit{in vitro} AR binding assay using purified AR. Data generated from these prevalidation studies could be used by the biostatisticians to develop the most reliable and robust statistical models for data analysis.

The Panel concluded also that, although the rat prostate cytosol (RPC) protocol was sufficiently detailed, this assay should not be a priority for further validation. Rather, the Panel was of the opinion that the simplest and most preferred assay would be one in which purified AR is fixed to multiwell plates. One commercial source of the AR (PanVera Corporation, Madison, WI, USA) is available, but this AR is a truncated protein and has not been evaluated for sensitivity and reliability.

The Panel recommended that the same reference substances should be used for validation of both \textit{in vitro} AR binding and \textit{in vitro} AR TA assays. Furthermore, the Panel recommended the inclusion of additional weakly positive reference substances representing the range of possible environmental exposures and an increase in the proportion of negative substances. The Panel also recommended that bicalutamide, a substance that binds to the AR but does not activate its
transcription, and finasteride, a commercially available 5α-reductase inhibitor which does not bind to the AR, be included as additional assay controls.

In regard to future validation studies, the Panel concluded that both inter- and intra-laboratory reproducibility must be assessed for the same reference AR preparation using an identical set of test substances.

**In Vitro AR TA Assays**

The Panel reviewed 17 different AR TA assays (15 mammalian reporter gene assays, 1 mammalian proliferation assay, 1 yeast strain reporter gene assay) in which 147 different substances had been tested in one or more of the assays. The source of the AR used in these assays included AR from the human, mouse, and rat. The luciferase and chloramphenicol acetyltransferase reporter genes were used in the mammalian cell line assays and the β-galactosidase reporter gene was used in the yeast strain assay.

It was the consensus of the Panel that no current *in vitro* AR TA protocol was optimal for assessing AR agonist and antagonist activities. However, the Panel concluded that one cell line, described in the *In Vitro* AR TA BRD, containing an endogenous AR and transduced with an adenovirus containing the reporter gene was the most promising approach for development of an assay to assess AR agonist and antagonist activity. The adenovirus method is straightforward and avoids time consuming procedures associated with transient transfection methodology. Important additional developments that are needed include the identification and use of a cell line that lacks high response levels of the glucocorticoid and progesterone receptors, and the use of a reporter vector that shows greater specificity for the AR.

There was consensus on the adequacy of the following minimum procedural standards provided in the *In Vitro* AR TA BRD:

- The transcriptional activation of the reference androgen must be demonstrated by a full concentration response curve.
- The reference androgen should be R1881.
- The active antagonist hydroxyflutamide was recommended as a positive control for antagonist studies.
- Test substances should be prepared preferably in water or absolute ethanol but DMSO could be used, if necessary. A set of solvent/vehicle-only controls (with solvent concentrations identical to those used with reaction mixtures containing test substances) must be performed in each set of assays.
- The solvent/vehicle volume must be the same as that used in the reaction mixtures containing the test substances.
- An androgen that is two orders of magnitude less potent than R1881 should be used as a positive control.
- The limit concentration should be 1 mM but the solubility characteristics of each test substance must be taken into consideration. Concern was expressed by some Panel members that this concentration might be excessive and that 30 μM would be adequate.
- The concentration range of test substances must consist of at least seven different concentrations spaced at one order of magnitude apart from each other (e.g., 1, 10, 100 nM,
ICCVAM Review of In Vitro Endocrine Disruptor Screening Assays

A-14

1, 10, 100 μM, 1 mM). However, if a lower maximum concentration is tested due to solubility constraints or excessive cytotoxicity, the number of concentrations tested can be reduced to account for the altered concentration range.

- Triplicate measurements should be performed at each test substance concentration level.
- Classification of a test substance as “positive” for agonist or antagonist activity in transcriptional activation assays should be based on the generation of a concentration response curve.
- Reference substances for agonism and antagonism assays must give appropriate responses based on historical data.
- The test report should include information on the test substance, the solvent used, the AR, the reporter plasmid, the cell line, the test conditions, the results, a discussion of the results, and a determination as to whether the substance is positive or negative.
- Replicate studies are not mandated but questionable data needs to be confirmed by re-testing of the substance.
- The assays should be performed following Good Laboratory Practice guidelines.

Additional or modified minimum procedural standards recommended by the Panel included:

- Serum free and phenol red free media should be used rather than charcoal stripped serum.
- The stability of the cell lines must be monitored using selection media.
- DHT should be used as an additional reference androgen.
- Levels of cytotoxicity must be evaluated in each assay.
- A nonselective reporter (e.g., MMTV) should be used.
- A suitable nonlinear regression model such as the Hill equation must be used to estimate the potency (EC50 or IC50 values) and slope of the concentration-response curve with a 95% confidence interval.
- Statistical analysis of the data should indicate agonist and inhibitory test chemical effects on transcription that meet or exceed the 2-3 fold induction or 50% inhibition level compared to the respective controls.
- Diagnostics need to be performed on the model by checking for suitability and normality of the curve. If necessary, suitable data transformations need to be performed.
- The test report should also include the type of method used for isolating the DNA for making constructs; the cell passage number; the volume of the test substance applied to the test mixture; information on controls for the activity of other steroid receptors and controls for cytotoxicity; source of plasticware and other materials used in the assay; entry and exit analytical assay results for all test article and control compounds; and the response in absolute units such as light units for luciferase activity with the error indicated and as fold induction, if this is deemed appropriate.

In terms of statistical analysis, the Panel concluded that nonlinear statistical models (e.g., the Hill equation) appear to be the most useful models for estimating the potency and the slope of the concentration-response curve for agonists and antagonists.

The Panel did not recommend the inclusion of a metabolic activation system for in vitro AR TA assays.
In terms of the list of substances recommended in the In Vitro AR TA BRD for future in vitro AR TA assay validation studies, the Panel identified a more limited list of potential candidate substances for use in prevalidation studies. This list included substances that could affect luciferase reporter gene transcription activity independent of the AR (e.g., by inhibiting RNA or protein synthesis).

Although recognizing that these in vitro endocrine disruptor assays are proposed as components of a screening test battery of test methods where the results will be used in making weight of evidence decisions, the Panel recommended determination of the predictive value of these assays for estimating in vivo responses. Therefore, the Panel recommended that substances proposed for validation of the in vivo test methods should also be evaluated in the in vitro assays included in the screening battery and, to the extent possible, and vice-versa.

The Panel encouraged the development of a centralized repository of substances with verified purity that could be distributed to laboratories developing or conducting validation studies. The purpose of this repository is to ensure the comparability of data generated during the validation of the different in vitro assays and to provide a source of coded samples for validation.
I. *In Vitro* Estrogen Receptor (ER) Binding Assays

1.0 Recommendations and Priority for Validation Studies
The *In Vitro* ER Binding BRD reviews the comparative performance, reliability, advantages, and disadvantages for different *in vitro* ER binding assays, and recommends a relative priority for further development and/or validation based on this information.

1.1 Considering the intended use of the assays as a toxicological screen, is the Panel aware of other advantages and disadvantages for the assays discussed in the BRD?
The Panel did not discuss any additional advantages and disadvantages not covered in the BRD.

1.2 Considering the intended use of the assays as a toxicological screen, does the Panel agree with the relative priority recommended for these sets of assays? Does the Panel recommend any changes in priority, or have specific recommendations for prioritization?
The Panel agrees with the BRD’s conclusions that assays using recombinant receptors (both subtypes) should be given the highest priority for assay standardization and validation. Human or rat receptors would be acceptable. For screening for possible effects in wildlife, recombinant receptors from other species might be more relevant. The Panel believes that a consistent, standardized preparation of the receptor is essential for quality control and in making valid comparisons across laboratories and experiments. The fluorescent polarization assay has not been in wide use and there are only limited data for comparison. Availability of specialized equipment and reagents is also of concern.

1.2.1 Is rat uterine cytosol the best source of estrogen receptors for the binding assays?
The rat uterine cytosol (RUC) is not considered the best source of ER for the ligand binding assay. A standardized preparation of the ER is of the utmost importance for quality control and comparison of results across laboratories.

1.2.2 Should the binding of substances to different receptor subtypes be addressed in the binding assays?
The use of either human or rat recombinant proteins, both α and β, is a high priority. Recombinant receptors from other species are recommended for screening substances that pose particular hazard to wildlife.

1.2.3 Should a metabolic activation system be included in the binding assays?
The inclusion of a metabolic activation system in *in vitro* ER binding assays is not recommended at this time. The type of metabolic activation system developed will depend on which *in vitro* assay(s) are considered validated for detecting endocrine disrupting substances. The Panel recommends, while validation is being conducted, that available information on the metabolism of the validation chemicals be compiled, including the degree to which metabolism is known to alter estrogenic activity. Once the importance of metabolic activation in the ability of substances to disrupt endocrine function has been demonstrated and validated in *in vitro* ER binding assays, appropriate methods for including metabolic activation in the assays can be developed and validated.
2.0 Minimum Procedural Standards for *In Vitro* ER Binding Assays

2.1 To facilitate assay standardization, the BRD proposes minimum procedural standards that should be incorporated into *In Vitro* ER Binding Assay protocols (Section 12.2). Does the Panel agree with the adequacy of the proposed procedural standards? If not, what changes should be made to each standard and why?

The Panel agrees with the critical methodological issues proposed in the BRD, and endorsed the fact that any assays using animals must be undertaken under the guidance of the relevant Institutional Animal Care and Use Committee (IACUC).

2.1.1 Binding Constant \( (K_d) \) of the Reference Estrogen

The Panel agrees that the dissociation constant must be determined with each set of assays and that 17β-estradiol should be used as the reference estrogen. Furthermore, the Panel recommends that the hexa-tritium labeled 17β-estradiol (i.e., \([2,3,6,7,16,17^-{3}H] \ 17\beta\text{-estradiol}\) be used as the ligand for all assays because it is the most potent naturally occurring estrogen in the human body, and because of the high specific radioactivity available commercially, which increases the sensitivity of both the ligand titration assay and the ligand competition assay considerably. Furthermore, there was consensus that recombinant ER preparations, particularly human ER, be employed in the validation and screening assays.

The Panel agrees that a detailed assay protocol must be provided for performing each type of assay (i.e., ligand titration and competition), with criteria for evaluation and acceptance of results, to demonstrate assay validation and transferability across laboratories. The ability of a laboratory operator to achieve a specific binding capacity and \( K_d \) value for 17β-estradiol of a reference receptor protein within accepted limits for that type of preparation is a critical measure of the robustness of the procedure at that institution. These data will be essential to the establishment of a Quality Assurance Program (Assay Proficiency) for the evaluation of endocrine disruptor substances at numerous laboratories. Also, the Panel recommends replicate determinations and replicate assays, and the establishment of decision criteria for evaluating inter- and intra-laboratory reproducibility across the range of chemicals selected for testing. Attention should be given to the level of nonspecific binding in the ER preparation selected.

The Panel recommends that straightforward procedures for determination of both the \( K_d \) value of the radio-labeled reference ligand \([2,3,6,7,16,17^-{3}H] \ 17\beta\text{-estradiol}\), and the \( K_d \) value of an unlabeled test substance, such as the ligand titration array (Raffelsberger and Wittliff, 1997), be considered. Software programs such as Compete® (Lundon Software, Inc., Cleveland Heights, OH), GraphPad Prism® (GraphPad Software, Inc., San Diego, CA), LIGAND (Munson and Rodbard, 1980) and OneSite® (Lundon Software, Inc., Cleveland Heights, OH), will determine the specific binding capacity and \( K_d \) values of the reference ER preparation, and analyze the competition results and compute the \( K_d \) value of the unlabeled putative estrogen mimic examined in the assay. Appendices 1 and 2 provide an example of the curves...
that might be generated using this approach. As shown in Appendix 1, the \( K_d \) value for the reference estrogen ([2,3,6,7,16,17-^3H]17\(^\beta\)-estradiol), determined by Ligand Titration Assay, is computed according to various models. This \( K_d \) value is used in the calculations of the Ligand Competition Array (Appendix 2) to arrive at an apparent dissociation constant (\( K_d \) value) of the unlabeled test substance (in this example, estrone) for the reference receptor preparation (in this example, recombinant ER\( \alpha \)). Note the good agreement in the \( K_d \) values computed for unlabeled estrone (4.0 versus 2.6 \( \times \) 10\(^{-9} \) M) with the two models and with the \( K_i \) value (1.96 \( \times \) 10\(^{-9} \) M). The IC\(_{50}\) value is also provided. As noted below, however, more study is necessary for specifying the precise statistical characteristics of ER binding data when fitting nonlinear regression curves and estimating pertinent parameters such as \( K_d \) and the IC\(_{50}\).

2.1.2 Reference Estrogen
The Panel agrees that only the native estrogen ligand, 17\(^\beta\)-estradiol, should be employed as the reference estrogen, for the reasons indicated.

2.1.3 Preparation of Test Substances
The Panel agrees with the BRD that test substances should be prepared in water, 95-100\% ethanol, or dimethyl sulfoxide (DMSO), depending upon their solubility (Section 12.2.3 of the BRD). The Panel recommends that preference should be given to 95-100\% ethanol and that a set of solvent (vehicle)-only controls (with solvent concentrations identical to those used with reactions containing test substances) must be performed in each set of assays. It is known that sex-hormone receptor preparations from various tissue origins, including recombinant expression systems, exhibit different performance characteristics in the presence of the same solvent, again emphasizing the need for solvent controls. In situations where more than one solvent could be used, preference should be given to water, then 95-100\% ethanol, and then DMSO, in that order.

2.1.4 Concentration Range of Test Substances
The Panel agrees that the limit concentration should be 1 mM but the solubility characteristics and potential toxicity of each test substance (e.g., denaturation of the receptor) must be taken into consideration. If the limit concentration is used, seven test substance concentrations at log intervals should be tested. If a lower maximum concentration is tested due to solubility constraints or excessive toxicity, the number of concentrations tested can be adjusted to account for the altered concentration range. Concern was expressed among the Panel that denaturation of the receptor could occur at high test substance concentrations, and that this needed to be considered when the results of positive assays are interpreted. One Panel member stated during the meeting that some substances (e.g., surfactants) at concentrations below 1 mM might produce results that will be erroneously interpreted as positive in receptor binding assays, because of the loss of tertiary structure of the receptor. Whatever limit dose is chosen, care must be taken to ensure that only soluble concentrations of the test substance are used.
Also, the Panel agrees that at least seven different concentrations of the test substance within the range proposed in the BRD should be examined to increase the likelihood of developing a competition curve satisfactory for IC\textsubscript{50} analysis. For substances exhibiting solubility problems, both 95-100% ethanol and DMSO should be evaluated as solvents, perhaps with gentle warming at 50-55°C, to achieve the higher concentrations.

2.1.5 Solvent and Positive Controls
The Panel agrees that it is essential that the solvent (vehicle) volume in the solvent control assays be the same as that used in the reactions containing the test substances, and further that the solvent (vehicle) volumes remain constant throughout the competition curve development.

Regarding the positive control substance, the Panel strongly agrees that a substance with a binding affinity of two or three orders of magnitude below that of 17β-estradiol should be used as a positive control.

In order to assess the sensitivity of the assay system in each laboratory, it is suggested that a positive control should be used that is known to be a weak binder. Such a positive control could be either a naturally occurring estrogen, such as estriol or coumestrol, or a synthetic estrogen mimic, like tamoxifen. This positive control should be tested at three different concentrations.

There was discussion about employing a reference substance to determine the lower limit of detection of the assay but no recommendation was made.

2.1.6 Within-Test Replicates
The Panel agrees that triplicate measurements should be performed at each dose level to increase the likelihood of developing a competition curve satisfactory for IC\textsubscript{50} analysis, particularly during prevalidation and validation studies.

2.1.7 Dose Spacing
The Panel agrees with the recommended dose spacing of one order of magnitude in the concentrations of the candidate estrogen mimics, and in the use of half-log doses in certain cases.

2.1.8 Data Analysis
The Panel agrees with the requirement to determine and compute both the B\textsubscript{max} (number of binding sites or specific binding capacity) and the K\textsubscript{d} value. There was general agreement that the approach presented in the BRD is acceptable for screening substances, which inhibit estrogen binding. However, the use of alternative approaches such as a ligand titration array (Raffelsberger and Wittliff, 1997), which provides simultaneous evaluation of a laboratory’s performance and determination of the estrogen binding properties (e.g., IC\textsubscript{50}, K\textsubscript{d} value, K\textsubscript{I} value) of both reference and test substances, is recommended. This approach is equally valid for androgen
receptor analyses of putative androgenic mimics. The study by Villeneuve et al. (2000), on the derivation and application of relative potency estimates based on \textit{in vitro} bioassay results could serve as a possible template for calculating EC_{50} values for partial agonists or for substances for which the slope of the binding curve is atypical.

In any case, more detail is needed on statistical models for nonlinear regression analysis to compute $K_d$, $K_i$, and IC_{50} values. This includes the nature of the statistical characteristics of the data (distribution, variance patterns, specific nonlinear models, etc.), and how to fit the models. When doing so, confidence limits must be calculated for $K_d$, $K_i$, and IC_{50} values. From these data, details on how to make pertinent and valid statistical inferences should be specified.

2.1.9 Assay Acceptance Criteria

In general, there is agreement among the Panel with each of the acceptance criteria presented in the BRD. However, it is highly recommended that a reference ER preparation with established binding parameters be employed for the determination of the $K_d$ value and specific binding capacity by the laboratories chosen for validation of ER-based procedures. The Panel recommends that evaluation of the same reference receptor preparation with an identical set of test substances by various laboratories (as well as by individuals within the same laboratory) involved in this key process will provide a means of assessing both inter- and intra-laboratory reproducibility. Distribution of sets of results from either androgen or estrogen-based procedures to laboratories involved in validation is recommended to assess and compare the uniformity in the mode of calculation of desired parameters.

The positive control response must fall within defined limits, and assays should be performed in compliance with standard Good Laboratory Practice guidelines.

2.1.10 Evaluation and Interpretation of Results

The Panel agrees with the approach presented in the BRD. Because the choice of a standard ER preparation will have a significant influence on the IC_{50}, $K_d$, and $K_i$ values determined, the Panel recommends that recombinant human ER be employed for the validation and screening assays.

There is general agreement among the Panel with the designation of “equivocal” for substances that do not bring about a 50 % reduction in specific estrogen binding, particularly because other protein molecules such as heat shock proteins, co-activators, and co-repressors are known to influence estrogen binding properties (e.g., Horwitz et al., 1996; McKenna et al., 1999; McKenna and O’Malley, 2002). Because these proteins might be unavailable or present in suboptimal concentrations in certain ER preparations, negating accurate assessment of a substance’s estrogen mimicry, discordance in results from ER\textsubscript{\alpha} and ER\textsubscript{\beta} assays should be considered in substance classification.
Furthermore, the classification of a test substance as “positive for binding” should be based on the use of statistical inferences pertinent to the positive characterization. This may require new research and development into valid statistical methods for making such a characterization. The state of the art for making statistical inferences with endocrine disruptor data is nascent and requires more detailed research and study.

2.1.11 Test Report
The Panel agrees with the detailed delineation of the test substance, the solvent/vehicle and the ER, but it recommends that the type of protein assay be specified and that the concentration of protein used in the reactions be reported. The Panel agrees also that the remaining information requested for test conditions, results, discussion of results and conclusion was adequate to achieve the stated goals of the survey and its validation.

The Panel recommends establishing a new range of reference IC$_{50}$, K$_d$ and K$_i$ values with a standardized ER preparation using a test set of substances. These data will be far more useful in the evaluation of putative estrogen mimics by various laboratories involved in the validation process, rather than using historical values of these parameters collected with various receptor preparations.

Historical data can also be used to assess the biological significance of results for a current test that has shown to be statistically significant.

2.1.12 Replicate Studies
The Panel agrees with the recommendation stated in the BRD.

2.2 Considering the intended use of the assays as a toxicological screen, are there other minimum procedural standards that should be included? If so, what are they and why?
The Panel recommends standardizing the type of protein assay and the conditions, and highly recommends that both the androgen and estrogen receptor-based assay reactions contain 10 mM sodium molybdate as well as a cocktail of protease inhibitors such as those described on page B3-5 of the BRD, to minimize degradation of the receptor protein.

Although the hydroxylapatite (HAP) procedure has been used by numerous investigators, it is fraught with problems, not the least of which is that the receptor-ligand complexes are bound to the matrix, require retention during washing, and must retain complex association during elution. These are important variables to control. In contrast, the U.S. Food and Drug Administration (FDA) approved the testing of human tissues with the radioligand (estrogen) binding assay using the Dextran-Coated Charcoal procedure. The latter procedure with dextran-coated charcoal allows the receptor-ligand complexes to remain in the original reaction medium while removing the unbound ligand. Published clinical cancer studies utilizing these FDA-approved procedures in Assay Proficiency Surveys (e.g., Fisher et al. 1980, 1983, 2001; Wittliff et al. 1981, 1998) indicate the reproducibility and transferability of this assay.
The Panel recommends the use of the ligand titration assay using dextran-coated charcoal as the preferred procedure.

3.0 Recommendations for In Vitro ER Binding Test Method Protocols for Validation Studies

3.1 A standardized In Vitro ER Binding Assay protocol using rat uterine cytosol (RUC) is provided in Appendix B of the BRD. This assay is proposed for validation studies by the U.S. EPA and other sponsors. Section 12.3 discusses additional detail that should be added based on the minimum procedural standards in Section 12.2. In addition, an example of the In Vitro ER Binding RUC Assay (based on the U.S. EPA protocol), which incorporates the recommended minimum procedural standards, is provided in Section 12 Annex of the In Vitro ER Binding BRD. Considering the intended use of the assays as a toxicological screen, would the current protocols, with the additions detailed in Section 12.2 and 12.3, provide a level of detail to appropriately minimize interlaboratory variability? If not, what revisions or additions should be made to the protocols?

The Panel is comfortable with the BRD protocol for the ligand binding assay, provided that it is amended to include the additional details presented in the discussion on minimum procedural standards (e.g., protease inhibitors, protein concentration assays [noting interference]) of the BRD and the points discussed in response to Questions 1 and 2.

3.2 In addition to the minimum procedural details listed in Section 12.2, are there other protocol elements that should be considered for other In Vitro ER Binding assays recommended for validation as a toxicological screen, including those protocols provided in Appendix B?

Assays are routinely performed using isotopes for comparative displacement binding assays. The Panel appreciates that some laboratories may have difficulties using isotopes because of licensing restrictions and efforts to limit production of isotope waste. Anisotropy for displacement of fluorescent estrogen may be used in non-isotope assays. However, experience is presently limited on the strengths and limitations of this end point.

There is also a concern about using a potent estrogen as the only standard reference, given that many of the agents that we wish to evaluate are at the weak end of the potency spectrum. The standard reference serves a role distinct from that of the positive control. The response of the positive control measures the assay’s performance and stability over time. The use of a concurrent positive control is a hallmark of in vitro tests used in the regulatory arena worldwide. The use of a concurrent positive control is a stated “requirement” in protocols submitted to ICCVAM. The measurements of positive control performance need to address the assay endpoint(s) of interest (e.g., RBA values). They provide the basis for assessing the acceptability of the assay trial and thus the use of data from “unknowns” tested concurrently. Positive control data provide one basis for comparison of assay performance across laboratories. The positive control selected must be able to demonstrate both an under and over response relative to its historical values. The positive control suggested in Question 2 would provide the ability to measure both over and under prediction.
3.3 Considering the intended use of the assays as a toxicological screen, is the Panel aware of other available standardized protocols for assays recommended for validation?

The Panel’s consensus is that purified recombinant ERα be the primary receptor used to develop the first assay for validation. To date, there are no estrogen mimics that are selective for either ERα or ERβ. In this assay, both ligand titration and competition end points should be developed for test chemicals. The ERα was suggested in response to Question 2 and the use of a ligand binding assay with dextran-coated charcoal is recommended to separate bound from free labeled 17β-estradiol. Among species comparisons could be facilitated by employing glutathione-S-transferase fusion proteins consisting of the d-e-f domains from the respective ERs (GST).

4.0 Recommended List of Substances to be Used for Validation of In Vitro ER Binding Assays

Section 12.4 of the BRD provides a list of substances recommended for use in validation studies of in vitro ER binding assays.

4.1 Considering the intended use of the assays as a toxicological screen, does the Panel agree with the selection criteria, adequacy and appropriateness of substances recommended for validation studies in terms of the following issues? If not, what substances should be added or deleted?

4.1.1 Number and Distribution of Substance Across the Range of Measurable ER Binding Activity, Including Negatives

4.1.2 The Number and Range of Substances by Chemical Class

4.1.3 The Number and Range of Substances by Product Class

The Panel endorses the list of chemicals provided in the BRD, but with the following caveats.

There are concerns about how well the list represented the kinds of substances that the U.S. EPA is interested in screening, including the diversity of chemical classes, the range of potencies. The Panel also indicates that it will not be possible to determine the specificity of the assay with a list of substances 90% of which are positive. The recommendation is that at least 25% of the test substances be negative for ER binding.
References


Appendix 1
Ligand Titration Assay

Data File: C303S.DAT
Protocol File: 3HE2A.PRO

File ID: hERalpha with 3HE2/tris
1.1.1 12:33:03

Protein (mg/ml): 0.2
Weighting: Unity
Nonspecific Binding: # Points = 6
Correlation Coefficient, R = 0.9974
Linear and Non-Linear Regressions: # Points = 6

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Data File: C303S.DAT
Protocol File: 3HE2A.PRO Assay Protocol

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Total Incubation Volume (ml) .................................................................0.1500
Separation Solution Added (e.g., DCC) (ml) ........................................0.3000
Aliquot Counted (ml) .................................................................0.3000
Efficiency of Dose Tubes (~) ............................................................100.0000
Efficiency of Total Bound and NSB Tubes (~) ......................................100.0000
Specific Activity Value .................................................................158.0000
Specific Activity Units ....................................................................DPM/fmol
Data File: C303S.DAT  
Protocol File: 3HE2A.PRO

Measured Data Table

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Appendix A

Data File: C303S.DAT
Protocol File: 3HE2A.PRO

![Graph 1](image1)

Data File: C303S.DAT
Protocol File: 3HE2A.PRO

![Graph 2](image2)
### Appendix 2

**Ligand Competition Array**

#### Summary Table

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Concentration File: YER303L6

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II. *In Vitro* Estrogen Receptor (ER) Transcriptional Activation (TA) Assays

1.0 Recommendations and Priority for Validation Studies
The *in vitro* ER TA BRD reviews the comparative performance, reliability, advantages, and disadvantages for different *in vitro* ER TA assays, and recommends a relative priority for further development and/or validation based on this information.

1.1 Considering the intended use of the assays as a toxicological screen, is the Panel aware of other advantages and disadvantages for the assays discussed in the BRD?
The Panel has not identified additional advantages and disadvantages over and above those discussed in the BRD.

1.2 Considering the intended use of the assays as a toxicological screen, does the Panel agree with the relative priority recommended for these sets of assays? Does the Panel recommend any changes in priority, or have specific recommendations for prioritization?
The Panel, while agreeing that a stably transfected cell line is an attractive test method for prioritization (because such assays would be more amenable to high throughput screening), is not convinced that the BRD made a strong enough argument for this approach as having the highest priority. Reasons for this conclusion are:
1. Stable cell lines are notorious for losing their stability over time and therefore require continuous selection.
2. Their availability is limited and a highly responsive stable cell line is difficult to isolate.
3. While stably transfected cell lines might be potentially less challenging to use, they could have inherent confounding issues such as the effect of multiple receptor subtype activation.

Therefore, before making such assays a priority, appropriate comparative data relative to assays using transiently transfected cell lines (which generally have a higher level of responsiveness) should be generated using a select set of test substances.

1.2.1 Which receptor types (species, isoform) are the best for *In Vitro* ER TA Assays?
The Panel expresses a general preference for the use of the human ERα and ERβ subtypes in *in vitro* ER TA screening assays. However, if patent issues preclude the utilization of human gene sequences in commercial applications, consideration should be given to rat and possibly porcine receptors. Also, the potential for evaluation of receptors (ERα, β, or γ) from species of environmental relevance (e.g., fish) should be considered.

1.2.2 Should preference be given to cells with endogenous ER, transiently transfected ER expression vectors, or stably transfected ER expression vectors?
The Panel does not think that there was enough information in the BRD to make a judgment as to the superiority of one cell system over another; too few positive and negative chemicals have been tested for agonism or antagonism activity in multiple *in vitro* ER TA assays using different mammalian cell lines. Furthermore, the *in vivo* endocrine disrupting activity of a chemical would most likely be tissue-, cell-,
and promoter-specific. Therefore, the intrinsic responsiveness of a cell line cannot be generalized based on the result of a single assay system, due to the potential differences in coactivator populations, cross-talk with other receptors, and other signal transduction pathways between cell types.

The availability of stable cell lines that are already established should be investigated.

1.2.3 Which response elements (species, sequence) are the best for the reporter vectors?

The Panel believes that the effect of the reporter construct itself should not be underestimated in the validation of these assays. Issues such as single versus multiple estrogen response elements (EREs), other enhancer elements, and different minimal promoters are of importance in the evaluation and optimization of an assay system. However, the vitellogenin ERE (vit ERE) consensus sequence is recommended based on its broad-based responsiveness, although concerns about the activity of the vit ERE in some mammalian cells have been expressed by the Panel. The use of a reporter construct containing multiple EREs is recommended to maximize the sensitivity of the resulting assay and to minimize missing weak responders.

The Panel is also of the opinion that the development of a series of transient transfection assays for individual receptor subtypes (e.g., ERα, β) is worthwhile, especially in the context of the use of chimeric receptor-reporter constructs which would allow for greater mechanistic specificity.

Some Panel members commented on the fact that the use of such chimeric ligand binding domain (LBD) constructs could potentially minimize effects due to differing promoters/EREs.

Estrogenic compounds can stimulate transcriptional activation of the AP-1 (fos-jun) complex (that bind to regulatory sequences in the promoter of various target genes to modulate transcription) through a cooperative interaction of the ER with this complex. The fact that different ER ligand complexes have different affinities for fos-jun and other co-activators should be a consideration in the selection and evaluation of cell lines and assays for determination of ER TA screening assays.

Suggestions and Recommendations:
1. As an important part of the validation process, a study needs to be conducted to determine if stably transfected cell lines really do perform better than transiently transfected cells. This study would involve a comparison of a stably transfected mammalian line with one transiently transfected with the ER and other reporter plasmids, along with one expressing an endogenous ER.
2. If stably transfected cell lines are used, there should be a standard procedure for ensuring the maintenance of minimum response criteria to selected standards, including 17β-estradiol. Furthermore, treatment with the required selection
antibiotic should be performed on a regular basis to ensure maintenance of the inserted receptor and/or reporter construct.

3. These assays do not measure toxicity. The incorporation of an appropriate measure of cytotoxicity into the assay system is recommended.

2.0 Minimum Procedural Standards for In Vitro ER TA Assays

2.1 Considering the intended use of the assays as toxicological screens, does the Panel agree with the adequacy of the proposed procedural standards recommended for In Vitro ER TA Assays?

2.1.1 Transcriptional Activation of the Reference Estrogen
The Panel agrees that the transcriptional activation-inducing ability of 17β-estradiol must be demonstrated and that consistency in the level of response is appropriate as a criterion for assay acceptance. There was a question of whether the level of response should be from a single dose or if it would be necessary to obtain a full dose response curve. The Panel generally felt a full dose-response curve would be more informative.

2.2.2 Reference Estrogen
17β-Estradiol is appropriate as the reference estrogen. The Panel recommends that preliminary studies be performed with multiple transactivation assays to statistically define assay performance expectations for 17β-estradiol dose response curves (i.e., maximum fold induction, EC₅₀ values, confidence limits). Concentrations to be tested for the reference positive control, 17β-estradiol, should range from 1 pM to 1 μM to establish a full dose-response curve. Clear guidance is needed with regard to expectations for the extent of response that should be observed, which can be determined based on preliminary studies.

2.1.3 Preparation of Test Substances
The test substances should be prepared in water, 95-100% ethanol, or dimethyl sulfoxide (DMSO), depending upon their solubility. Preference should be given to the solvent that allows testing of the maximal concentration of the test substance, without exceeding the limit dose. However, in situations where more than one solvent could be used, preference should be given to water, then 95-100% ethanol, and then DMSO, in that order. The Panel suggests that guidelines be provided with regard to the concentration of solvent in the stock solution. Even when using 95-100% ethanol or DMSO, substances to be tested could be prepared in stock solutions where their concentration approaches solubility limits. This could introduce variation from laboratory to laboratory. In addition, it should be stated that standards or positive controls need to be dissolved in the same solvent and to the same maximal concentration. One Panel member indicated a preference for 95-100% ethanol at concentrations of 0.01 to 0.1% (v/v) because some substances dissolved in DMSO have been observed to exhibit lower activity than when dissolved in ethanol.
The Panel concludes that it would be prudent to perform a prevalidation of \textit{in vitro} ER TA assays with the reference estrogen for assessment of the level of solvent that does not adversely affect assay response.

\subsection*{2.1.4 Concentration Range of Test Substances}

The Panel generally agrees that, for both agonism and antagonism assays, the limit concentration should be 1 mM as long as the solubility characteristics and potential cytotoxicity of each test substance is taken into consideration. However, concentrations greater than 10 \(\mu\)M are often problematic due to solubility issues in aqueous media, compounding the level of toxicity. Thus, one Panel member recommended 0.1 mM as the limit concentration. At a minimum, the solubility of the substance should be reported and the concentration used in the test should not exceed the limit of solubility.

If the limit concentration is used, the Panel generally agrees that seven test substance concentrations at log intervals should be tested. However, one Panel member suggested that it is likely that covering a concentration range of five orders of magnitude will be appropriate and that it is unclear what would be gained by using anything other than one order of magnitude between the doses. This is especially true since the assay results will only be assessed in a semi-quantitative manner (i.e., \(IC_{50}\) or \(EC_{50}\) values should not be used to rank compounds regarding possible potency).

If a lower maximum concentration is tested due to solubility constraints or excessive cytotoxicity, the number of concentrations tested can be adjusted to account for the altered concentration range.

In addition, a measure of cellular cytotoxicity incorporated into the assay could help define the upper limit for test material concentrations, similar to the Maximum Tolerated Dose (MTD) approach used in \textit{in vivo} studies. This type of assessment might mitigate the need to go to concentrations higher than 10 \(\mu\)M.

The Panel also recommends that a sequential testing strategy be adopted. For example, if the substance is positive for agonist activity, there would seem to be little value in testing it for ER antagonist activity or AR-mediated activities. A positive result in any of these assays will likely warrant further examination in \textit{in vivo} screens and tests other than transactivation assays. However, the validity of this approach should be evaluated in the prevalidation phase and a decision made on its applicability after sufficient data has been reviewed.

Assuming that each test substance is tested as an agonist and an antagonist, substances demonstrating a positive agonist response could also be tested with ICI 182,780 (the candidate ER antagonist) to make sure that the effect reflects a receptor-mediated activity. Assuming that ICI 182,780 does block the action of the
test substance under the test conditions used, it will also help to indicate the presence of toxicity if the signal level is significantly below that of ICI 182,780 alone.

During deliberations of the Panel, it was suggested that the concentrations (doses) of chemicals tested be similar for ER binding and ER transcriptional activation assays. However, the concentrations presented to the receptor in cell based systems could be markedly different from that presented to the receptor in media. Differences in chemical properties in serum or in solvent can markedly affect the uptake of chemicals by cells. Thus, the magnitude of response may not be directly comparable between cell-free binding assays and cell based TA assays. When considered important, radiolabeled test chemicals should be used to determine the amount of chemical taken up by the cells.

2.1.5 Solvent and Positive Controls
The Panel concludes that the recommendations in the BRD are appropriate. ICI 182,780 appears to completely block 17β-estradiol at 0.1 µM. However, the collective experience of the Panel is that obtaining ICI 182,780 might require permission from its producer in England and the maximum amount available might possibly be only 10 mg/year. One Panel member expressed reservations concerning the use of tamoxifen as a positive control. In addition, as with the reference standard for agonist activity, clear guidelines for the positive antagonist and the expected extent of antagonism when testing the substance should be provided. Perhaps running a parallel toxicity study on the same plates with a compound like Alamar Blue or a metabolism assay would add additional information.

During the deliberations of the Panel, it was suggested that a weak estrogen (e.g., estriol) should be included routinely in assays along with 17β-estradiol as a reference compound. Whether this will add necessary and sufficient information should be evaluated in prevalidation assays. If the inclusion of a weak estrogen improves the ability to interpret the results obtained with validation chemicals, or with unknowns that are weak estrogens, then the inclusion of a weak estrogen reference positive control is strongly recommended.

2.1.6 Within-Test Replicates
The Panel recommends that, initially, each test chemical concentration be tested in triplicate. The validity of this approach, however, should be evaluated with statistical consultation.

2.1.7 Dose Spacing
The Panel generally agrees with the recommended dose spacing of one order of magnitude in the concentrations of the candidate test substances but did not think the use of half-log doses in certain cases would be useful.
2.1.8 Data Analysis

2.1.8.1 Response Variable for Evaluating the Potential Agonism/Antagonism of a Substance

In Section 5-3 of the BRD, it is stated that there are several different definitions available for the "relative activity" of a test substance. The Panel recommends that a consensus be reached on one definition that can be considered as the standard definition for all future assays. This is vital because important decisions, including the final call (positive/negative agonism, and positive/negative antagonism) regarding a chemical are based on the chosen definition.

2.1.8.2 Assumptions Made in a Statistical Analysis

Most statistical procedures make certain assumptions regarding the underlying data. For instance, if ANOVA type methods are used, then it is necessary to demonstrate that there is no heteroscedasticity and that the data are approximately normal. The Panel recommends that before any statistical procedure is used, suitable diagnostics are performed to make sure all underlying assumptions regarding the procedure are true. If the assumptions are not true then suitable data transformations might be performed before analyzing the data.

2.1.8.3 Estimation of EC\textsubscript{50}/IC\textsubscript{50} Values and a "Steepness" Parameter

If it is appropriate to perform nonlinear modeling, then a suitable nonlinear model should be selected. In some cases, the Hill equation might be suitable. Once a model is selected, appropriate model diagnostics need to be performed to ensure that the model fits the data and the various underlying assumptions such as normality and homoscedasticity are true. Diagnostics should also be performed to detect curvature effects, typically by using standard asymptotic confidence intervals. However, in some situations, especially in the presence of curvature effects, these confidence intervals might not be appropriate. In such cases, a resampling procedure such as jackknife or bootstrap\textsuperscript{1} might be used.

2.1.8.4 Combining EC\textsubscript{50} and IC\textsubscript{50} Estimates from Different Laboratories

To obtain estimates of mean EC\textsubscript{50} and mean IC\textsubscript{50} values from different laboratories, the average across laboratories should not be computed but rather estimates should be obtained using mixed effects nonlinear models, treating laboratories as the random effects. This approach takes into

account within and between laboratory variability. This methodology also allows for a determination of corresponding standard errors.\(^2\)

### 2.1.8.5 Uniformity Trials

To understand the underlying variability in the data, which is important for proper data analysis, the Panel recommends undertaking a set of carefully planned comprehensive interlaboratory negative control studies.

### 2.1.9 Assay Acceptance Criteria

Reference substances for agonism and antagonism should give responses within appropriate confidence limits. These confidence limits should be determined in preliminary studies (see Section 2.1.2). The Panel recommends that guidelines be provided for a certain level of agonism or antagonism expected for the reference standards and that responses in these ranges should be required if the assay is to be accepted. There should also be a minimal fold (\(B_{\max}\)) and/or minimal fold/between experiment variance ratio for the assay. This will be needed for each assay type recommended.

Incorporation of an evaluation of cell cytotoxicity/cell proliferation should be included in the protocol to ensure that responses at non-toxic doses only are analyzed, and acceptance criteria need to be established for when cytotoxicity affects the performance of the assay.

The Panel recommends that the assays be performed following Good Laboratory Practice guidelines.

### 2.1.10 Evaluation and Interpretation of Results

The interpretation of positive results for a substance as an agonist or antagonist should incorporate some elements of a dose-response relationship in comparison to the reference standards. Simply classifying a substance as an ER agonist based on significance above the concurrent control without consideration of a dose-response is not sufficient. In addition, assay performance criteria must be within an acceptable range.

### 2.1.11 Test Report

In addition to the information required for the test report, as listed in the BRD, the complete DNA sequence of constructs and vectors used for receptor and reporter genes should be identified. All assay parameters regarding cells, plasmids, culture methods, transfection methods, detection of luciferase activity, etc. must also be reported. If it is a transfection assay, there is also a need to include a constitutive reporter gene assay to control for transfection efficiency between wells. The passage number of the cell line should be monitored, as well as the CO\(_2\) level during culturing.

and treatment. The EC$_{50}$ value for agonism or the IC$_{50}$ value for antagonism, together with corresponding confidence limits, must be reported.

2.1.12 Replicate Studies
The Panel concludes that the appropriate extent of replication should be determined after prevalidation studies have been conducted and the resulting data statistically evaluated. However, it was generally thought that replicate studies in a screening assay are probably not required as long as the expected response from the reference standards has been observed and a statistically meaningful dose response is observed for the test substance(s). If either of these provides data that do not conform to expectations, the assay should be repeated, as per standard practice. Defining the minimum standards for replication will need to wait until the extent of variation within a test has been carefully evaluated. In contrast, one Panel member believes that each substance should be tested at least three times in different experiments because it could be toxic in one assay (due to low cell density, fitness, etc.) and then the activity is detected in another assay. If repeated, the incorporation of more closely spaced dosing/treatment concentrations in the replicate assay based on the initial test results should also facilitate better analysis of the overall dose-response of the test substance.

2.2 Considering the intended use of the assays as a toxicological screen, are there other minimum procedural standards that should be included? If so, what are they and why?
This issue has been discussed in preceding sections, as appropriate.

General Statements or Comments in Regard to the BRD - The Panel concludes that:
1. There was inconsistency in the statements on pages 12-1 and 12-11 of the BRD concerning stable versus transiently transfected cell lines.
2. There was no discussion of individual assays for ER$\alpha$ and $\beta$.
3. In vitro ER TA assays are not a toxicological screen as stated in the questions to the Panel. They are simply a measure of transactivation. Further research is needed to understand the link between the results from this in vitro assay and a toxicological effect in an organism.

3.0 Recommendations for In Vitro ER TA Test Method Protocols for Validation Studies
Protocols provided by scientists with expertise in in vitro ER TA test methods were provided in Appendix B of the BRD. Section 12.3 discusses additional details that should be added, based on the minimum procedural standards in Section 12.2.

3.1 Considering the intended use of the assays as a toxicological screen, would the current protocols, with the additions detailed in Sections 12.2 and 12.3, provide a level of detail to appropriately minimize interlaboratory variability? If not, what revisions or additions should be made to the protocols?
The consensus among the Panel is that the protocols adequately described the needed procedures and that any laboratory with cell/yeast culture and basic molecular experience should be able to produce dependable results.
Some interlab variability may be expected due to laboratory specific culture techniques (for example, cell counting, determination of the percentage of confluence, ability to seed plates evenly, etc.). Perhaps standards or performance criteria should be specified for such activities.

3.2 In addition to the minimum procedural details listed in Section 12.2, are there other protocol elements that should be considered for In Vitro ER TA Assays recommended for validation as a toxicological screen, including those protocols provided in Appendix B? The Panel believes that the level of detail was generally sufficient, but recognizes that performance would depend on the experience of the staff in each laboratory. During Panel deliberations, one member suggested that additional procedural details should be added if volatile chemicals are tested. Another member suggested that problems with volatiles might be reduced if specifications are made for incubators without circulation fans in the chamber.

The Panel concludes that the following issues might need to be added or expanded on in the protocols:

1. Standards should be provided for uniform counting and plating of cells among wells and between experiments.

2. Discussion and review of methods should be included for making dextran coated charcoal (DCC)-stripped sera and perhaps even a recommendation for commercial sources.

3. Discussion and review of known sources of estrogen contamination in the laboratory should be included. This might include plastic ware as well as media additives and commercial prepared media. Some tubes, filter units, antibiotic mixtures, and pre-made media in polycarbonate bottles are examples of suspect and/or problematic items. Performance criteria should be established for determining background levels of hormone active contaminants (e.g., by comparing the reporter activity for ICI 182,780 versus the blank). This could also include methods to maintain a hormone free lab environment.

4. The issue of culturing some cell stocks in estrogen rich media and then withdrawing them to an estrogen free media may need to be expanded or emphasized. Suggested washing techniques, including the number of days for withdrawal, etc., need to be detailed. Again, performance criteria should be established to show that each experiment was conducted using estrogen free conditions (e.g., by comparing the reporter activity for ICI 182,780 versus the blank).

5. Another issue related to hormone active contamination is that while an estrogen free environment is required for reliable estrogen activity assays, it does not seem to be an issue for androgen receptor (AR) activity determinations. However, it should be noted that there is potential for cross talk between "estrogenic" media contaminants and other signaling pathways. Thus, it is not clear what effects this may have on androgen activity assays. Perhaps the AR methods should use hormone free procedures as a precaution (no phenol red, DCC sera, etc.).

6. The metabolism of chemicals selected for validation, or unknowns, is an important concern in cell-based assays. Oxidative (Phase I) or conjugation (Phase II) reactions
can convert pro-estrogens to active estrogens or inactivate parent chemicals that are active estrogens. Cells in primary cultures have inconsistent capacities for xenobiotic metabolism. Cell lines often have limited capacity for xenobiotic biotransformation. Given the number of chemicals involved and the number of enzymes potentially involved, the Panel suggests that the scope of effort to determine pathways, products, and activities of products is beyond what would be feasible in a validation study. However, the Panel recommends that available information on the P450 complement and Phase II enzyme complement be compiled for the cell lines employed in this validation process. The Panel also recommends that available information on the metabolism of the validation chemicals is compiled, including the degree to which metabolism alters estrogenic activity. It is further recommended that studies to obtain such information for systems ultimately employed in screening be planned and performed, when applicable. While metabolism could affect the magnitude of the signal of parent estrogens, it is unlikely to negate the possibility of detecting such activity. If metabolic conversion of proestrogens to an active derivative occurs at very low rates, then the estrogenic activity could be missed.

3.3 Considering the intended use of the assays as a toxicological screen, is the Panel aware of other available standardized protocols for assays recommended for validation?

One Panel member indicated the availability of a standardized protocol for the MVLN assay, which uses an MCF-7 cell line derivative known as MVLN. This cell line, which harbors an endogenous ER, has been stably transfected with the luciferase reporter gene under control of the vit ERE. Estrogen-specific transcription activity is directly related to luciferase activity. Cells, seeded into a microtiter plate, are treated with the test substance and incubated overnight. The following day the cells are lysed and luciferase activity in the supernatant is measured in a luminometer. A copy of the protocol has been added to Appendix B of the ER TA BRD.

4.0 Recommended List of Substances to be Used for Validation of In Vitro ER TA Assays

4.1 Does the Panel agree with the selection criteria, adequacy, and appropriateness of substances recommended for validation studies in terms of the following issues? If not, what substances should be added or deleted?

In general, the Panel agrees with the selection criteria, adequacy, and appropriateness of the chemicals chosen for the validation studies. However, several specific concerns were raised and the Panel made recommendation to address these concerns.

4.1.1 Number and Distribution of Substance Across the Range of ER TA Responses, Including Negatives

The basis in the BRD for the selection of chemicals to use in the validation of in vitro ER TA assays was not based on ER binding only. Rather, selection was based on the median EC$_{50}$ values of the chemicals to induce the expression of a reporter gene, as reported in published papers and publicly available reports. Table 12-1 in Section 12 (pages 12-9 through 12-10) of the BRD lists thirty-one substances that are recommended for validation of in vitro ER TA agonist assays. The median
EC$_{50}$s for these chemicals range from 8.85 (methoxychlor) to 0.000011 μM (17α-ethinyl estradiol), although no indication of the variation around these values was provided (many of the values were derived from a single study). This represents a six log range of EC$_{50}$ values from $10^{-5}$ μM to 10 μM. In addition, the list included one equivocal chemical and five chemicals that gave negative results when tested.

The Panel concludes that the distribution of the recommended agonist chemicals across the range of “potency” responses, based on the EC$_{50}$ values, for agonist activity in reporter gene assays was appropriate.

In Table 12-2 in Section 12 (page 12-10) of the BRD, 21 substances are listed as being recommended for validation of in vitro ER TA antagonism assays. Based on the published/publicly available data, the substances were categorized qualitatively as positive or negative for ER antagonist activity; 17 of the substances are positive and four are negative.

The Panel concludes that this group of recommended antagonist chemicals seemed appropriate, although selection is based only on their qualitative classification.

However, the Panel has some concerns and qualifications to these responses. Of the 31 chemicals recommended to validate in vitro ER TA agonist assays and of the 21 chemicals recommended to validate in vitro ER TA antagonist assays, only six chemicals are in common. These are α-zearalanol, zearalenone, phloretin, bisphenol A, coumestrol, and atrazine. The “scientific” basis for these chemicals being the ones that are common between these two assays is not apparent. Are they in common for some reason or is it just by chance? Is the basis for selection that they cover a range of relative binding affinity (RBA) values for binding to the ER?

The Panel recommends that consideration be given to choosing a set of chemicals that will be used and that the selection process be based on a solid scientific rationale such as a combination of existing ER binding, antagonist, or agonist data. (A particular chemical need not have published/public data available for its performance in all three assays, but should have data available for at least one or two such properties).

Of the 31 chemicals recommended to validate in vitro ER TA agonist assays and the 21 chemicals recommended to validate in vitro ER TA antagonist assays, only five are expected to be negative in agonist assays and only four are expected to be negative in antagonist assays.

The Panel recommends the inclusion of additional negative chemicals in the list to more effectively evaluate the specificity of the assays.

Of the 31 chemicals recommended for validation of in vitro ER TA agonist assays and of the 21 chemicals recommended for validation of in vitro ER TA antagonist assays, only 16 and eight chemicals, respectively, are on the list of 33 chemicals
recommended for validation of *in vitro* ER binding assays. (see Table 1 and Section 12.4, pages 12-11 through 12-16 of the *In Vitro* ER Binding BRD).

The Panel recommends that complete overlap exist for chemicals to be tested in both the *in vitro* ER binding and transcriptional assays, or at the very least, that a core of chemicals that is common to all *in vitro* assays be developed. The scientific basis for this selection should be distribution across a range of RBAs, EC$_{50}$ values for TA agonism activity, and positive/negative responses for TA antagonism activity.

The Panel does not expect that data for all three effects will be available for each chemical. However, the list should include chemicals covering RBA values ranging from $>10$ to $<0.0001$ plus negatives (see Table 12-1 of the *In Vitro* ER Binding BRD); chemicals with *in vitro* ER TA agonism potencies, as shown in Table 12-1 of the *In Vitro* ER TA BRD, ranging from $10^{-5}$ μM to $>1.0$ μM, including negatives; and substances classified as positive and negative for *in vitro* ER TA antagonism activity. During the Expert Panel meeting, there was discussion concerning the possible need for a prevalidation study with regard to identifying the most appropriate *in vitro* ER TA assays to use. If a prevalidation study is undertaken, it would be appropriate to conduct it using a smaller number of chemicals. Nevertheless, the basis of selection of such a smaller group of chemicals should also be based on a solid scientific rationale. Table 1 can serve as the starting point for chemical selection.

Another concern pertains to coordination of the selected substances with those being proposed for use by the Endocrine Disruptor Methods Validation Subcommittee (EDMVS) of the U.S. Environmental Protection Agency (EPA) for the *In Vivo* Screening Assays. The overlap of chemicals should also be reviewed for *in vivo* Tier I and Tier II studies under consideration by the EDMVS and the Organisation for Economic Co-operation and Development (OECD) Global Harmonization Program so that responses can ultimately be compared throughout the entire screening and testing battery.

The Panel recommends close collaboration and cooperation regarding the chemical selection process by ICCVAM with the *in vivo* test validation studies being reviewed by the EDMVS and OECD.

### 4.1.2 The Number and Range of Substances by Chemical Class

The 31 chemicals selected for the validation of *in vitro* ER TA agonism assays and the 21 chemicals selected for the validation of *in vitro* ER TA antagonism assays represent a wide range of chemical classes. However, a couple of notable deficiencies were identified. In particular, no phthalates or polychlorinated biphenyls were included. In addition, only two polycyclic aromatic hydrocarbons were selected, two for antagonism assays and only one for agonism assays. However, evaluation of these substances could follow the prevalidation or validation steps.
The Panel recommends that these deficiencies in the chemical list be considered as a revised list of substances is developed as recommended above. However, it may be more appropriate to address these issues following the initial prevalidation studies referred to above.

Specific compounds in Tables 12.1 and 12.2 of the BRD identified as chiral are (this may not be all): zearalenone, β-zearalenol, o,p’-DDT (dichlorodiphenyltrichloroethane), naringenin, and heptachlor. Specific substances in Tables 12.1 and 12.2 that may have multiple isomers present (positional isomers that may or may not be chiral): zearanol, chlordane (cis and trans), methoxychlor (p,p’ and o,p’), dicofol (p,p’ and o,p’). Chiral components that might be present are: both zearanol isomers, cis and trans chlordane, o,p’-methoxychlor, o,p’-dicofol. It is possible for one enantiomer to have agonist and another antagonist activities and for the racemate to be neutral.

The Panel recommends that chiral compounds be evaluated as the racemate and as individual enantiomers, as available. Efforts should be made to test only pure isomers when possible (e.g., cis and/or trans chlordane, p,p’-methoxychlor, p,p’-dicofol, etc.) and to provide analytical data from suppliers indicating what the isomer and/or enantiomer ratio is so data can be related to others in the validation study. The Panel felt that investigation of chiral issues may, in many cases, be deferred until prevalidation studies identify an optimal assay(s)/protocol(s) to validate further.

It is not clear that the Chemical Abstracts Service Registry Numbers (CAS RN) given for zearanol, chlordane, methoxychlor, and dicofol are the designation for the isomer mix or for one pure isomer.

The Panel recommends that CAS numbers should be checked since they are sometimes different for commercial grade mixtures compared to pure compounds.

4.1.3 The Number and Range in Substances by Product Class
The chemicals selected cover a range of products from the pharmaceuticals, natural products, chemical intermediates, and pesticides. Natural product chemicals appear at a somewhat higher frequency and pesticides seem appropriately represented.

The Panel feels that the range of products is appropriate for a validation study.
Table 1
Substances in Common Between *In Vitro* ER Binding Assays and *In Vitro* ER TA Agonism and Antagonism Assays

<table>
<thead>
<tr>
<th>Substance</th>
<th>Median EC50 (mM)*</th>
<th>Median RBA*</th>
<th>Agonist</th>
<th>Antagonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diethylstilbestrol</td>
<td>0.0000189</td>
<td>214</td>
<td>Agonist</td>
<td></td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>0.000098</td>
<td>100</td>
<td>Agonist</td>
<td></td>
</tr>
<tr>
<td>Estrone</td>
<td>0.00063</td>
<td>48</td>
<td>Agonist</td>
<td></td>
</tr>
<tr>
<td>Zearalenone</td>
<td>0.002</td>
<td>44</td>
<td>Agonist</td>
<td>positive</td>
</tr>
<tr>
<td>Coumestrol</td>
<td>0.0168</td>
<td>1.9</td>
<td>Agonist</td>
<td>negative</td>
</tr>
<tr>
<td>Estriol</td>
<td>0.0348</td>
<td>16</td>
<td>Agonist</td>
<td></td>
</tr>
<tr>
<td>4-tert-Octylphenol</td>
<td>0.05</td>
<td>0.20</td>
<td>Agonist</td>
<td></td>
</tr>
<tr>
<td>Genistin</td>
<td>0.075</td>
<td>0.56</td>
<td>Agonist</td>
<td></td>
</tr>
<tr>
<td>Phloretin</td>
<td>0.03</td>
<td>0.069</td>
<td>Agonist</td>
<td>positive</td>
</tr>
<tr>
<td>Bisphenol A</td>
<td>0.45</td>
<td>2.6</td>
<td>Agonist</td>
<td>positive</td>
</tr>
<tr>
<td>o,p'-DDT</td>
<td>0.66</td>
<td>0.013</td>
<td>Agonist</td>
<td></td>
</tr>
<tr>
<td>Naringenin</td>
<td>1.0</td>
<td>0.008</td>
<td>Agonist</td>
<td></td>
</tr>
<tr>
<td>o,p'-DDT</td>
<td>2.0</td>
<td>0.003</td>
<td>Agonist</td>
<td></td>
</tr>
<tr>
<td>Methoxychlor</td>
<td>8.85</td>
<td>0.001</td>
<td>Agonist</td>
<td></td>
</tr>
<tr>
<td>Progestrone</td>
<td>equivocal</td>
<td>0.0003</td>
<td>Agonist</td>
<td></td>
</tr>
<tr>
<td>Atrazine</td>
<td>negative</td>
<td>0.0003</td>
<td>Agonist</td>
<td>negative</td>
</tr>
<tr>
<td>4 Hydroxytamoxifen</td>
<td>positive</td>
<td>175</td>
<td>--------</td>
<td>positive</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>positive</td>
<td>3.1</td>
<td>--------</td>
<td>positive</td>
</tr>
<tr>
<td>4-Octylphenyl</td>
<td>positive</td>
<td>0.005</td>
<td>--------</td>
<td>positive</td>
</tr>
</tbody>
</table>

*Values from Tables in *In Vitro* ER Binding and *In Vitro* ER TA BRDs.*
Additional Panel Recommendations

The Panel’s recommendations are not only for in vitro ER TA assays but also some general comments that are applicable to the performance and use of these types of in vitro assays.

1. The Panel recommends the development of datasets for statistical analysis (i.e., confidence limits), to assess reliability and to determine the impact of variability. Pre-validation studies should be carried out to provide data for evaluation by the statisticians.

2. There is a need to assess the ability of these in vitro screens to predict in vivo responses. One way to accomplish this is to make sure that substances to be tested in the in vitro screens are also tested in the in vivo screens and tests so that information and the “weight of the evidence” can be assessed for particular chemicals.

3. More comprehensive in vitro methods are needed to detect endocrine disrupters. The regulatory community does not need to depend forever on the current assays. As methods develop and are refined, their utility in the screening process need to be evaluated. Different protocols need to be further optimized and the most reliable assays identified.

4. The Panel encourages the development of a centralized repository of chemicals with verified purity that can be used across assays.

5. It is well established that ER mediated gene expression is species, tissue, cell and promoter context specific. Consequently, the agonist and/or antagonist activities of a ligand cannot be generalized or extrapolated to all genes based on a single assay. Moreover, in vitro ER TA assays use artificial reporter genes (i.e., engineered with multiple ERE and minimal promoters), further limiting their utility for predicting in vivo ligand activity. Therefore, a sequential testing strategy is recommended for in vitro AR TA agonism/antagonism assays. If a substance induces a positive effect in any of these assays, testing in additional in vitro ER and AR binding or TA agonism/antagonism assays should not be conducted before proceeding to short term Tier 1 in vivo studies. The rational for this recommendation is that a positive response in these assays only demonstrates a functional consequence and in TA assays, an agonist/antagonist classification for this artificial response system. It is highly likely that the substance will elicit a broad spectrum of agonist and antagonist gene expression responses in vivo and therefore, further classification of the activity of the substance using TA assays will provide little additional information that will assist with prioritization and subsequent in vivo study design.

If the substance fails to induce an agonist response in an in vitro AR TA assay, antagonist activity should be investigated since some substances may only exhibit antagonist activity in the TA assay being used. Similarly, if a substance exhibits agonist or antagonist activity in an in vitro ER TA assay, it is questionable if testing for AR activity will provide significant additional information since the positive ER-mediated activity will trigger further short-term Tier 1 in vivo testing.

The Panel recommends that a sequential testing strategy be evaluated as part of a pre-validation assessment of in vitro AR TA assays in order to determine the value of performing
agonism and antagonism studies for estrogen and androgen receptors and how information from these assays are used to decide subsequent short-term Tier 1 \textit{in vivo} testing.

6. Standardization and validation across laboratories performing these studies for regulatory decision-making is critical and must occur before these assays are used for regulatory purposes. The work that is outlined in the BRD suggests that within laboratory variability for some of the assays is acceptable and the studies are reproducible. However, little between laboratory standardization has been performed. A formal validation process is needed in order to establish a “gold standard” study for use and not have many personal variants of similar assays.
III. *In Vitro* Androgen Receptor (AR) Binding Assays

1.0 Recommendations and Priority for Validation Studies

The AR Binding BRD reviews the comparative performance, reliability, advantages, and disadvantages for different *in vitro* AR binding assays, and recommends a relative priority for further development and/or validation based on this information.

1.1 Considering the intended use of the assays as a toxicological screen, is the Panel aware of other advantages and disadvantages for the assays discussed in the BRD?

Many of the advantages and disadvantages are presented in the BRD, and these, for the most part, are reasonable. However, additional comments by the Panel are found below.

The Panel rejects the two principal recommendations of assay protocols put forward in the BRD. Neither the rat prostate cytosol (RPC) nor the transient transfection of COS cells with the human AR expression vector were deemed to be optimal for *in vitro* AR binding assays. Rather, based upon scientific rationale, the Panel proposes and recommends that a high-throughput assay for AR binding be developed using purified, recombinant full-length AR protein. This recommendation is concordant with that of the *in vitro* ER binding Panel members for validation of binding assays based upon purified, recombinant receptor proteins.

For the purposes of organization, the responses to this question have been organized by the type of assay (i.e., cytosol-based, cell-based, and use of purified AR). Other comments/issues relevant to many of the assays are also summarized below. Recommendations, where applicable, are also included. For the purposes of the BRD, it might be useful to construct a summary table listing the type of assay along with the relative advantages and disadvantages of each assay.

A. **Cytosol-based Assays (RPC Assay):**

The RPC assay has historically been the assay most frequently used in published studies of AR binding. The experimental protocol for this assay was described in greatest detail within the BRD. This assay is currently being used by the U.S. Environmental Protection Agency (EPA) for testing the ability of test chemicals to bind to the AR. Although the Panel recognized several advantages of this assay, it was reluctant to endorse this assay for future studies based upon a significant number of disadvantages.

Among the advantages of the RPC assay was its description as the “gold standard” for *in vitro* assays of AR binding. As such, this is particularly useful as a reference. Another advantage that should not be minimized is the fact that the rat prostate expresses endogenous AR and the AR functions to regulate specific genes in the prostate. Although this latter advantage may seem obvious, it is significant with respect to other, heterologous cell-based assay systems in which the AR is expressed from a foreign expression vector.
A significant number of disadvantages were recognized within the RPC assay. This assay was viewed as an inappropriate use of animals in a single type of assay for which the small (500-700 mg) ventral prostate tissue was harvested. The assay is time consuming, for, among other reasons, the animals must be castrated 18-24 h prior to the harvest of tissue to reduce the binding of endogenous androgen to the receptor. The RPC is an assay of rat AR and not human AR. The AR is notoriously labile in in vitro systems, including the RPC assay, and significant methodological precautions must be taken to avoid its degradation. The RPC assay measures ligand binding, and not any functional aspect of the AR. A cell-free binding assay by nature cannot distinguish between agonists and antagonists. The in vitro binding of chemicals and ligands to the AR in the RPC assay occurs at 4°C (i.e., it does not occur under physiologic conditions of temperature or intracellular environment). RPC is a crude tissue cytosol preparation that contains many proteins in addition to AR, including other endogenous steroid receptors that may interfere with the assay. In addition, some metabolism of the reference ligand and/or test chemical may occur even in cytosol preparations incubated at 4°C.

Although the RPC assay has been widely used for many years to assess the binding properties of the rat AR, the Panel recommended that other AR binding assays be considered and developed in place of the RPC assay.

B. Cell-based Assays (COS + hAR Assay):
A second assay described in the BRD was based upon AR binding studies conducted in heterologous cells (e.g., COS monkey kidney cells) that were transfected with a human AR expression plasmid to express the receptor. The advantage of such a system is that it models whole cell, physiologic conditions for ligand binding. The fact that only the AR and not other receptors are expressed in transfected COS cells is an advantage. Moreover, the AR in this assay system is most often human, but the AR of other species can also be expressed in the COS cells to assess the binding of chemicals that may be relevant to a particular animal, fish or amphibian species.

Although numerous research laboratories have utilized this assay to characterize basic functional properties of the human AR, a number of disadvantages can be cited relevant to its use in evaluating and validating the binding of chemicals to AR. As mentioned above, the hAR expression vector must be transfected into COS cells. In addition to being labor intensive, this procedure has the potential of being highly variable between laboratories, especially in the absence of detailed methodological protocols. Only a fraction (which also can be quite variable) of the cells will express AR, and the expression is most often artificially high within individual cells. When gene expression occurs following transient transfection, the gene is not subject to normal restrictions of chromatin structure. COS cells do not normally express AR and therefore, the intracellular environment is artificial compared to an androgen-responsive cell. If polymorphisms for the hAR have functional significance, the effects of this genetic variability will not be appreciated in cells that express a single form of AR from an expression vector. In an intact cell system, access of different
ligands to the AR may be determined by differential kinetics of transport across the cell membrane, of metabolism, and of binding to cellular components. Similarly, it may not be clear if metabolism of the test chemical has taken place to a metabolite that has no binding, some binding or even higher binding to AR. The derivation of COS cells from monkey kidney also suggests that species differences in the degree and type of metabolism that takes place in these cells may not accurately reflect the human situation in target cells.

In summary, the Panel suggests that the use of a stable-transfected cell line would preclude some of the difficulties inherent to the use of transient transfection assays. However, the Panel recognizes that stable cell lines are also prone to instability over time in culture. The Panel recommends consideration of other human cells lines that might be amenable to establishing transient or stable transfection/expression of the human AR.

C. Assay Using Purified hAR:
A third assay system that was not included in the BRD, but which garnered the most support among members of the Panel, was the development of an in vitro, high-throughput AR binding assay based upon the use of a purified, recombinant human AR. By definition, this assay would be the most efficacious and time-saving of the potential assay systems. It eliminates any problems associated with the use of animals or cells. Large amounts of recombinant protein could be produced in bulk and supplied as a homogeneous, uniform preparation to all test laboratories. Assurances regarding the steady production and availability of recombinant AR protein would, however, need to be ascertained from potential suppliers. The use of purified, recombinant protein can be readily adapted to high-throughput methods of analysis. Disadvantages regarding the potential absence of biologically significant post-translational modifications of the recombinant AR protein and the absence of a putative biologically relevant environment during the conduct of the binding assays were noted.

The Panel enthusiastically recommends, with highest priority, the development of an assay using purified, recombinant AR from human, rat or another mammalian species.

1.2 Considering the intended use of the assays as a toxicological screen, does the Panel agree with the relative priority recommended for these sets of assays? Does the Panel recommend any changes in priority, or have specific recommendations for prioritization?
As stated previously, the Panel strongly encourages the development of an AR binding assay based upon the use of a purified, recombinant form of AR.

1.2.1 In considering prioritization, is RPC the best source of AR for binding assays?
No. The Panel considers the disadvantages of the RPC assay to be such that this assay should not be assigned a high priority. The disadvantages that were cited above include the fact that RPC contains other steroid receptors that may interfere
with the assay for AR binding and that ligand metabolism may take place in cytosol preparations. The RPC cannot substitute for human AR, or for AR in those wildlife where significant exposure to androgenic chemicals may occur. Considering the number of disadvantages attributed to the RPC assay, alternatives should be sought.

1.2.2 Should the binding of substances to different receptor subtypes be addressed in the binding assays?
Yes. This is prudent if it is known that subtypes with known functional significance exist. Although only a single type of AR is known to exist in the human, this may be particularly relevant to other non-mammalian species, such as the rainbow trout.

1.2.3 Should a metabolic activation system be included in the binding assays?
No, at least not currently in the context of routine AR binding assays. The Panel considers the evaluation of the binding of specific test chemicals to AR to be the first priority such that the binding of chemical derivatives of the parent substance resulting from metabolism were irrelevant to the present mandate of the proposed studies. Moreover, there is currently no definition of an activation system appropriate for each tissue or species of concern. The Panel recognizes the usefulness of having a system in which the binding assay was coupled to metabolic activation, if there are indications of an AR-binding chemical that was generated as a metabolite of the parent substance.

NOTE: The Panel bases its recommendations of AR binding assays on the basis of scientific considerations and relevance. However, it is critical to point out that there are other issues that influence the implementation of our recommendations. Most significantly, the human AR cDNA sequence is protected by at least two different U.S. Government patents. Furthermore, the commercial/non-academic use of hAR in cis-trans functional assays of AR is further governed by a license granted to Ligand Pharmaceuticals (San Diego, CA). The rat AR cDNA sequence may/is also protected by a patent. Although a commercial source of recombinant AR-ligand binding domain protein is currently available, the reliability of this preparation in binding assays has not been established. A full-length recombinant form of AR from any species is presently not available. An AR sequence from a species closely related to human may be necessary to allow the development of such an assay.

The appropriate government agencies should investigate the status of patents and licenses attendant to the use of the human and rat AR and should provide guidance for the use and development of AR assays in the public/private domains.
2.0 Minimum Procedural Standards for In Vitro AR Binding Assays

2.1 To facilitate assay standardization, the BRD proposes minimum procedural standards that should be incorporated into In Vitro AR Binding Assay protocols (Section 12.2). Considering the intended use of the assays as a toxicological screen, does the Panel agree with the adequacy of the proposed procedural standards? If not, what changes should be made to each standard and why?

The Panel agreed with the critical methodological issues proposed in the BRD for in vitro AR binding assays, and endorsed the fact that any assays using animals must be undertaken under the guidance of the relevant IACUC.

2.1.1 Binding Constant (K_d) of the Reference Androgen

There was consensus that a specific binding capacity, B_max, and the dissociation constant, K_d, values for the reference receptor protein is a critical measure of the robustness of the procedure. The K_d should be clearly established for the reference androgen in each assay and all test laboratories should be able to generate comparable data within acceptable limits. At present, data from different laboratories do not establish a well-defined, highly replicated K_d for R1881 in any of the test systems for AR binding. A minimum number of concentrations of ligand should be identified for generating a K_d. The Panel recommends adoption of seven concentrations of ligand for analysis, as is implied later in the BRD.

The Panel agrees that a detailed assay protocol must be provided for performing each type of assay, accompanied by criteria for evaluation and acceptance of results, to demonstrate assay validation and transferability across laboratories. The ability of a laboratory operator to obtain a B_max and K_d value for the reference androgen of a reference receptor protein within accepted limits for that type of preparation is a critical quality control parameter in that laboratory. These data will be essential to the establishment of a Quality Assurance Program for endocrine disruptor substance evaluation at numerous laboratories. The Panel recommends that straightforward procedures for determination of both the K_d value of the radiolabeled reference androgen, and the K_d value of an unlabeled test substance, such as the ligand titration array (Raffelsberger and Wittliff, 1997), be considered. Software programs such as Compete® (Lundon Software, Inc., Cleveland Heights, OH), GraphPad Prism® (GraphPad Software, Inc., San Diego, CA), LIGAND (Munson and Rodbard, 1980) and OneSite® (Lundon Software, Inc., Cleveland Heights, OH), will determine the specific binding capacity and K_d values of the reference AR preparation, and analyze the competition results and compute the K_d value of the unlabeled test substance examined in the assay. The Panel concludes that additional studies are necessary to specify the precise statistical characteristics of AR binding when fitting nonlinear regression curves and estimating pertinent parameters such as K_d and the IC_{50}.

2.1.2 Reference Androgen

The choice of a reference androgen is, in part, dependent on the assay being used. If the assay is based on a purified AR, then using the natural ligand [i.e., 5α-
dihydrotestosterone (DHT)] would make the most sense and should be the preferred ligand for standardization. If the test is based on a crude cytosolic preparation or on a cell line, then selecting a molecule that does not undergo significant metabolism is important.

The use of R1881 is based upon its properties as a high affinity AR ligand, its lack of metabolism and its low nonspecific protein binding in whole cell and crude cellular extracts. Thus, in this case, R1881 seems appropriate as a ligand. However, R1881 is a synthetic substance and may not recapitulate all of the properties of the endogenous ligands, testosterone or DHT. R1881 will also bind to the progesterone receptor (PR) in binding assays based on cells or tissues that contain this receptor, as for instance RPC. Since the specific binding of R1881 to AR is confounded by the presence of PR in the sample (e.g., RPC), triamcinolone acetonide should be used to block binding of R1881 to PR.

During the Panel deliberations, none of the members expressed any knowledge of known interference between triamcinolone acetonide and other chemicals. However, the Panel does not believe that it has sufficient understanding to predict if the potent synthetic glucocorticoid, triamcinolone acetonide, will interfere in the subsequent evaluations of androgen mimics. An alternative might be to use the synthetic progestin, promegestone (R5020) that has a high affinity for PR, as a means of diminishing the contribution of PR binding. Another alternative would be to use mibolerone. This ligand interacts less avidly with PR. Therefore, if an assay is chosen based on radioactivity measurement and uses PR-containing cells or tissue, consideration should be given to the use of mibolerone, rather than R1881. This would avoid the concomitant use of triamcinolone acetonide to block binding to PR in the AR binding assay.

### 2.1.3 Preparation of Test Substances

All test substances should be standardized and prepared according to rigorous quality controls for purity and concentration. The test substances should be prepared in water, 95-100% ethanol, or dimethyl sulfoxide (DMSO), depending upon their solubility. Preference should be given to the solvent that allows testing of the maximal concentration of the test substance, without exceeding the limit dose. However, in situations where more than one solvent could be used, preference should be given to water, then 95-100% ethanol, and then DMSO, in that order. The Panel strongly agrees that a set of solvent (vehicle)-only controls (with solvent concentrations identical to those used with reactions containing test substances) must be included in each set of assays. In the experience of the Panel, many test substances will require DMSO as a solvent, and again solvent-only controls must be performed. In situations where more than one solvent could be used, preference should be given to water or 95-100% ethanol, in that order. Preparation of stock solutions should be performed under rigorous quality control standards. The stability of stock solutions should be determined on an on-going basis and it may be necessary to prepare some chemical solutions fresh at each time of use.
The Panel recommends that the U.S. EPA establish an inventory of high purity chemicals that can be provided to laboratories as required for validation and test studies.

2.1.4 Concentration Range of Test Substances
In general practice, substances should be tested over a wide range of concentrations. It is desirable to have a concentration high enough to produce some effect in an assay even though very high concentrations (mM range) of a test chemical may be unrealistic when compared to levels found in the environment or to those obtained after normal exposure. The Panel agrees with the recommendations that at least seven different concentrations of the test substances within the range 1 nM to 1 mM should be examined to increase the likelihood of developing a competition curve satisfactory for IC$_{50}$ analysis. Furthermore, it is important to have at least one concentration below the IC$_{50}$ of the test substance. The limit concentration should be 1 mM, taking into consideration the solubility characteristics and possible toxicity (e.g., denaturation of the receptor) of the test substance. If a lower maximum concentration is tested due to solubility constraints or excessive toxicity, the number of concentrations tested can be adjusted to account for the altered concentration range. The concentration range should be governed by practical considerations of the chemistry of each substance, which determine its solubility in a specific solvent.

2.1.5 Solvent and Positive Controls
A set of solvent controls, with solvent concentrations identical to those used with the reaction mixtures containing the test substance must be included in each set of assays. Solvents should be the standard ones used (i.e., water, 95-100% ethanol, DMSO). The solvents should not have any effect on AR binding at the concentrations used in the assays and should be of utmost purity. The solvent volumes must remain constant throughout the concentration range tested. The positive control substance should have a binding affinity within two orders of magnitude of the limit of sensitivity of the assay. This control is critical to the assessment of the reproducible sensitivity of the assay within and between laboratories and is of particular relevance in determining the ability to assign substances with low AR binding affinity as different from no binding. A second positive control (e.g. cyproterone acetate) is recommended since this substance has an RBA within the range of 1-10% of the RBA of the reference androgen.

2.1.6 Within-Test Replicates
The IC$_{50}$ value should be based on triplicate measurements at each dose level.

2.1.7 Dose Spacing
The Panel agrees with the recommended dose spacing of one order of magnitude in the concentrations of the candidate test substances, and in the use of half-log doses in certain cases.
2.1.8 Data Analysis

The Panel recommends the essential requirement to determine and compute both the $B_{\text{max}}$ and the $K_d$ value for AR binding in each assay. Alternative approaches such as ligand titration array provide a simultaneous evaluation of a laboratory’s performance and determination of the AR binding properties (e.g., IC$_{50}$, $K_d$, and $K_i$ values) of each test substance. It may be useful to determine the noncompetitive, competitive and uncompetitive nature of AR binding with specific chemicals that demonstrate unexpected binding curves. More detail is needed on statistical models for nonlinear regression to assess $K_d$, $K_i$, and IC$_{50}$ values. The mode of calculation and assumptions for the statistical methods must be justified. This includes the nature of the statistical characteristics of the data (distribution, variance patterns, specific nonlinear models, etc.) and how to fit the models. When doing so, confidence limits must be calculated for $K_d$, $K_i$ and IC$_{50}$ values. From these, details on how to make pertinent and valid statistical inferences should be specified.

A possible approach for developing these statistical characteristics is to conduct a set of carefully-designed, comprehensive interlaboratory negative control studies. These would enable better understanding of the underlying statistical variability in AR binding data.

2.1.9 Assay Acceptance Criteria

There is agreement among the Panel with each of the BRD acceptance criteria. In addition, the Panel recommends that the assays be performed in compliance with standard Good Laboratory Practice guidelines.

2.1.10 Evaluation and Interpretation of Results

The approach presented in the BRD is accepted by the Panel. The designation of “equivocal” for substances that do not bring about a 50% reduction in specific AR binding is accepted. The classification of a test chemical as “positive for binding” requires the use of statistical methods.

2.1.11 Test Report

All of the BRD recommendations are accepted by the Panel. In addition, the assay used for protein determination should be specified and the concentration of protein used in the reactions reported. The chemical and radiochemical purity and the supplier of the radiolabeled androgen should be stated. A new range of IC$_{50}$, $K_d$, and $K_i$ values with a standardized AR preparation using a selected set of test substances should be established.

2.1.12 Replicate Studies

The Panel agrees with the recommendations contained in the BRD.

2.2 Considering the intended use of the assays as a toxicological screen, are there other minimum procedural standards that should be included? If so, what are they and why?

Specific recommendation regarding the type of protein assay and the conditions would
be useful. The Panel highly recommends that both the androgen and estrogen receptor-based assay reactions contain 10 mM sodium molybdate as well as a cocktail of protease inhibitors such as those described on page B3-5 of the BRD. Although the hydroxylapatite (HAP) procedure has been used by numerous investigators for separation of free and bound ligand, and is recommended for the RPC assay, problems with this separation procedure may arise. One such problem is that the receptor-ligand complexes are bound to the matrix, their retention is required during washing and the association of receptor-ligand complexes must be retained during elution. These represent significant variables that must be controlled. By contrast, the U.S. Food and Drug Administration (FDA) approved the testing of human tissues with the dextran-coated charcoal procedure in combination with a ligand titration assay. The latter procedure with dextran-coated charcoal allows the receptor-ligand complexes to remain in the original reaction medium while removing the unbound ligand. The results may be far more reproducible with this method than those obtained with the HAP assay.

3.0 Recommendations for In Vitro AR Binding Test Method Protocols for Validation Studies

3.1 A standardized In Vitro AR Binding Assay protocol using rat prostate cytosol (RPC) is provided in Appendix B of the BRD. This assay is proposed for validation studies by the U.S. EPA and other sponsors. Section 12.3 discusses additional details that should be added, based on the minimum procedural standards in Section 12.2. In addition, an example In Vitro ER Binding RPC Assay (based on the U.S. EPA Protocol) is provided in Section 12 Annex of the BRD. Considering the intended use of the assays as a toxicological screen, would the current protocols, with the additions detailed in Sections 12.2 and 12.3, provide a level of detail to appropriately minimize interlaboratory variability? If not, what revisions or additions should be made to the protocols?

The Panel is in agreement regarding the lack of an existing, standardized, acceptable protocol for an AR binding assay. Little effort and no synthesis were put forward by the BRD with respect to Question 3. The text on pages 12-8/12-9 of the BRD merely provided a list of the four documents in Appendix B and indicated that there was a need to review these protocols “for completeness and adequacy for their intended purpose.” Appendix B1 is a detailed description of the protocol presently being used by the U.S. EPA to validate the RPC assay. Appendix B2 is a brief description of the COS cell binding assay. The information, as provided, does not allow another researcher to reproduce the work nor does it provide the rationale for inclusion of most of the steps. Appendix B3 is similar in scope to B1, but is much less well written and has numerous severe omissions/errors. This protocol should not receive further consideration. Appendix B4 does not provide a protocol, but rather gives a valuable list of general concerns, cautions and guidelines on how to put such an assay together.

The standardized protocol for the RPC assay is provided in great detail. Although the RPC assay has been designated as the “gold standard”, this is the more difficult of the assays to perform in a standardized format. The relative simplicity of the transfected cell assay (COS + hAR/rAR) is amenable to high throughput and requires simple methods and minimal volumes and variations of buffers and solutions. If this assay is to be pursued further, a
standard transfection protocol based upon commercially available transfection reagents and a standardized cell line would be necessary for these assays. The production of a stable cell line expressing AR would avoid the problems inherent in transient transfection assays.

Perhaps, the simplest and least amenable assay to inconsistency would be one in which the AR protein would be fixed in multiwell plates and tracer and test ligands added in appropriate amounts to develop data appropriate for the accurate calculation of the $K_d$, $K_i$, and IC$_{50}$ values, or other pertinent data analyses. The desirability of moving away from radioactive tracer ligands and toward more environmentally and safer fluorescent ligands also needs to be considered.

### 3.2 In addition to the minimum procedural details listed in Section 12.2, are there other protocol elements that should be considered for other In Vitro AR/ER Binding Assays recommended for validation as a toxicological screen, including those protocols provided in Appendix B?

- If a radionuclide is to be used as the tracer ligand, its chemical and radiochemical purity must be stated.
- The maximal time of storage at -80°C/-20°C of cytosol, cells, or other material used as the source of AR should be indicated.
- The type of tubes/culture dish for homogenization and storage of cytosol or of cells should be indicated.
- Details are missing from the COS-cell binding assay. Some of these include preparation, purity and stability of the AR vector, more detailed timing on cell transfections, confluency of cells, transfection efficiencies (what these should be or if it makes a difference). Some rationale for the choice of timing, incubation conditions, etc., should also be given, especially since equilibrium conditions are sought.
- If a cytosolic protein preparation is to be used, a cocktail of protease inhibitors, rather than a single inhibitor, is to be used to increase stability of the AR.
- The simplest and least amenable assay to inconsistency would be one in which the AR protein would be fixed in multiwell plates and tracer and test ligands added in appropriate amounts to develop data for binding analyses.
- The desirability of moving away from radioactive tracer ligands and toward more environmentally and safer fluorescent ligands also needs to be considered in assay development.
- In developing such an assay, it is important to avoid situations that may render the assay less readily acceptable at the international level (e.g., having to comply with patent regulations and regulations regarding the use of radionuclides).

### 3.3 Considering the intended use of the assays as a toxicological screen, is the Panel aware of other available standardized protocols for assays recommended for validation?

Pan Vera Corporation (Madison, WI, USA) is selling an ‘AR Ligand Binding Domain: Activity Assay’ based on radioactivity measurement. The sensitivity and reliability of this assay are not apparent. Furthermore, the present Pan Vera AR assay uses only the ligand binding domain recombinant protein; this is much less desirable than the use of the full-length AR in either an in vitro or in vivo assay. There is no indication that a full-
length recombinant AR will be available in the near future. The present ER, progesterone receptor (PR) and glucocorticoid receptor (GR) competitive binding assays from Pan Vera Corporation are based upon full-length recombinant proteins and do not use radioactivity, rendering them much closer to the idealized assay described above. Given the development of non-AR assays, it would be surprising that Pan Vera Corporation does not have under development the type of assay discussed above as the optimal one. The Panel is not aware of any other assay under development that would meet the desired criteria.

4.0 Recommended List of Substances to be Used for Validation of In Vitro AR Binding Assays

4.1 Section 12.4 provides a list of substances recommended for use in validation studies of In Vitro AR Binding Assays. Considering that the intended use of the assays are as a toxicological screen, does the panel agree with the selection criteria, adequacy and appropriateness of substances recommended for validation studies, in terms of the following issues? If not, what substances should be added or deleted?

Generally:
• The Panel members essentially accept and/or recommend the list of test chemicals for validation of the assays.
• The same range and types of substances are recommended for validation of both AR binding and TA (transcriptional assays), if both assay types are selected for further validation.

Specifically:
• Weak-positive reference chemicals, which comparably represent the breadth of environmental chemicals, should be available.
• There are several “obvious” substances missing from the list. Anti-androgenic chemicals such as flutamide (or hydroxyflutamide if used in vitro) and bicalutamide that bind to AR but do not activate its transcriptional activity, should be used as model chemicals. Finasteride (the commercially available 5α-reductase inhibitor) does not bind to AR and should be added as a negative control.
• An assumption has been made regarding the mode of action with AR, such as competitive ligand binding (i.e., substances bind to the same site as endogenous androgens). As mentioned previously, consideration should also be given to non-competitive and uncompetitive mechanisms.
• One or more of the estrogens (ethinyl estradiol, estrone, or diethylstilbestrol) could be omitted from the list, as 17β-estradiol is already included.

4.1.1 The Number and Distribution of Substances Across the Range of Measurable AR Binding Activity, Including Negatives
A total of 31 chemicals are recommended in the AR binding BRD. They are almost equally divided among those with higher binding affinities in the range of 10 – 0.1 RBA relative to the R1881 and those with considerably lower binding affinities in the range of 0.01 – 0.0001 RBA. However, only three of the chemicals listed are negative and it is necessary to expand this number.
4.1.2 The Number and Range of Substances by Chemical Class

Of the 31 chemicals listed in the BRD, 21 are steroids of endogenous biological origin or are pharmaceuticals. Many of the other chemicals are organochlorines. Chemicals in the class of polychlorinated biphenyls, phthalates, and heavy/organo metals are not represented and should be.

4.1.3 The Number and Range of Substances by Product Class

The steroids are represented by 21 chemicals and the remainder represents pesticides, herbicides, and fungicides. Additional polychlorinated biphenyls, phthalates, and heavy/organo metals should be included.
IV. *In Vitro* Androgen Receptor (AR) Transcriptional Activation (TA) Assays

1.0 Recommendations and Priority for Validation Studies

The *In Vitro* AR TA BRD reviews the comparative performance, reliability, advantages, and disadvantages for different *in vitro* AR TA assays, and recommends a relative priority for further development and/or validation based on this information.

1.1 Considering the intended use of the assays as a toxicological screen, is the Panel aware of other advantages and disadvantages for the assays discussed in the BRD?

Advantages and disadvantages of these assays are discussed below.

1.2 Considering the intended use of the assays as a toxicological screen, does the Panel agree with the relative assessments of the protocols as described in the BRD? Should any of these be considered for further evaluation? What specific aspects have not been addressed that should be considered in the formulation of the ideal protocol for screening potential AR agonists and antagonists?

Only one type of assay was recommended in the BRD for further study. This resulted in part from the expected limitations imposed by patent restrictions on the AR clone. The recommended protocol involves the use of the endogenous AR and a stably integrated reporter. Only one assay referenced in the BRD meets these criteria and made use of an MDA-kb2 cell line. The MDA-MB-453 cell assay discussed is not a stable cell line. An analysis of these assays required a review of the original publications as fold induction of luciferase activity with 5α-dihydrotestosterone (DHT) was provided for the MDA-MB-453 cells, but the response to cortisol was not provided. Both of these cell lines are complicated by the lack of steroid receptor specificity in transactivation of the mouse mammary tumor virus luciferase (MMTV-Luc) reporter and by a lack of specificity in terms of androgen induction by the AR.

MDA-kb2 is a breast cancer cell line with endogenous AR and glucocorticoid receptor (GR) and has a stably integrated MMTV-luciferase reporter (Wilson et al. 2002) and derives from MDA-MB-453 cells. The response was 3-9 fold for DHT and 1-19.5 fold for dexamethasone. Hydroxyflutamide was used to inhibit AR agonists to differentiate activities mediated by AR and GR. A disadvantage of the assay is that weak AR agonist activity could be difficult to detect due to the weak response to DHT (only up to 9 fold). After 40 passes of the cells, the luciferase response to 1 nM DHT dropped to 5-6 fold. Thus, relatively low cell passage numbers would be required for transactivation assays and a need to continually monitor the cell line for genetic drift and loss of activity. Another potential problem is that hydroxyflutamide has only a moderate binding affinity for AR so its inhibitory effect is lost in the presence of high agonist activity; 1 µM hydroxyflutamide did not inhibit the activity of 10 nM DHT. In addition, it might be difficult to differentiate activity mediated by GR from the ineffectiveness of hydroxyflutamide to inhibit the agonist response. The reporter vector was activated by 17β-estradiol by the AR. Overall, the method may fail to detect weak AR agonists present at relatively low concentrations and is complicated by the presence of endogenous GR.
The issue of estradiol activation of the AR in the in vitro assays is complicated by the fact that there is no physiological evidence to support that estradiol is an AR agonist. Therefore, the question arises whether the positive in vitro response to estradiol as an AR agonist is meaningful. The AR is known to be promiscuous in terms of ligand binding and can accommodate a wide range of steroids. For various mechanistic reasons that are beyond the scope of this report, estradiol often results in a positive response in vitro. An optimal assay would show that estradiol is not an AR agonist at concentrations below 100 nM; however, thus far the majority of assays show estradiol as an AR agonist at concentrations of 10 nM or higher. It is recognized that agonists working through this in vitro mechanism may be false positives compared to in vivo results. Ideally, the in vitro assays should predict in vivo activity.

MDA-MB-453 cells are human breast cancer cells that contain endogenous AR and GR. These are not stably transfected cells in contrast to what is indicated in the BRD. For each assay, cells are transduced with a recombinant adenovirus containing the MMTV-Luc reporter. The response was 24 fold with 0.1 nM DHT and 248 fold with 1 µM dexamethasone (Hartig et al. 2002). The level of induction by DHT meets the suggested minimum performance criteria of fold induction by the control androgen. No method was proposed to account for the high transactivation of the reporter by GR. Presumably, this would be done by selectively inhibiting AR mediated activity with hydroxyflutamide. The presence of endogenous GR and its high activity complicates the assessment of AR agonist activity. The presence of GR would probably not interfere with assessing antagonist activity unless the AR antagonist interacts with GR. AR and GR agonists would be identified simultaneously using these cells. The use of adenovirus infection is advantageous in that virus infections are relatively straightforward. Development of the recombinant adenovirus is complex and the recombinant virus must be made available by the U.S. Environmental Protection Agency (EPA) to laboratories, which might limit applicability of the assay. Despite the limitations of this adenovirus infection protocol, it was deemed the best method of those presented in the BRD to proceed with further evaluation. This decision was tempered by inherent limitations resulting from the presence of GR in the cells. The Panel suggests that additional studies be performed to develop this assay using cells that lack the GR and to develop an adenovirus vector for a different androgen responsive reporter vector that shows greater specificity for the AR.

The other stable cell line (protocol B6) presented also had low fold induction with DHT. All the remaining protocols involve transient transfection and are subject to patent restrictions. Below are outlined some of the major advantages or disadvantages of these assays.

ADVANTAGES AND DISADVANTAGES
1. Different cell lines - presence of other steroid receptors and cell metabolism
One of the primary differences among the transient transfection protocols provided in the BRD is the use of different cell lines. For transient and stable transfection experiments, the optimal cell line would be one that expresses only the AR either endogenously or after transfection. HepG2, HeLa, and Chinese hamster ovary (CHO) cells express relatively high levels of GR, making it more difficult to specifically assess
AR activity by different ligands. It should be noted that while the presence of the GR is likely required for a viable cell, some cell lines such as monkey kidney CV1 cells have a sufficiently low level of GR that does not interfere with assays for AR transcriptional activity. Three of the protocols (B2, B5 and B6) utilize CHO cells which respond to cortisol suggesting the presence of GR. Except for the use of hydroxyflutamide to selectively inhibit AR mediated transactivation as described above, the protocols do not provide a method to differentiate reporter gene transactivation between AR and GR. The presence of the GR in the MBA cell line complicates its use as a screening assay for detecting AR agonist activity. This complication can be addressed by co-incubating with an AR antagonist such as hydroxyflutamide but this would require a parallel set of assays for all test substances, essentially doubling the effort. The LNCaP cell proliferation assay should not be considered since this cell line contains a mutant AR that does not discriminate agonists from antagonists. The assays proposed to measure increases or inhibition of cell proliferation are not transcriptional activation assays and could be impacted through multiple cellular pathways that do not necessarily involve the AR, and thus could not be recommended. Additional complications with cellular proliferation assays for the evaluation of endocrine activity is that cellular proliferation may be a consequence of non-AR receptor mediated mechanisms through the activation of cellular signaling cascades (e.g., phorbol esters like tetradecanoylphorbol-13 acetate (TPA) through protein kinase C).

The cell lines also differ in metabolic activity. For example, HepG2 cells derived from liver cells may retain some metabolic activity that could bioactivate or bioinactivate test substances. On the other hand, most metabolic activity, specifically P450 activities, might be lost when liver cells are cultured. Residual activity could confound interpretation of in vitro results. Differences in cell metabolism make R1881 the control agonist of choice as suggested in the BRD although DHT should be included as an additional positive control. A potential complication of R1881 is that it is less stable than DHT in solution. Control stocks must be prepared frequently and maintained in the dark in ethanol at -20°C. For yeast, there are potential differences in metabolism from mammalian cells and different chemical transport activities by transporters such as P-glycoprotein homologues. Thus, some parent substances may not gain access to the yeast cell to interact with the AR.

**Recommendation:**
CHO, HepG2, and HeLa cells are less advisable due to the presence of the GR. HepG2 cells have some metabolic activity which could inactivate test substances and/or agonist ligands. CV1 cells have relatively low metabolic activity and no detectable endogenous GR, but also no endogenous AR. There is evidence that CV1 cells metabolize the parent forms of several environmental antagonists to their active forms that interact with AR. The control androgen should be R1881, as recommended in the BRD, due to possible metabolism of natural androgens, but DHT should be included as an additional control. However, the inclusion of a metabolic activation system in in vitro AR TA assays is not recommended at this time. The type of metabolic activation
system developed will depend on which *in vitro* assay(s) are considered validated for detecting endocrine disrupting substances.

2. **Stable versus transient expression**

Cell lines with stably integrated reporter vectors and endogenous AR are advantageous because there are fewer patent issues. It should be noted, however, that cell lines with stably integrated AR plasmids are subject to AR patents restrictions since the AR plasmid DNA was integrated. A potential problem with stable cell lines is that they are not completely stable and tend to lose the integrated plasmids. They usually require continuous drug selection that may be expensive. The advantage of stable cell lines is that time consuming transfection procedures are not required. Variance in signal response tends to be less in stable versus transient transfection assays. On the other hand, transient transfection methods have been streamlined in recent years through the use of multi-well plates and commercially available, highly efficient transfection reagents that have simplified the process and improved reproducibility. Transient transfections require the continuous expansion of DNA plasmids although less amounts of these plasmids are needed as protocols are scaled to multi-well plates.

**Recommendation:**

Transiently transfected cell lines would be advisable due to their greater sensitivity if patent issues can be resolved. If patent issues persist, cells with endogenous AR with a stably expressing reporter vector are optimum. It may be necessary to demonstrate 10-20 fold induction by a control agonist in order to detect weak agonists. Other considerations are stability of the stably transfected cell line, and absence of the complicating factor of endogenous GR. The Panel suggests an approximate minimally acceptable level of 10-20 fold to allow for sufficient sensitivity to detect weak agonists. The stable cell line should contain a reporter with a response element that is relatively specific to the AR. If stable cell lines can be developed with sufficient sensitivity, these would be advisable but probably must be maintained in drugs to select for cells containing the integrated plasmids. A central laboratory for the generation and disposition of stable lines should be pursued, since during the course of generating these lines, integration of transfected receptor constructs may occur at different locations within the genome leading to potentially unique response profiles across laboratories. A central source of adenovirus vector would be needed for the adenovirus protocol because propagation of the virus can be technically challenging. Thus far, a particular stable cell line could not be recommended because of weak induction of luciferase activity by the control agonist.

3. **Reporter plasmid specifically for androgen-bound AR**

The optimal androgen responsive reporter vector would not allow for cross specificity with other receptor subtypes such as GR and the progesterone receptor (PR), as exhibited by MMTV. An androgen specific consensus human response element (HRE) is preferred and the use of multiple HREs in the construct could increase sensitivity. The MMTV reporter vector was used in the majority of the transient (protocol B1, B2, and others) or stable (MDA-MB) assays presented in the BRD. Protocols B5 and
B6 use four copies of the rat ventral prostate prostatic binding protein gene subunit C3 linked to the luciferase gene. None has been shown to be specifically activated by the androgen-bound AR. In almost all the protocols listed, 17β-estradiol activated the reporter indicating that AR can activate the MMTV and other reporters in the presence of a variety of ligands, depending on its concentration. This is a potential problem in screens that assay relatively high doses of substances and could result in false positives. Other reporters not included in the report could have greater specificity for activation by the androgen-bound AR. Establishing the optimum reporter that demonstrates specificity for AR and for the androgen-bound AR requires further investigation. Some reporters that have been investigated include the sex-limited protein gene and probasin. However, even these can show activation by other receptors.

**Recommendation:**
Other reporter vectors different from MMTV that show specificity for activation by the androgen-bound AR should be investigated. Possibilities include the rat prostate C3 gene promoter and enhancer promoter regions for the prostate specific antigen gene, sex limited protein gene, and probasin, although these reporters may be less sensitive than MMTV. Ideally, the reporter should not be activated by the AR in the presence of 17β-estradiol or cortisol. The alternative is to indicate a cutoff concentration, otherwise all estrogenic substances may be identified as AR agonists. This may be difficult since the cutoff concentration for steroid hormones will differ from the cutoff values for unknown environmental substances.

4. **Sensitivity to detect low concentration substances in screening**
The transient transfection assays were more sensitive than stably transfected lines. Stable cell lines often have less than 10 fold induction with the control agonist whereas transient transfection can have 50-100 fold induction. Low induction by the control agonist may make reliable detection of antagonists more difficult and the detection of weak agonists impossible. During the deliberations of the Panel, it was suggested that stable cell lines with generally lower fold induction could reliably detect AR antagonists. The CHO stable cell line (protocol B6) had only 5 fold induction with the control agonist which would be too weak a response for testing unknown substances. The HeLa cell assay of Wang and Fondell (2001) had 4.5-7 fold induction with 100 nM DHT, which is unacceptably too low and would not be useful in detecting weak or strong agonists or antagonists. The CHO assay with CAT activity of 100 fold with 0.1 nM DHT was 35 fold with cortisol, reflecting the complication of endogenously expressed GR. Fold induction for the MDA stable cell line ranged from 3-10 fold.

**Recommendation:**
Based on the experience of the Panel, an induction of 10-20 fold should be achieved by the control agonist in a concentration range of 0.1-1 nM R1881 or DHT to allow for the detection of weak agonists. An effective means of separating out confounding effects of other steroid hormone receptors is essential.
5. **Suitability for large scale screening**
   All of the protocols could be scaled to multiwell plate assays. This is a requirement for the optimal assay as it allows for large-scale screening and the use of transfection reagents that would otherwise be too costly.

*Recommendation:*
The assay should be established in a multiwell format.

6. **Ability to detect a weak active agonist or antagonist in a complex mixture**
   This could be difficult using a stable cell line that typically has less than 10 fold induction with the control agonist. Detection of weak acting substances may require an assay with at least a 10-20 fold induction with the control androgen at a concentration of 0.1 nM DHT or R1881.

*Recommendation:*
The most sensitive assay would be achieved using transient cotransfection assays because the sensitivity of these assays exceeds that of the stable lines. However, considering the potential patent restrictions on the AR and the cis/trans cotransfection methodology, the optimum assay would be a cell line with endogenous AR that is sufficiently sensitive to detect weak acting agonists and antagonists. Further research should be directed toward making more sensitive stable cell lines.

7. **Ability to discriminate agonist and antagonist**
   Each of the assays, except for the yeast assay, discriminate AR agonists and antagonists. All cell assays documented in the BRD demonstrated the antagonist activity of casodex (bicalutamide) and hydroxyflutamide in the presence of an AR agonist such as DHT. Each also indicated agonist activities of DHT, testosterone and other known agonists. However, AR agonist activities were also reported for cortisol, dexamethasone, and 17β-estradiol, none of which are AR agonists *in vivo*. These latter responses reflect difficulties due to lack of specificity of the reporter and AR ligand binding.

*Recommendation:*
All assays showed 17β-estradiol was an AR agonist, which is not observed *in vivo*. This results primarily from lack of specificity of the MMTV reporter to ligand activation of the AR and a lack of absolute AR specificity for binding steroids. Many of the assays showed AR agonist activity of cortisol which is neither an agonist nor an antagonist and results in part from the presence of GR. All of the assays showed DHT as an agonist and hydroxyflutamide and casodex to be antagonists. The ideal AR screening assay will not show AR activation by 17β-estradiol or cortisol.

8. **Sufficient fold induction by androgen to detect antagonist activity**
   A reporter assay should show at least 10-20 fold induction with 0.1-1 nM of the control agonist. It was not possible to evaluate fold induction for many of the assays in the BRD. Transient transfection experiments would be superior to stable cell lines in terms of sensitivity (i.e., extent of fold induction).
**Recommendation:**
Thus far, most stable cell lines show less than 10 fold induction. Transient transfected cells or adenovirus infected cells are more sensitive and would be advantageous.

9. **Transferability, patent/proprietary issues**
The AR protein and coding sequence have been patented. Ligand Pharmaceuticals (San Diego, CA) holds an exclusive license based on the U.S. patent and Karo Bio AB (Huddinge, Sweden) has licensing rights on the European patent. The AR patent covers any AR vectors that have the recombinant derived human AR sequence. The patents therefore apply to cells stably transfected with a human AR plasmid. Presently, Ligand Pharmaceuticals will not allow other companies to use this technology. They also have patent rights over the cis/trans duel transient transfection assays. It needs to be clarified whether these patents nullify all of the transient transfection protocols proposed. Under the present conditions, while the protocols could be repeated in independent academic labs, they could not be repeated by commercial companies. If the gold standard protocol does not require transferability to commercial laboratories, transient transfection methods could be further considered. It is stated on page 6-9 in the BRD that patent issues preclude the use of transient transfection assays. It should be noted that this also applies to cell lines with an integrated AR plasmid. The patent restrictions on many of the assays are a significant disadvantage. It needs to be clarified whether a gold standard assay should be available to commercial companies involved in developing AR screening assays.

**Recommendation:**
A stable cell line with endogenous AR and stably expressed reporter would avoid patent issues unless there is a patent associated with the reporter. The MMTV lacks specificity for activation by AR and also for low androgen specificity for AR activation. However, a more suitable reporter may lead to additional patent problems. The U.S. government supported the original research that determined the AR coding sequence. The U.S. EPA could investigate their ability to use its rights under that funding to get a license from Ligand Pharmaceuticals to make the transient transfection methodology using the AR expression plasmid a viable alternative.

10. **The use of yeast in endocrine disruptor screening**
The yeast assay B4 is complicated by the possibility of unusual metabolism of ligands, by problems associated with cellular uptake and transport of steroids and test substances, and by an inability to distinguish agonists from antagonists. The BRD is correct that yeast would not be the optimal approach for these assays.

**Recommendation:**
Yeast assays should be avoided.

11. **Reproducibility and expense of transfection methods**
There was insufficient information to compare the assays described in the BRD in terms of reproducibility within and between labs. Most methods use expensive but highly
effective commercially available transfection reagents. These can be cost effective using the multiwell plate format. All of the methods proposed appear to be applicable to multiwell technology. The calcium phosphate transfection method is the least expensive but requires precise handling and close attention to details and may not be easily transferable between laboratories, but should be amenable to the multiwell format.

**Recommendation:**
The use of multiwell plates is recommended to keep down transfection costs and to provide for large scale screening. Reproducibility would need to be evaluated.

12. **Endogenous mutant AR with loss of androgen specificity**
A cell proliferation assay using the human prostate cancer cell line LNCaP was mentioned, although the protocol was not provided. This cell line is unacceptable due to a mutation in the endogenously expressed AR that alters its ligand specificity.

**Recommendation:**
A cell line must express an AR with wild-type sequence.

13. **Are results directly applicable to humans and wildlife species?**
The results with all of the mammalian-based cell assays are similarly applicable to *in vivo* conditions, although some cells metabolize ligands more efficiently than others. It would be optimal to have minimal metabolism of control steroids. On the other hand, it would be advantageous to have metabolism of substances that reflects what occurs *in vivo*. In almost all of the assays, the human AR was used, making the data relevant to human. Data obtained using the human AR should also be relevant to wildlife species as the ligand binding domain of the AR across species is highly conserved. Mammalian cells should be used for the assay of human AR activity as human AR has not been shown to be active in fish cells, for example. This most likely reflects the low sequence homology of the AR amino terminal region that contains the major activation domain of the AR. Thus far, every active substance examined in both wildlife and mammalian assays has been detected in mammalian assays. However, this is not true for wildlife assays. For example, flutamide is not detected as an AR antagonist in some fish assays despite finding hydroxyflutamide as the primary metabolite. No substances have been reported that are only positive in wildlife assays. The data support what is known about the function of these hormones in both sex differentiation and development and AR action in adults in mammals and other species. All currently available AR transient and stable transfection assays suffer from the same weakness, that they may not accurately predict response in humans or whole animals because *in vitro* assays cannot adequately measure absorption, metabolism, distribution, and elimination, as well as target-tissue specific factors that influence AR function.

**Recommendation:**
Depending on the extent of cell metabolism of the test substances, results from transient or stable transfection assays should be applicable to humans and wildlife. Assays should
use the human AR that requires transcriptional analysis be performed in mammalian cells.

14. Are controls provided to test for cytotoxicity when assessing antagonists?
   About half of the protocols provide β-galactosidase control vectors as a control for cytotoxicity. A more appropriate control would be to include transfection of a constitutively active luciferase vector such as CMV-Luc or pSG5-Luc. This is advantageous over β-galactosidase in controlling for cytotoxicity because the use of luciferase vectors also controls for direct effects of the test substances on luciferase enzymatic activity. If only β-galactosidase is used, the possibility exists that the test substance directly inhibits β-galactosidase activity and has nothing to do with gene expression. In contrast, a direct inhibitor of luciferase may be mistaken for an AR antagonist if β-galactosidase is used as the enzyme assay. By using luciferase as the cytotoxicity control, direct effects on enzyme activity and on cytotoxicity are included in the control assay. Tests for cytotoxicity are especially important at high concentrations of test ligands so it may be appropriate that cytotoxicity and luciferase activity tests are limited to samples at concentrations over 1 µM. Controls should also include those for the vehicle used for hormone and chemical additions.

Recommendation:
Control plasmids such as CMV-Luc or pSG5-Luc should be included to assess cytotoxicity and inhibition of luciferase activity. Alternatively, cytotoxicity tests might be limited to substances that show antagonist activity since it might apply to substances at high concentrations.

15. Other endogenous steroid receptors that complicate the assays
   A complication of using the stable cell line MDA-kb2 is that it contains sufficiently high levels of GR to interfere with the assay. This could also be a complication of HepG2 and HeLa cells depending on which reporter vector is used. Because the MMTV Luc or CAT reporters respond to GR better than they do to AR, it becomes more difficult to conclude that a response is significant.

Recommendation:
Cell lines should be used that lack relatively high levels of other steroid receptors, in particular the GR and PR. These receptors share with the AR a similar DNA binding domain sequence allowing them to activate in many cases the same enhancer-promoter sequence.

16. Are the results in general agreement with other assays and known activities?
   Almost all the assays show that 17β-estradiol activates AR. However, there is no evidence to support that this substances activates AR in vivo. The optimum assay would be expected to not show AR activation by 17β-estradiol. Better protocols are needed that allow for AR activation only by known androgen agonists.
Recommendation:
Most of the assays show cortisol and 17β-estradiol as AR agonists. Cortisol activity results in part from the presence of GR in some cell lines, and in part because of the lack of specificity of response elements in the reporter vector. AR agonist activity of 17β-estradiol does not agree with its known in vivo activity. The results raise the question at what concentration should a substance be considered a real AR agonist.

17. What is the minimum acceptable fold induction for a control agonist?
For most of the assays presented in the BRD, it was not possible to determine the fold induction achieved by the control androgen. At least a 10-20 fold induction would be considered acceptable. Otherwise, it may be difficult to assess weak agonists or antagonist activity.

Recommendation:
Induction should be at least 10-20 for a control AR agonist at 0.1-1 nM R1881 or DHT.

18. Can the assay be accommodated in a multi-well format necessary for large scale screening?
All of the assays could be accommodated by this format.

Recommendation:
A multi-plate assay is required.

19. Error range, variance
RELIABILITY = REPRODUCIBILITY WITHIN AND BETWEEN LABS
It was not possible to determine this from the data provided in the BRD.

Recommendation:
The data should be within 20% error.

20. Statistical considerations
a. Recommendations for agonist and antagonist classification
Substances can display both AR agonist and antagonist activities depending on the concentration, the assay system, or the presence of endogenous androgen. In some instances, the duel activities are real and reflect endogenous activities. One example is hydroxyflutamide, a classical AR antagonist, which at high concentrations has agonist activity. Whether this occurs in vivo is not clear but could be reflected in the hydroxyflutamide withdrawal syndrome, where prostate cancer patients improve after removing treatment with the antagonist. Another example is the drug medroxyprogesterone acetate (MPA), which when administered to pregnant women can induce clitoromegaly in a female fetus (an agonist response) and hypospadias in a male fetus (an antagonist response). A substance like MPA with weak AR agonist activity can be androgenic in the developing female fetus where low androgen levels are present. However, a weak agonist may be antiandrogenic in the developing male
fetus because, by being a weaker agonist than DHT, it binds AR and elicits a weaker agonist response, appearing as an antagonist.

For substances that display agonist activity, the effective concentration (EC) should be provided that increases transcriptional activity by 2-3 fold. For substances that display antagonist activity, the IC25-IC50 could be used (i.e., the concentration required to inhibit ligand-induced transcriptional activity by 25-50%). In some cases, antagonist activity may be detected but not achieve 25-50% inhibition. It is not clear whether decreases in transcriptional activity of 10% or less should be considered physiologically relevant. For antagonist assays, the concentration of agonist (i.e., R1881 or DHT) used to assay antagonist activity should be concentrations of R1881 or DHT that induce approximately 75% transcriptional response in the assay system but not more than 1 nM. In some cases, dose response relationships may not be observed due to sample impurities or metabolism of the substance. In such situations, further analysis is suggested using more purified preparations or cell-free \textit{in vitro} competitive binding assays. The results from Tier 1 screening assays should be considered together in the decision of whether Tier 2 testing is required or not.

\textbf{b. Recommendations for statistical analysis}

A critical aspect of the analysis of \textit{in vitro} and \textit{in vivo} Tier 1 screening assays is to assess the results from all screening assays \textit{in toto} and subjectively establish whether evaluation by Tier 2 testing is necessary. There appear to be two major considerations in the evaluation of \textit{in vitro} AR transcriptional activation data. First is agonist or antagonist potency. The EC at which a 2-3 fold increase in transcriptional activity is observed or a 25-50% decline in gene expression (IC25-IC50 concentration) could be used to rank order potency. The second consideration of equal importance is whether the response varies with dose and if so, how steep is the dose-response curve, as discussed further below. The reported data for these assays should be the concentration where gene expression is increased by 2-3 fold or decreased by 25-50%, and the slope of the tangent line to the dose-response curve. As such, the reported data from these assays will minimize the tendency to label substances as significant \textit{in vitro} endocrine disruptors and maximize their input toward assessing the results from all Tier 1 screens.

The goal in the \textit{in vitro} screening studies is to determine the concentration of a substance that induces an alteration in gene expression that is biologically meaningful. Here, it is assumed that a 2-3 fold increase or 25-50% decrease in response is indicative of a potential \textit{in vivo} response. This approach, combined with determining how steep the dose-response curve is from this point downward (or upward for agonists), should provide sufficient information from these assays together with results from other \textit{in vitro} and \textit{in vivo} assays to assess whether Tier 2 testing is warranted. The ultimate decision as to whether to proceed to Tier 2 testing should not be a quantitative assessment where results from each of the assays in the Tier 1 screen are given a score and a weight. Data from individual assays in the Tier 1 screen should be assessed \textit{in toto}. 
i. **Response variable for evaluating the potential agonism/antagonism of a test substance**

On page 5-3 of the BRD, several definitions are indicated for relative activity of a test substance. Classification of a test substance as an agonist or antagonist depends on how relative activity is defined. Estimates of quantities such as EC$_{50}$ and IC$_{50}$ values for a given test substance depend on the definition used for relative activity. Meaningful comparisons of EC$_{50}$/IC$_{50}$ across studies and chemicals require that all studies use the same definition for relative activity. For instance in Table 12-1 on page 12-11 of the BRD, median EC$_{50}$ values are provided. The median EC$_{50}$ for a given chemical is meaningful only if all the participating labs used the same definition for relative activity. Thus, definition of the response variable of interest should be standardized for future assays. This is vital because all important decisions, including the final determination of positive/negative agonism, and positive/negative antagonism, regarding a chemical are based on the chosen definition.

ii. **Assumptions made in a statistical analysis**

Most statistical procedures make certain assumptions regarding the underlying data. For instance, if ANOVA type methods are used then it must be determined that there is no heteroscedasticity and that the data are approximately normal. The Panel recommends that before any statistical procedure is used, suitable diagnostics be performed to verify that all underlying assumptions regarding the procedure are true. If the assumptions are not true, then suitable data transformations might be performed before analyzing the data.

iii. **Estimation of EC$_{50}$/IC$_{50}$ and steepness parameter**

Section 12.2.11 (pages 12-5 and 12-7) of the BRD state that EC$_{50}$ and IC$_{50}$ values should be reported along with their confidence intervals. The standard approach is to fit the data with a suitable non-linear model, such as a Hill equation, which gives an objective estimate of the EC$_{50}$/IC$_{50}$ values, as well as a confidence interval. In most cases, an objective estimate of the EC$_{50}$/IC$_{50}$ values based on a Hill equation or other suitable nonlinear model is required. If it is appropriate to perform nonlinear modeling based on a suitable dose response curve, a suitable nonlinear model should be used. Suitable model diagnostics should be performed to ensure that the model fits the data and the various underlying assumptions such as normality and homoscedasticity are true. Also, diagnostics should be performed to detect curvature effects and a suitable estimation procedure should be used for estimating confidence intervals for the parameters of interest. In some cases, especially in the presence of curvature effects, the standard asymptotic confidence intervals might not be appropriate.
In such cases, a resampling procedure such as jackknife or bootstrap might be used.\(^3\)

iv. **Combining EC\(_{50}\) and IC\(_{50}\) estimates from different labs**

To obtain estimates of mean EC\(_{50}\) and mean IC\(_{50}\) values from different laboratories, the average across the laboratories should not be simply computed. Instead, estimates using mixed effects nonlinear models, treating laboratories as the random effects, should be used. This approach takes into account within and between laboratory variability. This methodology also allows for a determination of the corresponding standard errors.\(^4\)

v. **Positive agonists and antagonists**

In situations where it is not possible to obtain a nonlinear model, the Panel recommends performing a statistical trend test. If the trend is significant, confidence intervals should be computed at each dose. If a confidence interval contains 10% of the maximum response, that would suggest that this substance should be further evaluated.

vi. **Uniformity trials**

To understand the underlying variability in the data, which is important for proper data analysis, the Panel recommends conducting a set of carefully planned comprehensive, interlaboratory negative control studies.

**Conclusions to Question 1**

Theoretically, stable transfection assays are preferable to transient transfection assays for high throughput screening. A stably transfected reporter system allows for non-radioisotopic detection using a reporter gene. Stably transfected lines would need to be established that allow for sufficient sensitivity and reproducibility across laboratories. Stable assays will require an agonist response to 0.1-1 nM control androgen such as R1881 or DHT that is sufficient to detect weak androgens. Induction of at least 10-20 fold would facilitate this detection; however, lower fold induction may be adequate. The Panel recommends further analysis of the adenovirus infection method using MDA-MB-453 cells, noting that while these cells contain endogenous AR, they also contain significant levels of GR, complicating analysis of potential AR agonist or antagonist activity.

This recommendation does not preclude the use of transient transfection methodology; however, potential patent restrictions require further clarification. Transient transfection assays can be developed for high-throughput screening and are advantageous because of higher fold induction.


and can include appropriate controls for transfection efficiency and toxicity, and can be designed to be more receptor specific. However, transient transfection assays may require greater cost and effort, are technically more difficult for laboratories that do not routinely do this type of assay, and are limited by patent restrictions.

Yeast-based assays are not acceptable because of reduced ability to detect certain substances either because of an inability of the substances to cross the yeast cell wall or because of active transport mechanisms. The yeast-based assay also does not accurately discriminate between agonists and antagonists. Stable assays with greater sensitivity should be pursued in cell lines that are not complicated by other endogenous steroid receptors.

2.0 Minimum Procedural Standards for In Vitro AR TA Assays

2.1 Considering the intended use of the assays as toxicological screens, does the Panel agree with the adequacy of the proposed procedural standards recommended for in vitro AR TA assays?

2.1.1 Transcriptional Activation of the Reference Androgen
The Panel agrees that the transcriptional activation-inducing ability of R1881 must be demonstrated and that consistency in the level of response is appropriate as a criterion for assay acceptance. A full dose-response curve should be generated.

2.1.2 Reference Agonist and Antagonist
The gold standard reference agonist for validation should be R1881 due to possible metabolism of natural androgens in different cell lines. DHT should also be included in all assays. The concentration of the agonist selected should be within the linear region of the dose-response curve of 50 - 70% induction. The concentration of the agonist selected, as well as the ability to identify significant effects, will depend on the assay, but should be within 0.1-10 nM DHT or R1881.

The reference antagonist should be hydroxyflutamide (not flutamide). Casodex (bicalutamide) should be included in the list, although casodex can be difficult to obtain. There should be 70-90% inhibition in the presence of 0.1 nM or 1 nM R1881 or DHT. Depending on the reporter, the reference androgen concentration should be 0.1 or 1 nM R1881 or DHT for maximal induction. The inhibitory concentration (IC$_{25}$-IC$_{50}$) of hydroxyflutamide is approximately 500 nM. The IC$_{25}$-IC$_{50}$ should be defined in terms of the androgen concentration against which it is inhibiting. Antagonist activity should be expressed in terms of the IC$_{25}$-IC$_{50}$, or the response rate at the p <0.05 level of significance.

2.1.3 Preparation of Test Substances
The test substances should be prepared in water, 95-100% ethanol, or dimethyl sulfoxide (DMSO), depending upon their solubility. Preference should be given to the solvent that allows testing of the maximal concentration of the test substance, without exceeding the limit dose. However, in situations where more than one
solvent could be used, preference should be given to water, then 95-100% ethanol, and then DMSO, in that order. It would be prudent to perform a pre-validation of the transcriptional activation assay with the reference androgen for assessment of the level of solvent that does not adversely affect assay response. Substances should be dissolved at 1-10 mg/mL in water, 95-100% ethanol, or DMSO and solubility verified. Appropriate solvent controls should be included in all screening assays.

2.1.4 Concentration Range of Test Substances
For both agonism and antagonism assays, the limit concentration should be 1 mM but the solubility characteristics and potential cytotoxicity of each test substance must be taken into consideration. If the limit concentration is used, seven test substance concentrations at log intervals should be tested. If a lower maximum concentration is tested due to solubility constraints or excessive cytotoxicity, the number of concentrations tested can be adjusted to account for the altered concentration range. A measure of cellular cytotoxicity will help define the upper limit for test material concentration similar to a Maximum Tolerated Dose (MTD) approach used in in vivo studies. This may mitigate the need for the higher concentrations which appear excessive in these in vitro systems. However, it is important to consider that agonist activities detected only at doses >1 μM for endogenous steroids or test substances should not be considered significant unless it is a pharmaceutical. At high concentrations, nonspecific interactions occur that could lack physiological relevance, depending on the exposure concentration. It is important to keep in mind the sensitive dose response relationships of endocrine activity.

2.1.5 Solvent and Positive Controls
In each experiment, there should be at least two positive controls, DHT and R1881. Controls for cytotoxicity should be performed for all samples that show apparent antagonist activity. This will involve the higher concentrations of ligands. Controls for cytotoxicity can be accomplished by including an internal constitutively active control reporter plasmid such as CMV-Luc. Levels of cytotoxicity exceeding 10% are unacceptable. Results are not useful at concentrations where substances are cytotoxic. If cell lines are used that contain other endogenous steroid receptors, inhibitors must be added that selectively inhibit ligand binding to that receptor. AR agonist activity could be selectively inhibited using hydroxyflutamide; however, this approach requires substantially more work. Hydroxyflutamide or bicalutamide (casodex) controls in the absence of added agonist could be included in antagonist experiments to control for possible endogenous androgen activity in the media used for the assay.

2.1.6 Within-Test Replicates
Each test chemical concentration should be tested in triplicate.

2.1.7 Dose Spacing
The Panel agrees with the recommended dose spacing of one order of magnitude in the concentrations of the test substances.
2.1.8 Data Analysis

A uniform method for expressing response should be established. The data should be expressed in absolute units such as light units for a luciferase assay. The data can be multiplied by a scaler for convenience (i.e., \( x \times 0.001 \)). The data can in addition be expressed as fold induction but fold induction alone is not sufficient. The use of modern plate readers with high sensitivity can lead to fluctuations in background levels that can have profound effects on the apparent fold induction. For agonist activity, the EC\(_{50}\) value, the lowest effective concentration where there is a 50% increase in response to the stimulatory ligand, may not be appropriate. EC\(_{50}\) values can be misleading if the substance is not a full agonist or if the substance cannot be tested at high enough concentrations due to solubility limits or toxicity. EC\(_{50}\) values depend on the conditions of the assay and can vary between laboratories even under standardized conditions. Instead, the lowest observed effective concentration (LOEC) at which a significant (2-3 fold) response is observed over background could be reported. Percent of control would not be acceptable unless the absolute relative light units (RLU) are given for the control (i.e., such that the RLU for all responses can be easily calculated). However, percent of control may be acceptable for comparing multiple experiments in which maximal induction levels vary. For transient transfections that include a proper constitutively expressed control for transfection efficiency and toxicity, the data could be expressed as corrected units. Control values should be monitored to ensure that assay responsiveness remains within historically accepted limits. Cells may lose their effectiveness over time and may need to be reestablished, and DNA used in transient transfections may degrade over time. An internal standard reporter vector is not required for stable cell lines but should be required for transient transfections. The benefit of an internal standard is that it can also be used to monitor for chemical toxicity.

2.1.9 Assay Acceptance Criteria

Test chemical entry and exit assays for all dose formulations must be within 10% of the intended concentration. An entry assay assesses the identification and concentration of the test article in the dose formulation prior to the start of the experiment, whereas the exit assay assesses these same parameters at the end of the experiment. The need for these analyses may depend on existing analytical methods for their assessment; complex mixtures may not be appropriate for these analytical analyses. The 10% level is based on standard analytical chemistry assessments of dosing solutions to insure the concentration is actually the concentration that was used. Compliance with standard Good Laboratory Practice (GLP) guidelines is advisable. An unacceptable experiment would have replicate variability exceeding 25-30%, cytotoxicity measurements exceeding 10% of the response level or positive and/or negative control levels that do not meet predetermined criteria, including fold induction of 20x, internal replicate variability of 20% or less, inappropriately negative or positive response. A valid experiment would have appropriate responses from positive (DHT, R1881) and negative (solvent) controls. The response should be within the acceptable limits as defined by historical data. If the response is outside the historically established range, it is not acceptable. This will also help the laboratory
monitor assay drift. Toxicity should be monitored. Concentrations of chemicals that cause 10% toxicity should not be considered. The transient transfection efficiency would be useful to know; however, it would not need to be established in every assay.

**Specificity of response:**
The reporter gene assay should show activation by the classical androgens DHT and testosterone, and by the synthetic androgens R1881 and mibolerone. Ideally, the assay should not show agonist activity with 17β-estradiol or cortisol up to concentrations of 10 nM. The assay should show the classical antagonist response of hydroxyflutamide and casodex and should not allow response to other receptors. This requires that the GR and PR are expressed at low levels in the test cell line and that an AR selective reporter vector is used. The MMTV reporter used in the majority of the assays presented in the BRD is nonselective and can be activated by other hormones if the appropriate receptors are present.

**Sensitivity to detect weakly active substances:**
Thus far, stable cell lines may lack the sensitivity to detect weakly active substances. This deficiency likely requires a transient transfection assay that demonstrates at least 10 fold induction with the control androgens and levels of variance that allow detection of alterations in gene expression of at least 2 fold stimulation or 25-50% inhibition of activity. It is difficult to determine what fold change in reporter gene activity is indicative of a change in gene expression in vivo. However, a 2-3 fold increase or 25-50% inhibition would imply a significant change in AR functional activity.

**What is the minimum fold induction acceptable for active androgens?**
The minimum acceptable induction is 2-3 fold over the no hormone control. For many of the assays presented in the BRD, this was difficult to evaluate and some were clearly not acceptable. A positive response would be at least 2 fold over background levels. Alternatively, rather than set cutoff limits based on fold induction or antagonism, a more statistical approach might be considered.

**Acceptable variance:**
The percent coefficient of variation (%CV) should not be greater than 20%. This is calculated as standard deviation/mean x 100.

**2.1.10 Evaluation and Interpretation of Results**
An acceptable limit for acceptance as a positive or negative response would be a change, relative to the control, of 2-fold induction for an agonist and 25-50% inhibition for an antagonist.

**2.1.11 Test Report**
The test report should include the recommendations in the BRD plus the following changes and additions:
a. Information should be included on controls for the activity of other steroid receptors and controls for cytotoxicity. The source of plasticware and other materials used in the assay should be listed. The cell passage number should be recorded.

b. Chemical names of known test substances and structures are appropriate.

c. The solvent does not require justification unless it is other than water, 95-100% ethanol or DMSO. The solvent used should be indicated. For the AR source, the supplier should be indicated if it is a noncommercial source.

d. Procedures for making constructs should indicate only the type of method used for isolating the DNA, not the detailed procedure.

e. The structure of the response elements in the reporter vector should not be needed, simply the name and reference.

f. The methodology for making the reporter plasmid should not be required.

g. The reference androgen should only need a rationale if it is other than DHT or R1881. The assays should require the use of the standard recommended androgens.

h. The concentration and volume should be indicated for the test substance.

i. At least two replicates of the experiments are needed and the assays performed in triplicate. More experiments are required if the experiments are not in agreement.

j. The response should be indicated in absolute units such as light units for luciferase activity with the error indicated, and in addition, as fold induction if this is deemed appropriate.

k. Statistical analysis of the data should indicate agonist and inhibitory test chemical effects on transcription that meet or exceed the 2-3 fold induction or 50% inhibition level compared to the respective controls.

2.1.12 Replicate Studies

Replicate studies are not mandated but questionable data needs to be confirmed by re-testing the substance. In such situations, the incorporation of stricter treatment concentrations in follow-up assays based on the initial experiment should facilitate better analysis of the overall dose-response of the test material.

2.5 Considering the intended use of the assays as a toxicological screen, are there other minimal procedural standards that should be included? If so, what are they and why?

1. An internal standard reporter vector such as CMV-Luc should be used to control for cytotoxicity and direct effects of the test substance on luciferase enzyme activity. However, other methods could also be acceptable. Cytotoxicity controls should only be needed for high concentrations of substances that show apparent antagonistic effects.

2. GLP guidelines should be required. The use of GLP will improve overall results and minimize potential sources of error. These include making sure the balance and pipettes measure accurately, reagents are not past expiration, and minimizing mistakes in data transfer or transcribing from one location to another. Entry and exit assays for test article and control substances should be included as out-of-normal results cannot be accurately interpreted without them. However, due to the associated costs and lack of appropriate
analytical standards for many substances that will be tested, this approach should not be a requirement for screening assays.

3. Methods for establishing and propagation of a stable cell line are available in the scientific literature.

4. The use of charcoal-stripped serum should not be required if the cells can be maintained during the assay in serum free media. Propagation of the cells should not require charcoal-stripped serum. In cell assays that require the presence of serum during hormone incubations, serum that has been stripped of endogenous hormones would allow for a more sensitive assay.

5. Some information should be provided concerning the stability of stably expressing cell lines. Stable cell lines should be sufficiently stable to retain the integrated plasmids and response to control agonists and antagonists. Details about the drug requirements for maintaining the stable cell lines should be indicated. The MDA cell line described in the BRD appeared to be stable for at least 80 passages. Stability of any cell line should be closely monitored and ultimately a cutoff passage number should be determined. The cost of drugs necessary to maintain a stable cell line may be less than the costs of reagents necessary for a transient transfection screening assay.

6. Steroid or chemical metabolism should be established for positive responses. This could be done by including additional entry and exit assays for control ligands and test chemicals using satellite cultures of the cells plus media before and after culture. This approach would not add much to the cost as most of the cost is spent in the initial set-up and validation of each assay.

### 3.0 Recommendations for In Vitro AR TA Test Method Protocols for Validation Studies

Protocols provided by scientists with expertise in in vitro AR TA test methods are provided in Appendix B of the BRD. Section 12.3 discusses additional details that should be added, based on the minimum procedural standards in Section 12.2.

### 3.1 Considering the intended use of the assays as a toxicological screen, would the current protocols, with the additions detailed in Section 12.2 and 12.3, provide a level of detail to appropriately minimize interlaboratory variability? If not, what revisions or additions should be made to the protocols?

In general, the details on the effectiveness of the different assays were scarce in the BRD. Important details needed to compare different assays include fold induction by the control androgen, intra- and inter-assay coefficients of variability, stability of cell responsiveness over time and passage number, and a standardized method for comparing potencies of agonists and antagonists in the different assays.

### 3.2 In addition to the minimum procedural details listed in Section 12.2, are there other protocol elements that should be considered for In Vitro AR TA Assays recommended for validation as a toxicological screen, including those protocols provided in Appendix B?

The Panel recommends that the adenovirus infection method be further explored with the goal to eliminate activation by endogenous GR and use a more selective reporter. The other mammalian-based assays may also be appropriate for use in validation studies. The yeast-based assay has inherent limitations and complications related to the presence of a yeast
cell wall and active transport mechanisms that differ from those found in mammalian cells. The yeast-based assays do not discriminate between agonists and antagonists and should not be considered. Many of the mammalian-based assays are limited by several major considerations. There are patent issues associated with the transient co-transfection assays and cell lines that have the AR plasmid stably integrated as a result of transfection. If the patent issues cannot be resolved, almost all of the assays proposed will not be useful. On the other hand, it may be that a transient cotransfection assay could be used as a gold standard by which other assays would be judged in terms of response. One of the stable cell lines (protocol B6) is also subject to patent issues because the AR plasmid was transfected. The usefulness of the MDA stable line assay by Wilson et al. (2002) was complicated by the presence of endogenous GR, use of the MMTV-Luc reporter, and the low fold induction to the control androgen agonist (less than 10 fold) and the positive responses to 17β-estradiol and cortisol.

3.3 Considering the intended use of the assays as a toxicological screen, is the Panel aware of other available standardized protocols for assays recommended for validation?

**Assay 1:**
The N/C two-hybrid interaction assay in mammalian cells makes use of GAL4 and VP16 fusion proteins with the AR ligand binding domain and the AR N-terminal region. Assays are also being developed using the GAL4-AR ligand binding domain expressed with full-length AR. These assays have been modified for use in HeLa cells in a multiwell format (He et al. 2000). The advantage of the assay is that it distinguishes agonists and antagonists and can be performed in a multiwell format. The assay does not have false positives resulting from 17β-estradiol or cortisol because it depends on the androgen-specific interaction between the NH₂- and carboxyl-terminal regions of the AR. Limitations of the assay are that it is subject to the same patent restrictions that apply to other transient cotransfection assays that use the AR expression vector and that apply to stable cell lines with an integrated AR plasmid. The N/C assay has greater than 20 fold induction with 0.1 nM DHT, a sensitivity significantly greater than that achieved by stable assays presented in the BRD. The reporter vector is a GAL-Luc reporter with which no other steroid receptors are active.

**Assay 2:**
Other naturally occurring androgen response elements may have greater specificity to activation by the androgen-bound AR as opposed to activation by AR binding of 17β-estradiol. Without this specificity, assays using MMTV-Luc, while highly sensitive, have the disadvantage of false positives. Other response elements may have lower response than that achieved by the MMTV reporter (50-100 fold for MMTV-Luc). An assay such as this would also be subject to AR patent restrictions and may be further complicated by restrictions on specific reporter vectors.
4.0 Recommended List of Substances to be Used for Validation of *In Vitro* AR TA Assays

4.1 Does the Panel agree with the selection criteria, adequacy and appropriateness of substances recommended for validation studies for agonists and antagonists? If not, what substances should be added or deleted?

The listed substances are primarily steroids and pesticides of known AR agonist or antagonist activity. The objective of this transcriptional screening assay is the correct identification of substances that act as AR agonists or antagonists. As such, the chemicals with known AR agonist or antagonist activity could be abbreviated to those listed below to determine the ability of the test system to correctly identify their activity and their correct rank order. In addition, it is equally important to determine the ability of the test system to correctly identify known and predicted confounders, such as chemicals whose activity would be expected to alter luciferase production or activity independent of AR binding. In this way, confidence in the test system to correctly identify androgen active substances and correctly identify indirect or cytotoxic activity is maximized. The assay of liquid and gaseous volatiles is apparently not subject to screening in these test systems, otherwise modifications to the basic protocols might be needed.

**Recommendations for Substances to be used in Pre-Validation Studies of In Vitro AR TA Agonists and Antagonists Assays**

- R1881
- DHT
- testosterone
- androstenedione
- dexamethasone
- cortisol
- 17β-estradiol
- progesterone
- medroxyprogesterone acetate
- hydroxyflutamide
- casodex (bicalutamide)
- cyproterone acetate
- fluoxymesterone
- Linuron
- p,p'-DDE (1,1 Dichloro-bis[4-chlorophenyl]ethylene)
- finasteride
- possibly other weak agonists yet to be determined

**Substances to be Included that have Known or Predicted Activity that Could Affect Luciferase Transcription**

- cycloheximide (*protein synthesis inhibitor*)
- actinomycin D (*RNA synthesis inhibitor*)
- sodium azide (*cytotoxicant*)
- specific inhibitors of luciferase activity (*none known to the panel*)
- TPA (*ligand independent activation*)
It should not be necessary to include chemicals from other classes such as heavy metals, acids, bases, insoluble solids or reactive agents. Instead, it is critical to demonstrate accurate detection of known agonists and antagonists and to interpret cytotoxicity and indirect effects on luciferase synthesis and activity. Based on the possibility of ligand independent activation of AR cell systems, the phorbol ester TPA could be included as a negative control for agonism. The addition of a classic metabolic inducer like phenobarbital or a protein synthesis inhibitor like cycloheximide as controls for antagonism might also be worthwhile. For full validation efforts, a more diverse set of chemicals such as that presented in the BRD could be considered.

Some of the suggested substances may not be readily available commercially. Substances on the list should be available from commercial sources, although hydroxyflutamide and casodex can be difficult to obtain. The U.S. EPA could provide this standard set of chemicals for validation purposes.

Some of the substances listed in the BRD are not the active forms of the chemical, including flutamide, methoxychlor, procymidone, vinclozolin and DDT. The U.S. EPA would need to provide the active forms such as HPTE (from methoxychlor) and M2 (from vinclozolin).

The list should include substances such as cortisol, 17β-estradiol and progesterone that rule out activity of other endogenous steroid receptors and also substances with known or predicted confounding mechanisms.

4.1.1 The Number and Distribution of Substances Across the Range of Measurable AR/ER Binding Activity, Including Negatives
There is a need for weak substances but most that are listed in the BRD are precursors of the active forms and most of the active forms are not readily available.

4.1.2 The Number and Range of Substances by Chemical Class
There is a need to reduce the number of substances within various chemical classes and increase the number of chemical classes and/or predicted confounding mechanisms that are controlled for with the recommended cytotoxicity control procedure.

4.1.3 The Number and Range of Substances by Product Class
This was not considered by the Panel.
Summary

It is the overall conclusion of the Panel that no specific protocol was optimum for assessing AR agonist and antagonist activities. Major problems with the protocols presented in the BRD include:

1. Confounding effects of other endogenous steroid receptors in stable cell lines
2. Questions concerning the robustness of stable cell lines to detect weak androgens
3. AR patent issues relating to the transient and stable cell lines that utilize AR expression vectors and the cis-trans cotransfection methodology, and
4. Specificity of reporter vector response elements to reveal AR mediated transcriptional activation or inhibition.

With these considerations in mind, of the protocols provided by the BRD, the Panel concludes that the adenovirus infection method provides the most promising avenue for assessing AR agonist and antagonist activities and should be further considered. This assay was considered advantageous because it avoids time consuming transfection procedures as adenovirus infection protocols are straightforward. The assay showed a robust response of up to ~80 fold induction (as indicated during the meeting). Important aspects in the improvement of this protocol would be the identification and use of a cell line that lacks high response levels to the glucocorticoid and progesterone receptors. It would also make use of a reporter vector that shows greater specificity for the AR. The lack of absolute specificity for androgen binding by the AR is reflected in a general lack of specificity in hormone response in these in vitro assays; however, this does not parallel the in vivo situation. An ideal in vitro protocol would accurately reflect what is known about the in vivo physiological properties of steroid hormones. It was determined that nonlinear statistical models (e.g., the Hill equation) be used to estimate potency and steepness of the dose-response curve for full agonists and antagonists and that trend analysis be used to establish the significance of data that does not follow classical dose response relationships. The Panel recommendation for the list of chemicals for use in validation contained fewer substances than that suggested in the BRD. During the deliberations at the meeting, it was indicated that the U.S. EPA will consider supplying chemicals for validation studies, so additional weak acting agonists and antagonists could be included.
Additional Comments on the BRD

viii-ix:
ER and ER are not isoforms. They represent the products of different genes and in some regions have little homology.

ES-3:
It is not necessarily difficult to reproducibly transfect the same amount of DNA. It requires close adherence to the protocol and high quality cells. Also, cells that are stably transfected with an AR plasmid are also subject to patent restrictions, in contrast to what is stated on this page. The only way around this is to use the endogenously expressed AR with a transfected reporter plasmid. The reporter plasmid may or may not have additional patent restrictions.

1-6:
The AR cDNA does not contain an androgen responsive promoter, but rather, a promoter (CMV) that is responsive to numerous ubiquitous transcription factors insuring a high rate of transcription of the AR in the transfected cell.

1-7:
In the paragraph beginning, “In a series of deletion …”, should be changed to “…, while the ligand binding domain served an inhibitory function in the absence of androgen binding”.

1-9; line 4:
The AR mutation in the LNCaP cell line “would definitely impact” on its use in screening assays, not “might impact”. The LNCaP cells could not be used because this mutant AR has lost its specificity for binding androgen.

1-10:
It has not been shown that the AR dissociates from corepressor proteins on the binding of agonist. Also, androgen response elements are not always located “upstream” but are often within intron regions. Also “… including those necessary for cell proliferation, normal ‘male’ fetal development, or adult homeostasis.” On this page, the AR gene is on the long arm of the X chromosome at q11-12, not the short arm as stated (see Quigley et al. 1995).

1-11; line 5 from the bottom:
...antagonist.

1-12; last paragraph:
The “AR system” is not highly conserved in vertebrates. The fish AR has not been shown to be active in mammalian cells. This is most likely due to low sequence homology in the NH2-terminal region of the AR in vertebrates. This contrasts the ER where the rainbow trout ER is active in mammalian cells. The ligand binding domain is relatively highly conserved so this could be reworded to say “the ligand binding domain” rather than the AR “system”.

Endocrine Dis disruptor Expert Panel Report
1-13; last line:
Relevance is defined…

2-7; 6th line from bottom:
Luminescence is measured in a luciferase assay, not fluorescence.

3-2:
It is hydroxyflutamide not flutamide that is the AR antagonist although flutamide is given to prostate cancer patients. In most places in the text, flutamide should be replaced with hydroxyflutamide. Flutamide does not bind the AR and must be metabolized in vivo to the active form of the AR antagonist.

6-4:
The Poulin et al. 1991 reference is missing from the reference list.

6-4:
What is 17-alpha oxidase? The two endogenous steroid hormone biosynthetic enzymes that alter steroids at the 17 position are 17-beta-hydroxysteroid dehydrogenase (oxidizes testosterone to androstenedione) and 17-ketosteroid reductase (reduces androstenedione to testosterone). If liver metabolizing enzyme activity is meant here, are the authors referring to CYP2C11 activity? What is the product of the reaction and subsequent androgen agonist activity of the product (i.e. potential to interfere with the assay)?

6-7:
MDA-MB453-kb2 only has the reporter vector integrated and expresses endogenous AR. Otherwise it would not escape patent restrictions.

6-9:
AR patent issues also apply to cell lines with an integrated AR plasmid, no matter which plasmid was used as long as it contains the recombinant human AR sequence.

7-5:
For the data in Table 7-2 to be meaningful, the concentration of androgen should be indicated. The more androgen used in the studies, the higher amount of antagonist is required for inhibition. Unless all of the assays used the same concentration of androgen, the data from the different assays are not directly comparable.

12-2:
Binding of testosterone or DHT to TeBG could potentially be a problem in the assays if serum is included in the cell culture medium during the hormone incubations.

14-1:
Androgen is not technically a male hormone. It is a class of male hormones. The male hormones are testosterone and DHT. Just like estrogen is not a hormone. The hormone is 17β-estradiol (see 14-3).
14-4:
It should be hypospadias and this anomaly only applies to males.

A2-7:
MDA-MB-453 is not a stable cell line. The cells must be transduced with adenovirus carrying the MMTV-luciferase reporter for each assay.

A3:
In several manuscripts, a truncated constitutively active human AR (AR1-660) was used to estimate cytotoxicity. This constitutively active human AR induces transactivation of the MMTV-Luc reporter and serves as an ideal cytotoxicity control as both nonspecific effects on transcription and luciferase enzyme activity are assessed. Alternatively, a CMV-Luc construct could be used to accomplish the same objective.
References


APPENDIX B

Evaluation Guidance to the Expert Panel for the Review of
In Vitro Test Methods for Detecting Endocrine Disruptors

B-1 Evaluation Guidance for Estrogen Receptor and Androgen Receptor Binding Assays .........................................................B-3

B-2 Evaluation Guidance for Estrogen Receptor and Androgen Receptor Transcriptional Activation Assays ..........B-7
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Evaluation Guidance to the Expert Panel for the Review of

*In Vitro* AR/ER Binding Assays

A. **General Instructions for the Expert Panel**

The Panel is charged with reviewing the information and data provided in the Background Review Documents (BRDs) and developing conclusions and recommendations on the following:

1. *In vitro* AR/ER binding assays that should be considered for further evaluation in validation studies, and their relative priority for further evaluation.
2. The adequacy of the minimum procedural standards recommended for *in vitro* AR/ER binding assays.
4. The adequacy and appropriateness of substances recommended for validation studies of *in vitro* AR/ER binding assays.

An outline of specific items to be addressed by the Panel is provided in **Section B** below. The Panel is charged with developing a written report that summarizes its recommendations and conclusions for each question.

All members of the Test Method Evaluation Group, including Secondary Reviewers (as outlined in the Panel Group spreadsheet), are asked to answer all four sets of Evaluation Guidance Questions and submit responses to the Question Leader (see Questions Leader assignments below). Panel Members are also welcome to respond to questions for the other two Test Methods where they are not a designated reviewer. The Question Leader is responsible for compiling comments and developing a draft response for their question. The Breakout Group Chair is responsible for compiling each question’s draft response into an overall draft position for the Breakout Group. This draft position will be circulated to each member of the Panel before the May review meeting for comment. The revised draft position will be presented and discussed at the Expert Panel review meeting in May.

**Proposed Evaluation Guidance Question Leaders**

*In Vitro* ER Binding BRD:

Chair: George Daston
Question 1: Nira Ben-Jonathan
Question 2: Bob Combes and James Wittliff
Question 3: John Giesy and John Harbell
Question 4: Stephen Safe
Statistician: Walter Piegorsch

*In Vitro* ER Transcriptional Activation BRD:

Chair: John Stegeman
Question 1: Grantley Charles
Question 2: Ellen Mihaich and Tim Zacharewski
Question 3: Tom Wiese
Question 4: James Yager
B. Questions for Evaluating the In Vitro AR/ER Binding BRDs

1. In Vitro AR/ER Binding Assays: Recommendations and Priority for Validation Studies

1.1 The respective BRDs review the comparative performance, reliability, advantages, and disadvantages for different in vitro AR/ER binding assays, and recommend a relative priority for further development and/or validation based on this information (Section 6.0). Considering that the intended use of the assays are as a toxicological screen, is the Panel aware of other advantages and disadvantages for the assays discussed in the BRDs?

1.2 Considering that the intended use of the assays are as a toxicological screen, does the Panel agree with the relative priority recommended for these sets of assays? Does the Panel recommend any changes in priority, or have specific recommendations for prioritization? In considering prioritization,

1.2.1 Are rat uterine cytosol and rat prostate cytosol the best sources of estrogen receptors and androgen receptors, respectively, for the binding assays?

1.2.2 Should the binding of compounds to different receptor isoforms be addressed in the binding assays?

1.2.3 Should a metabolic activation system be included in the binding assays?

2. Minimum Procedural Standards for In Vitro AR/ER Binding Assays

2.1 To facilitate assay standardization, the BRDs propose minimum procedural standards that should be incorporated into in vitro AR/ER binding assay protocols (Section 12.2). Considering that the intended use of the assays are as a toxicological screen, does the Panel agree with the adequacy of the proposed procedural standards? If not, what changes should be made to each standard and why?

2.1.1 Binding Constant (Kd) of the Reference Androgen/Estrogen
2.1.2 Reference Androgen/Estrogen
Should the reference androgen be an endogenous one rather than a synthetic androgen like R1881? In AR binding assays containing the progesterone receptor (PR) in addition to the AR, triamcinolone acetate is added to prevent the binding of R1881 to the receptor without interfering with the binding of either R1881 or test substances to the AR. Is enough known to predict that triamcinolone acetonide will not interfere with future test substances if this compound is routinely used in the assay?

2.1.3 Preparation of Test Substances
2.1.4 Concentration Range of Test Substances
2.1.5 Solvent and Positive Controls
2.1.6 Within Test Replicates
2.1.7 Dose Spacing
2.1.8 Data Analysis
2.1.9 Assay Acceptance Criteria
2.1.10 Evaluation and Interpretation of Results
2.1.11 Test Report
2.1.12 Replicate Studies

2.2 Considering that the intended use of the assays are as a toxicological screen, are there other minimum procedural standards that should be included? If so, what are they and why?

3. Recommendations for In Vitro AR/ER Binding Test Method Protocols for Validation Studies
3.1 A standardized in vitro AR binding assay protocol using rat prostate cytosol (RPC) and a standardized in vitro ER binding assay protocol using rat uterine cytosol (RUC) are provided in Appendix B of their respective BRDs. These two assays are proposed for validation studies by the U.S. EPA and other sponsors. Section 12.3 discusses additional details that should be added, based on the minimum procedural standards in Section 12.2. In addition, an example of an in vitro ER Binding RUC assay (based on the U.S. EPA protocol), which incorporates the recommended minimum procedural standards is provided in Section 12 Annex of the “In Vitro ER Binding BRD”. Considering that the intended use of the assays are as a toxicological screen, would the current protocols, with the additions detailed in Section 12.2 and 12.3, provide a level of detail to appropriately minimize interlaboratory variability? If not, what revisions or additions should be made to the protocols?

3.2 In addition to the minimum procedural details listed in Section 12.2, are there other protocol elements that should be considered for other in vitro AR/ER binding assays recommended for validation as a toxicological screen, including those protocols provided in Appendix B?
3.3 Considering that the intended use of the assays are as a toxicological screen, is the Panel aware of other available standardized protocols for assays recommended for validation?

4. **Recommended List of Substances to be Used for Validation of In Vitro AR/ER Binding Assays**

4.1 **Section 12.4** provides a list of substances recommended for use in validation studies of *in vitro* AR/ER binding assays. Considering that the intended use of the assays are as a toxicological screen, does the Panel agree with the selection criteria, adequacy and appropriateness of substances recommended for validation studies, in terms of the following issues? If not, what substances should be added or deleted?

4.1.1 The number and distribution of substances across the range of measurable AR/ER binding activity, including negatives.

4.1.2 The number and range of substances by chemical class.

4.1.3 The number and range of substances by product class.
Evaluation Guidance to the Expert Panel for the Review of
*In Vitro* AR/ER Transcriptional Activation (TA) Assays

A. General Instructions for the Expert Panel

The Panel is charged with reviewing the information and data provided in the Background Review Documents (BRDs) and developing conclusions and recommendations on the following:

1. *In vitro* AR/ER TA assays that should be considered for further evaluation in validation studies, and their relative priority for further evaluation.
2. The adequacy of the minimum procedural standards recommended for *in vitro* AR/ER TA assays.
4. The adequacy and appropriateness of substances recommended for validation studies of *in vitro* AR/ER TA assays.

An outline of specific items to be addressed by the Panel is provided in Section B below. The Panel is charged with developing a written report that summarizes its recommendations and conclusions for each question.

All members of the Test Method Evaluation Group, including Secondary Reviewers (as outlined in the Panel Group spreadsheet), are asked to answer all four sets of Evaluation Guidance Questions and submit responses to the Question Leader (see Questions Leader assignments below). Panel Members are also welcome to respond to questions for the other two Test Methods where they are not a designated reviewer. The Question Leader is responsible for compiling comments and developing a draft response for their question. The Breakout Group Chair is responsible for compiling each question’s draft response into an overall draft position for the Breakout Group. This draft position will be circulated to each member of the Panel before the May review meeting for comment. The revised draft position will be presented and discussed at the Expert Panel review meeting in May.

**Proposed Evaluation Guidance Question Leaders**

*In Vitro* ER Binding BRD:

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<tr>
<th>Chair</th>
<th>Nira Ben-Jonathan</th>
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<td>Question 1</td>
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<td>Question 3</td>
<td>Steve Safe</td>
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<td>Statistician</td>
<td>Walter Piegorsch</td>
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*In Vitro* ER TA BRD:

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<th>John Stegeman</th>
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<tr>
<td>Question 3</td>
<td>Tom Wiese</td>
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</table>
In Vitro AR Binding BRD:
Chair: Terry Brown
Question 1: Thomas Gasiewicz
Question 2: Anne Marie Vinggaard
Question 3: Bernard Robaire
Question 4: Tohru Inoue
Statistician: Walter Piegorsch

In Vitro AR TA BRD:
Chair: Elizabeth Wilson
Question 1: William Kelce
Question 2: William Kelce
Question 3: Kevin Gaido
Question 4: Elizabeth Wilson
Statistician: Shyamal Peddada

B. Questions for Evaluating the In Vitro AR/ER TA BRDs
1. In Vitro AR/ER TA Assays: Recommendations and Priority for Validation Studies
   1.1 The respective BRDs review the comparative performance, reliability, advantages, and disadvantages for different in vitro AR/ER TA assays, and recommend a relative priority for further development and/or validation based on this information (Section 6.0). Considering that the intended use of the assays are as a toxicological screen, is the Panel aware of other advantages and disadvantages for the assays discussed in the BRDs?

   1.2 Considering that the intended use of the assays are as a toxicological screen, does the Panel agree with the relative priority recommended for these sets of assays? Does the Panel recommend any changes in priority, or have specific recommendations for prioritization? In considering prioritization,
   1.2.1 What receptor types (species, isoform) are the best for the transcriptional activation assays?
   1.2.2 Should preference be given to cells with endogenous ER, transiently transfected ER expression vectors, or stably transfected ER expression vectors?
   1.2.3 Which response elements (species, sequence) are the best for the reporter vectors?

2. Minimum Procedural Standards for In Vitro AR/ER TA Assays
   2.1 To facilitate assay standardization, the BRDs propose minimum procedural standards that should be incorporated into in vitro AR/ER TA assay protocols (Section 12.2). Considering that the intended use of the assays are as a toxicological screen, does
the Panel agree with the adequacy of the proposed procedural standards? If not, what changes should be made to each standard and why?

2.1.1 Transcriptional Activation of the Reference Androgen/Estrogen
2.1.2 Reference Androgen/Estrogen
2.1.3 Preparation of Test Substances
2.1.4 Concentration Range of Test Substances
2.1.5 Solvent and Positive Controls
2.1.6 Within Test Replicates
2.1.7 Dose Spacing
2.1.8 Data Analysis
2.1.9 Assay Acceptance Criteria
2.1.10 Evaluation and Interpretation of Results
2.1.11 Test Report
2.1.12 Replicate Studies

2.2 Considering that the intended use of the assays are as a toxicological screen, are there other minimum procedural standards that should be included? If so, what are they and why?

3. **Recommendations for In Vitro AR/ER TA Test Method Protocols for Validation Studies**

3.1 Protocols provided by scientists with expertise in *in vitro* AR/ER TA test methods are provided in Appendix B of the respective BRDs. Section 12.3 discusses additional details that should be added, based on the minimum procedural standards in Section 12.2. Considering that the intended use of the assays are as a toxicological screen, would the current protocols, with the additions detailed in Section 12.2 and 12.3, provide a level of detail to appropriately minimize interlaboratory variability? If not, what revisions or additions should be made to the protocols?

3.2 In addition to the minimum procedural standards listed in Section 12.2, are there other protocol elements that should be considered for *in vitro* AR/ER TA assays recommended for validation as a toxicological screen, including those protocols provided in Appendix B?

3.3 Considering that the intended use of the assays are as a toxicological screen, is the Panel aware of other available standardized protocols for assays recommended for validation?

4. **Recommended List of Substances to be Used for Validation of In Vitro AR/ER TA Assays**

4.1 Section 12.4 provides a list of substances recommended for use in validation studies of *in vitro* AR/ER TA Assays. Considering that the intended use of the assays are as a toxicological screen, does the Panel agree with the selection criteria, adequacy and appropriateness of substances recommended for validation studies, in terms of the following issues? If not, what substances should be added or deleted?

4.1.1 The number and distribution of substances across the range of measurable AR/ER transcriptional activity, including negatives.
4.1.2 The number and range of substances by chemical class.
4.1.3 The number and range of substances by product class.
APPENDIX C

Expert Panel Evaluation Meeting Agenda
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Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)
Expert Panel Evaluation of the Validation Status of
In Vitro Test Methods for Detecting Endocrine Disruptors:

Estrogen Receptor and Androgen Receptor Binding
and Transcriptional Activation Assays

Agenda

Meeting Venue: Sheraton Imperial Hotel, Imperial Ballroom
Research Triangle Park, North Carolina
Date: May 21-22, 2002

Objectives for the Expert Panel:

The Panel is charged with reviewing the information and data provided in the Background Review Documents (BRDs) and developing conclusions and recommendations on the following:

1. *In vitro* estrogen receptor (ER)/androgen receptor (AR) binding and transcriptional activation (TA) assays that should be considered for further evaluation in validation studies, and their relative priority for further evaluation.
2. The adequacy of the minimum procedural standards recommended for *in vitro* ER/AR binding and TA assays.
3. The adequacy of available *in vitro* ER/AR binding and TA test method protocols for use in validation studies.
4. The adequacy and appropriateness of substances recommended for validation studies of *in vitro* ER/AR binding and TA assays.
ICCVAM Review of In Vitro Endocrine Disruptor Screening Assays

ICCVAM Endocrine Disruptor Expert Panel Public Meeting
Sheraton Imperial Hotel
Research Triangle Park, NC
Imperial Ballroom

Tuesday, May 21, 2002

8:30 a.m. Opening Session

1. Call to Order and Panel Introductions (Panel Chair, Dr. George Daston)

2. Overview of the ICCVAM Test Method Evaluation Process and Charge to the Panel (Dr. William Stokes)

3. Overview of ER and AR In Vitro Binding and Transcriptional Activation Assays (Dr. Vickie Wilson)

9:05 a.m. ER Binding Assays: Evaluation of the ER Binding Background Review Document (Drs. Daston, Ben-Jonathan, Combes, Giesy, Harbell, Safe, Wittliff, and Piegorsch)

Overview of the ER Binding Background Review Document

Question #1: Recommendations and Priority for Validation Studies
PANEL DISCUSSION OF QUESTION #1

Question #2: Minimum Procedural Standards for In Vitro ER Binding Assays
PANEL DISCUSSION OF QUESTION #2

10:30 a.m. Break

10:45 a.m. ER Binding Assays (Continued)

Question #3: Recommendations for In Vitro ER Binding Protocols for Validation Studies
PANEL DISCUSSION OF QUESTION #3

Question #4: Recommended List of Substances to be used for Validation of In Vitro ER Binding Assays
PANEL DISCUSSION OF QUESTION #4

12:05 p.m. Public Comment

12:30 p.m. Lunch Break
1:30 p.m.  ER TA Assays: Evaluation of the ER TA Background Review Document  
(Drs. Stegeman, Charles, Mihaich, Wiese, Yager, Zacharewski, and Peddada)

Overview of the ER TA Background Review Document

Question #1: Recommendations and Priority for Validation Studies  
PANEL DISCUSSION OF QUESTION #1

Question #2: Minimum Procedural Standards for In Vitro ER TA Assays  
PANEL DISCUSSION OF QUESTION #2

2:55 p.m.  Break

3:10 p.m.  ER TA Assays (Continued)

Question #3: Recommendations for In Vitro ER TA Protocols for Validation Studies  
PANEL DISCUSSION OF QUESTION #3

Question #4: Recommended List of Substances to be used for Validation of In Vitro ER TA Assays  
PANEL DISCUSSION OF QUESTION #4

4:30 p.m.  Public Comment

5:00 p.m.  Adjourn for the day
Wednesday, May 22, 2002

8:30 a.m.  AR Binding Assays: Evaluation of the AR Binding Background Review Document  
(Drs. Brown, Gasiewicz, Inoue, Robaire, Vinggaard, and Piegorsch)

Overview of the AR Binding Background Review Document

Question #1: Recommendations and Priority for Validation Studies  
PANEL DISCUSSION OF QUESTION #1

Question #2: Minimum Procedural Standards for In Vitro AR Binding Assays  
PANEL DISCUSSION OF QUESTION #2

9:55 a.m.  Break

10:10 a.m.  AR Binding Assays (Continued)

Question #3: Recommendations for In Vitro AR Binding Protocols for Validation Studies  
PANEL DISCUSSION OF QUESTION #3

Question #4: Recommended List of Substances to be used for Validation of In Vitro ER Binding Assays  
PANEL DISCUSSION OF QUESTION #4

11:30 a.m.  Public Comment

12:00 p.m.  Lunch Break

1:00 p.m.  AR TA Assays: Evaluation of the AR TA Background Review Document  
(Drs. Wilson, Gaido, Kelce, and Peddada)

Overview of the AR TA Background Review Document

Question #1: Recommendations and Priority for Validation Studies  
PANEL DISCUSSION OF QUESTION #1

Question #2: Minimum Procedural Standards for In Vitro AR TA Assays  
PANEL DISCUSSION OF QUESTION #2

Question #3: Recommendations for In Vitro AR TA Protocols for Validation Studies  
PANEL DISCUSSION OF QUESTION #3

3:05 p.m.  Break
3:20 p.m.  AR TA Assays (Continued)

Question #4: Recommended List of Substances to be used for Validation of In Vitro AR TA Assays
PANEL DISCUSSION OF QUESTION #4

4:00 p.m.  Closing Comments

5:00 p.m.  Adjourn
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APPENDIX D

Expert Panel Meeting Minutes

\textbf{Introduction}

A public meeting of an independent Expert Panel was convened on May 21-22, 2002, at the Sheraton Imperial Hotel, Research Triangle Park, North Carolina, to review the current status of \textit{in vitro} methods used to measure estrogen and androgen receptor binding and estrogen and androgen transcriptional activation assays. The meeting was organized by ICCVAM and the National Toxicology Program (NTP) Interagency Center for the Evaluation of Toxicological Methods (NICEATM) and sponsored by the National Institute of Environmental Health Sciences (NIEHS) and the NTP. A comprehensive report of the peer review panel is provided as an attachment to these minutes.

The following scientists served on the expert panel:

- George Daston, Ph.D., (Panel Chair), Research Fellow, Miami Valley Laboratories, Procter & Gamble, Cincinnati, Ohio
- Nira Ben-Jonathan, Ph.D., Professor of Cell Biology, Neurobiology and Anatomy, University of Cincinnati Medical School, Cincinnati, Ohio
- Terry Brown, Ph.D., Professor, School of Public Health, Johns Hopkins University, Baltimore, Maryland
- Grantley Charles, Ph.D., Toxicology and Environmental Research & Consulting, Dow Chemical Co., Midland, Michigan
- Robert Combes, Ph.D., Professor, FRAME, Nottingham, United Kingdom
- Kevin Gaido, Ph.D., Scientist II, CIIT, Research Triangle Park, North Carolina
- Thomas Gasiewicz, Ph.D., Professor, Dept. of Environmental Medicine, University of Rochester School of Medicine, Rochester, New York
- John P. Giesy, Ph.D., Professor, Michigan State University, East Lansing, Michigan
- John W. Harbell, Ph.D., Vice President and Chief Scientific Officer, Institute for \textit{In Vitro} Sciences, Inc., Gaithersburg, Maryland
- Tohru Inoue, M.D., Ph.D., Director, Center for Biological Safety Research, National Institute of Health Sciences, Tokyo, Japan
- Ellen M. Mihaich, Ph.D., D.A.B.T., Senior Environmental Toxicologist, Rhodia, Inc., Raleigh, North Carolina
- Shyamal Peddada, Ph.D., Biostatistics Branch, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina
- Walter Piegorsch, Ph.D., Professor, Dept. of Statistics, University of South Carolina, Columbia, South Carolina
ICCVAM Review of In Vitro Endocrine Disruptor Screening Assays

Appendix D

Expert Panel Meeting Minutes

Bernard Robaire, Ph.D., Professor, Dept. of Pharmacology & Therapeutics, McGill University, Montreal, Quebec, Canada
Stephen Safe, Ph.D., Professor, Dept. of Veterinary Physiology & Pharmacology, Texas A&M University, College Station, Texas
John Stegeman, Ph.D., Senior Scientist, Chairman, Biology Dept., Woods Hole Oceanographic Institution, Woods Hole, Massachusetts
Anne Marie Vinggaard, Ph.D., Senior Scientist, Institute of Food Safety & Toxicology, Danish Veterinary & Food Administration, Soborg, Denmark
Tom Weise, Ph.D. Assistant Professor, Environmental Health Science, Tulane University, New Orleans, Louisiana (not present at the meeting)
Elizabeth Wilson, Ph.D., Professor of Pediatrics, Biochemistry & Biophysics, University of North Carolina, Chapel Hill, North Carolina
James L. Wittliff, Ph.D., F.A.C.B., Professor of Biochemistry & Molecular Biology, University of Louisville, Louisville, Kentucky
James D. Yager, Ph.D., Senior Associate Dean of Academic Affairs, Professor, Environmental Health Sciences, School of Public Health, Johns Hopkins University, Baltimore, Maryland
Tim Zacharewski, Ph.D., Associate Professor, Dept. of Biochemistry & Molecular Biology, Michigan State University, East Lansing, Michigan

The following ICCVAM agency representatives were present:
- Dr. Karen Hamernik, (Endocrine Disruptor Working Group - EDWG) U.S. Environmental Protection Agency
- Dr. David Hattan, (EDWG) U.S. Food and Drug Administration
- Dr. Jerold Heindel, (EDWG) National Institute of Environmental Health Sciences
- Dr. Abigail Jacobs, (EDWG) U.S. Food and Drug Administration
- Dr. Leonard Schechtman, (EDWG) (ICCVAM Chair) U.S. Food and Drug Administration
- Dr. William Stokes, (EDWG) Director, NICEATM and Principal ICCVAM Agency Representative, National Institute of Environmental Health Sciences

The following additional members of the ICCVAM Endocrine Disruptor Working Group (EDWG) were present:
- Dr. Paul Brown, U.S. Food and Drug Administration
- Dr. Sally Perreault-Darney, U.S. Environmental Protection Agency
- Dr. Julius Thigpen, National Institute of Environmental Health Sciences

The following NICEATM Staff were present:
- Mr. Brad Blackard, ILS, Inc.
- Ms. Sue Brenzel, ILS, Inc.
- Ms. Loretta Frye, National Institute of Environmental Health Sciences
- Ms. Christina Inhof, ILS, Inc.
- Ms. Linda Litchfield, ILS, Inc.
- Ms. Debbie McCarley, National Institute of Environmental Health Sciences
- Mr. Steve Myers, ILS, Inc.
- Mr. Michael Paris, ILS, Inc.
The purpose of this meeting was to evaluate the validation status of in vitro test methods for detecting endocrine disruptors. The Expert Panel was asked to evaluate four background review documents (BRDs) prepared by National Toxicology Program Interagency Center for the Evaluation of Toxicological Methods (NICEATM).

The four BRDs reviewed and discussed were:
- Current Status of Test Methods for Detecting Endocrine Disruptors: In Vitro Estrogen Receptor (ER) Binding Assays
- Current Status of Test Methods for Detecting Endocrine Disruptors: In Vitro Estrogen Receptor Transcriptional Activation (ER TA) Assays
- Current Status of Test Methods for Detecting Endocrine Disruptors: In Vitro Androgen Receptor (AR) Binding Assays
**Introductions**

Dr. George Daston, Panel Chair, called the meeting of the Expert Panel (Panel) to order at 9:00 a.m. and asked each attendee to state their name and affiliation. Dr. Daston stated that the public would be given the opportunity to speak at various times during the meeting. Each speaker from the public would be limited to seven minutes, and anyone addressing the group should state their name for the benefit of the transcriptionist.

Dr. William Stokes, Executive Secretary for the Expert Panel, read the Statement of Conflict of Interest and explained policies and procedures regarding confidentiality and avoidance of conflict of interest, as follows:

“The members of this expert panel serve as individual scientists and not as representatives of any organization. Each member is to exercise judgment as to whether a potential conflict of interest might exist relative to one or more of the topics being discussed due to his or her occupational affiliation, professional activity or financial interest. Should there be a potential conflict of interest, the member is to recuse him or herself from participating in the discussion of panel recommendations and/or decisions on the topic. You will be signing a conflict of interest certification which declares that during this panel meeting you did not participate in discussion of panel recommendations and/or decisions that involve a particular matter that could have a direct and predictable effect on: 1) Any organization, institution or university system in which a financial interest exists for yourself, spouse, parent, minor child or partner. 2) Any organization in which you, your spouse, parent, minor child or partner serves as an officer, director, trustee or employee or is otherwise similarly associated. 3) Any organization with which you, your spouse, parent, minor child or parent [sic] is negotiating or have any arrangements concerning prospective employment or other such associations. Panel members are asked to identify at the beginning of this meeting the nature of any such conflicts.”

Dr. Elizabeth Wilson (University of North Carolina) responded that she was one of the scientists involved in cloning the androgen receptor (AR) in 1998. “This resulted in the awarding of a patent for the androgen receptor sequence to the University of North Carolina in Chapel Hill. This patent has also been awarded to the University of Chicago.” Dr. Wilson went on to say “And at the moment Ligand Pharmaceuticals holds an exclusive license on this patent. And because of that potential complication, I plan to offer my comments in terms of scientific expertise, but I will recuse myself from any decisions relating to protocols.”

Dr. Robert Combes (FRAME, UK) responded that although he did not have a financial conflict of interest, he did have a bias against the use of animal tests that would affect his recommendations. He works for an organization that promotes non-animal methods “and therefore, if there is a recommendation, a choice between two assays that are scientifically equivalent, but one uses less animals or no animals at all, then I would promote the one that doesn’t use animals or is more welfare conscious. So I don’t think I need to recuse myself.”
Overview of the ICCVAM Test Method and Evaluation Process

Dr. Stokes, (Director, NICEATM, NIEHS) provided a brief background on ICCVAM and NICEATM, and described the purpose of the meeting. He explained that this is an Expert Panel rather than a Peer Review Panel because no specific methods have been standardized and evaluated in validation studies.

ICCVAM was established as an ad hoc committee in 1994 in response to revisions in the 1993 NIH Revitalization Act (P.L. 103-43) that mandates that the NIEHS develop criteria for validation and regulatory acceptance of test methods, and develop a process to achieve regulatory acceptance of scientifically valid methods. The ad hoc committee issued its report in 1997, and the ICCVAM committee was formally established that year to implement P.L. 103-43 directives. In 2000, the ICCVAM Authorization Act (P.L. 106-545) established ICCVAM as a permanent committee.

Member agencies of ICCVAM include those involved in regulatory and research activities (CPSC; DOA; DOI; DOT; EPA; FDA; OSHA) and those involved in non-regulatory research (ATSDR; DOD; DOE; NCI; NIEHS; NIOSH; NLM; NIH, OD). NICEATM is located at NIEHS and is responsible for providing operational and technical support to ICCVAM.

The purposes of ICCVAM, as set forth in P.L. 106-545, are to:
- Increase efficiency and effectiveness of Federal agency test method review;
- Eliminate unnecessary duplicative efforts and share experiences between Federal regulatory agencies;
- Optimize utilization of scientific expertise outside the Federal Government;
- Ensure that new and revised test methods are validated to meet the needs of Federal agencies;
- Reduce, refine, or replace the use of animals in testing, where feasible.

The duties and responsibilities of ICCVAM are to:
- Review and evaluate new, revised or alternative test methods;
- Facilitate interagency and international harmonization of test methods;
- Facilitate and provide guidance on test method development, validation criteria, and validation processes;
- Facilitate acceptance of scientifically valid test methods;
- Submit test method recommendations to Federal agencies;
- Consider petitions from the public for review and evaluation of validated test methods.

An ICCVAM Endocrine Disruptor Working Group (EDWG) comprised of government scientists that is co-chaired by Drs. David Hattan and Marilyn Wind, worked with NICEATM to develop the questions that were addressed to the panel. This group also recommended experts to serve on the panel and the members reviewed the BRDs for completeness. The EDWG will review the recommendations proposed by the Expert Panel and develop draft ICCVAM recommendations. ICCVAM recommendations and the Panel’s report will be forwarded to the U.S. EPA and other Federal Agencies for consideration.
The background and history of the ICCVAM evaluation of *in vitro* estrogen receptor (ER) and androgen receptor (AR) methods were described. In 2000, the U.S. Environmental Protection Agency (U.S. EPA) requested that ICCVAM conduct an independent scientific peer review of *in vitro* ER and AR binding and transcriptional activation methods. In March 2001, ICCVAM published a Federal Register notice requesting data and information on these methods, and the nomination of experts that might serve on the peer-review Panel. At the same time, the four BRDs - *in vitro* ER binding; *in vitro* ER transcriptional activation; *in vitro* AR binding; *in vitro* AR transcriptional activation were being prepared by NICEATM. During this review it was noted that there were no standardized test methods that had undergone formal validation studies. In April 2002, a Federal Register notice announced the dates of this meeting, the availability of the BRDs, and a request for public comments.

**Charge to the Expert Panel and Organization of the Review**

Dr. Stokes explained the charge to the Expert Panel. The Panel was requested to review the BRDs and provide conclusions and recommendations on the following:

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

**Overview of the biology of estrogen and androgen receptor binding and transcriptional activation assays**

Dr. Vickie Wilson, (Research Biologist, Reproductive Toxicology Division NHEERL, U.S. EPA) provided an overview of the biology of estrogen and androgen receptor binding and transcriptional activation assays. She discussed the concept of the binding of a hormone ligand with a receptor, resulting in a conformational change of the receptor. The ligand-receptor complex dimerizes and is then able to bind to a DNA response element resulting in the transcription or inhibition of the transcription of a gene. Ultimately a protein is produced that has some biological function in the organism. The receptor binding assays measure whether a test substance binds in place of the natural hormone to the receptor. The transcriptional activation (TA) assays measure the next step in the pathway, namely, the transcription of a gene. A compound that initiates transcription, following receptor binding, is known as an agonist while one that blocks transcription after binding to the receptor is known as an antagonist.

**Receptor Binding Assays**

Dr. Vickie Wilson described two general types of receptor binding assays. In the first type of assay, a saturation binding experiment is performed in which increasing amounts of radiolabeled hormone are added to the receptor until binding is saturated. This experiment allows for the determination of the equilibrium dissociation constant for a radioligand (K_d), an indicator of the binding affinity of the radiolabeled hormone to the receptor, and for the maximum number of binding sites in the receptor preparation (B_max). The second type of experiment is a competitive
binding experiment in which increasing amounts of the test substance are added to the receptor in the presence of a single concentration of the radiolabeled reference hormone that is usually at or just below the $K_d$ value. The components are allowed to come to equilibrium, the bound radioligand is separated from the free radioligand, and the quantity of radioligand bound receptor is determined at each concentration of test compound. An IC$_{50}$, which is the molar concentration of test substance that reduces the binding of the radiolabeled hormone to the receptor by 50%, can be calculated. The relative binding affinity (RBA) of the test substance, which is the ratio between the IC$_{50}$ of the substance and the IC$_{50}$ of the reference hormone, can then be calculated. To determine if the observed binding inhibition is truly due to competitive inhibition; assays can be performed to experimentally determine the affinity of the unlabeled substance ($K_i$) to the receptor. Similar experiments can be performed for estrogen and androgen binding substances.

Dr. Vickie Wilson then described examples of the different kinds of binding curves that can be obtained when substances displace the hormone from the receptor. She stressed the problems associated with the testing of relatively high concentrations of the test substance and the situation that can occur when one obtains a precipitous decline in the binding over a very narrow range in concentration of the test substance. Under the latter conditions, it might be necessary to determine the $K_i$ value experimentally. In this case, increasing concentrations of the test substance are added to several different concentrations of radiolabeled hormone to generate a number of lines in a double reciprocal plot. The pattern of the lines indicates the type of inhibition, for example, competitive inhibition versus non-competitive inhibition. The slopes of the lines are then plotted and the intercept of the line on the X-axis is the $K_i$. Dr. Vickie Wilson then briefly discussed the various sources of the ER receptor and some of the general strengths and limitations of binding assays.

**Transcriptional Activation Assays**

Dr. Vickie Wilson described four major categories of TA assays and the methods of transfecting the receptor (androgen or estrogen) and reporter gene (luciferase) into the cell lines. Transcriptional activation is quantified by the measurement of an androgen- or estrogen-responsive promoter attached to a reporter gene such as luciferase. For antagonism assays, Dr. Vickie Wilson emphasized the importance of first measuring the TA of the reference ligand to determine the linear part of the dose response curve and to establish the appropriate concentration of the reference ligand to use in subsequent assays. Then increasing concentrations of the test substance are added to the cells that are simultaneously being exposed to a specific concentration of the reference ligand. She emphasized the need for the use of media controls and performance of a cytotoxicity assay to determine that decreases in reporter gene activity are not due to cell death. Dr. Vickie Wilson discussed some of the strengths and limitations of the different types of transcriptional activation assays. In closing, she stated that data evaluation is critical and criteria need to be established to determine whether a compound is positive or negative.

**Organization of the Panel review**

During the course of the meeting the Panel addressed the questions concerning the completeness and utility of the BRD and the performance of each particular assay. Four sub-groups of the
Expert Panel were responsible for addressing the questions for each BRD, and drafting responses for consideration by the entire Panel.

Prior to the presentations and discussions by each of the four groups, Dr. Barbara Shane (NICEATM, ILS) provided a brief summary of the assays described in the BRD, the minimum procedural standards for an assay, and the substances suggested for validation of the assay(s).

Each group presented their draft responses for each of the questions assigned for their BRD. After each presentation, the Panel discussed the draft positions and offered additional comments and suggestions. The Chairman summarized the discussion for each question and sought consensus from the Panel on the topic. Public comments were accepted following the Panel’s discussion of each BRD.

A. Estrogen Receptor (ER) Binding Assays

Primary reviewers: G. Daston, Group Chair (Procter & Gamble); N. Ben-Jonathan (University of Cincinnati); R. Combes (FRAME, UK); J. Harbell (Institute for In Vitro Sciences, Inc.); S. Safe (Texas A&M University); J.L. Wittliff (University of Louisville); W. Piegorsch (University of South Carolina).

Summary of the ER Binding Background Review Document

Dr. Shane described the approach used to compile the BRDs. She stated that the on-line databases searched for publications on ER binding were Medline, Cancerlit, Toxline, Agricola, NIOSHTIC, Embase, CABA, Biosis, and Life Sciences. The key words screen, tests, batteries, bind, ligand, estrogen, and receptor were used in the search. This yielded 260 records of which 74 contained relevant information for inclusion in the BRD. The data abstracted from all records included the assay description, substance name, CASRN, and citation. Where available, the substance purity, \( K_i \) (µM), \( IC_{50} \) (µM), standard deviation of \( IC_{50} \) (µM), relative binding affinity (RBA), and highest dose tested for negative data (µM), were included in the BRD. If the RBA was not provided in the record, it was calculated from the available information in the report.

The database contained information on 14 assays with data for 635 unique chemicals. Of these chemicals, 235 (37%) were tested in 2 or more assays, and 51 (8%) were tested in 7 or more assays. The chemicals were assigned to chemical and product classes; 17 chemical classes and 7 product classes each had at least 10 entries. The most frequent chemical class was the polychlorinated biphenyls; the most frequent product class was pharmaceuticals.

The 14 assays included uterine cytosol from the mouse, rabbit, and rat; MCF-7 cell cytosol; intact MCF-7 cells; purified human (h) and rat (r) receptors, hER\( \alpha \), hER\( \beta \), or purified hER\( \alpha \) using a fluorescent polarization assay (hER\( \alpha \)+FP), rER\( \alpha \), and rER\( \beta \); and glutathione (GST) constructs containing the “def” (binding domain) domains of the receptor from anole, chicken, human, mouse, and rainbow trout. All assays used radioactivity to measure binding except the fluorescent polarization (hER\( \alpha \)+FP) assay, which used a fluorescently labeled estrogen.
Comparative performance analyses were performed following log transformation of positive RBA values of substances tested in two or more assays. The data was analyzed quantitatively using two- and three-way ANOVA and qualitatively for relative sensitivity by comparing the different RBA values of each substance in each assay to that of the substance in the rat uterine cytosol (RUC) assay. It was concluded that the numbers of substances tested in multiple assays was too limited for an adequate comparison to be conducted. Comparative inter-laboratory reproducibility analyses concluded that there was little variation in RBA values of the same substance tested in different labs and in different assays. However, this conclusion was based on data obtained with potent substances only.

The three assays with the most promise use purified human ER’s. Either the human ERα (hERα) or human ERβ (hERβ) proteins with radiolabelled 17β-estradiol or the ERα protein with a fluorescently-labeled estrogen (hERα+FP) are the most appropriate assays. The RUC assay could be used for comparison purposes. These assays were recommended in the BRD because of their greater sensitivity, direct relevance to humans, and their elimination of the use of animals, and in the case of the fluorescent polarization assay, the elimination of radioisotopes. A revised U.S. EPA RUC protocol incorporating minimum procedural standards was also proposed in the BRD. For future validation studies, 35 substances were suggested for testing.

1. **Recommendations and Prioritization of Assays for Validation Studies**

The Panel agreed that assays using recombinant human or rat estrogen receptor alpha or beta should have the highest priority for validation and standardization. Recombinant receptors from other species would be more relevant for screening for possible effects in wildlife. A standardized preparation of the receptor is essential for quality control and to enable comparison across laboratories.

In general, the ERα and ERβ proteins produce similar results, and the differences between them are primarily quantitative. There are a few examples of substances that bind to only one of the ERs. The Panel recommended that once a basic assay using a recombinant ER, either ERα or ERβ, is developed, other types of ERs could be substituted in the protocol. However, there is a preference for the use of recombinant hER.

Despite the suggestion in the BRD that the rat uterine cytosol (RUC) assay be used as a “benchmark” assay, the Panel identified a number of disadvantages. These include its bias towards ERα, animal welfare considerations, the difficulties of standardizing a cytosolic preparation from an animal due to the effect of age, weight, strain, etc., of the animals and the use of many animals even though this is an *in vitro* assay. Despite these drawbacks, there is much information using RUC for ER testing, and therefore the Panel recommended that this test be considered for comparative purposes only. In addition the Panel recommended that the minimum performance criteria that have been developed for this assay (see later) be applied when validating the other assay types using recombinant ERα or ERβ proteins.

The Panel recommended that receptors for species other than human and rat should be
considered for ecotoxicological concerns. There have been no species identified for use in general ecotoxicology screening, but the identification of such an environmentally relevant ER is important and should be considered in future plans. There are three ERs in fish, but it is not known how results with human and rat ERs reflect the binding of substances to any of these fish proteins. Amphibian liver ER has been proposed for the testing of substances that could alter endocrine disruption in amphibians and reptiles. Some European laboratories have made reference preparations of these non-mammalian ERs.

The Panel acknowledged that while an assay using fluorescent polarization (FP) would be advantageous, this assay currently is not in wide use, and there are limited data available for comparison with other methods. The FP assay also requires specialized equipment and a fluorescently tagged estrogen. A fluorescent estrogen would obviate the use of radioactivity, the use of which is being phased out in many European countries. Although the FP method has many attributes, the Panel recommended that methods that use radioactivity should be used for the present.

The Panel agreed that incorporation of metabolic activation capabilities into the test system should be considered. However, it would be difficult to obtain the ideal in vitro system, since most in vitro metabolic activation systems only contain enzymes and co-factors for phase I metabolism, which generates molecules that have binding activity. As there is no significant phase II (detoxifying) activity in many of these preparations, inclusion of metabolic activation would be expected to generate false positives. Another difficulty would be how to incorporate such a system into the assays. Thus, although the inclusion of a metabolic activation system would be desirable, the Panel did not recommend it until extensive development of this aspect of the assay was undertaken in the future.

While there are no known patent issues pertaining to hERs, there are some commercial assays that use these proteins. The question of patent issues should be investigated because they could affect any test system that would be selected.

2. Minimum Procedural Standards for In Vitro ER Binding Assays

The Panel was in agreement regarding most of the procedural standards in the BRD but also proposed revisions to the following standards:

**Dissociation Constant of the Reference Estrogen:**
- The dissociation constant must be determined with each set of assays. Hexa-tritium labeled 17β-estradiol- (i.e., 2,3,6,7,16,17-3H) 17β-estradiol should be used as the ligand for all assays because it is the most potent naturally occurring estrogen in the human body, and because it is available commercially with a high specific activity. Such a potent preparation will increase considerably the sensitivity of both the ligand titration array and the ligand competition assays.

**Preparation of Test Substances:**
- Test substances should be prepared in water, 95-100% ethanol, or dimethyl sulfoxide (DMSO), depending upon their solubility. Preference should be given to the solvent that
allows testing of the highest concentration of the test substance, without exceeding the limit dose.

**Concentration Range of Tests Substances**

- It was recommended that the highest dose tested should depend on the solubility constant \((K_{m})\) of the substance. This concentration may or may not be 1mM as originally proposed in the BRD. The substance at the highest dose will then be diluted by seven orders of magnitude in log decrements to obtain the relevant dilutions of the test substance for the assay. This will permit the generation of a dose response curve.

**Solvent and Positive Controls:**

- A set of solvent (vehicle)-only controls (with solvent concentrations identical to those used with reactions containing test substances) must be included in each set of assays.
- The solvent (vehicle) volumes must remain constant throughout the concentration range tested.
- A naturally occurring estrogen, such as estriol or coumestrol, or an estrogen mimic, tamoxifen is recommended as a positive control.
- The positive control should be tested at 3 dose levels whenever each assay is run.
- Because it is anticipated that many of the substances that will be tested in the future will be weak, the inclusion of a weakly positive control substance should be considered if only one positive control substance will be used. The routine use of a weakly positive control would establish the lower level of sensitivity of the assay, and confidence in low-level responses. However, no recommendation of a specific substance was made.

**Within-Test Replicates:**

- Triplicate measurements should be performed at each dose level.

**Data Analysis:**

- More details are needed on statistical models for non-linear regression to assess \(K_d\), \(K_i\), and \(IC_{50}\) values.
- The statisticians noted that it is also important to calculate standard errors or other confidence levels associated with the \(K_d\), \(K_i\), and \(IC_{50}\) values and that these calculations may not be trivial.
- The use of alternative approaches such as the ligand titration array, which provides simultaneous evaluation of a laboratory’s performance and determination of the estrogen binding properties (e.g., \(K_d\), \(K_i\), and \(IC_{50}\) values) of both reference and test substances, was recommended.

**Assay Acceptance Criteria:**

- A detailed assay protocol must be provided for performing each type of assay (i.e., ligand titration and competition), with criteria for evaluation and acceptance of results, with demonstrated assay validation and lab transferability.
- Achieving a specific binding capacity and \(K_d\) value for the reference receptor protein is a critical measure of the robustness of the procedure. These data are essential to the establishment of a Quality Assurance Program (assay proficiency).
A reference ER preparation, with established binding parameters must be employed for the determination of the $K_d$ value and specific binding capacity by the laboratories chosen for the validation of the ER binding assay.

**Evaluation and Interpretation of Results:**
- The method of calculation of the statistical parameters and assumptions must be justified. The classification of a test substance as “positive for binding” will require the use of statistical models. Historical data can also be used to assess the biological significance of results for a current test that has shown to be statistically significant.
- The Panel did not come to a clear consensus on the definitions of a weak response or a negative response. However they were agreeable on an equivocal response.

**Test Report:**
- Solubility information should be included in the test report
- A description of the justification for the chemical concentrations used must be included in the report.
- A clear identification of the test chemical by name and Chemical Abstracts Service Registry Number (CASRN) is required. The chemical structure may also be desirable in some cases, especially where the substance is chiral or if the CASRN points to a substance that is not pure. A proposal was originally made to use the IUPAC name for the chemical but it was noted that it is often difficult to determine this nomenclature and the common name would be sufficient.
- The Panel recommended establishing a new range of reference $K_d$, $K_i$, and IC$_{50}$ values with a standardized ER preparation using a test set of substances.

The Panel recommended the following additional minimum procedural standards:
- The assay used for protein determination should be specified and the concentration of protein used in the reactions reported.
- 10 mM sodium molybdate as well as a cocktail of protease inhibitors should be employed to minimize degradation of the receptor protein during the assay.
- Dextran-coated charcoal is preferred over the hydroxyapatite procedure for separating the free from the bound radiolabeled 17$\beta$-estradiol.
- In performing the binding assays, a range of 50-100 fM of hER$_{\alpha}$, which corresponds to 5-10 pg/mg of extracted protein, was recommended. There is less experience with hER$_{\beta}$, so no protein range could be recommended.

There was extensive discussion regarding the need for, and use of, concurrent positive controls during the performance of the assays. With the exception of one member of the Panel, the Panel agreed that concurrent positive controls are essential. The purpose of the positive control is to measure the performance of the test and of the laboratory. The reference ligand, 17$\beta$-estradiol, should not be used as the positive control in the ER assays because it would then be compared against itself. Although there was agreement with the need for positive controls and the need for consistency among ER and AR assays, there was no consensus regarding the minimum numbers and types of controls to be used, specifically as they related to substances with low activity. The advantage of including control substances that would be expected to elicit low and mid-range
responses would be the ability of determining the limit of detection of the test in the laboratory on a specified day. This would aid in concluding whether a test result is called positive or negative. The ideal situation would be the inclusion of three or four positive controls spanning a range of different binding affinities to measure test and laboratory performance. This is especially important because of the increasing variability in the response as one moves towards the lower end of the dose-response curve. Recommended positive controls were estrone and estriol, which are one and two logs less potent \textit{in vitro} than 17\beta-estradiol. Reasons presented for limiting the assays to one positive control substance is cost and level of effort.

The Panel recommended that each BRD contain a separate paragraph or section describing pertinent statistical analysis, and especially the evaluation of low-activity chemicals. However, the biostatisticians on the Panel stated that currently insufficient data are available to address all the statistical or data evaluation issues that would enable them to recommend specific statistical analyses. Before specific statistical procedures and action levels can be identified, more details are needed about the methods and their performance criteria. It will be necessary to evaluate confidence limits, standard errors etc., to better understand the data. Different data and statistical analyses will be required depending on whether the test will be used simply as a yes/no indicator, than if the results will be used in a quantitative manner.

Classification of a substance as positive will require a formal statistical procedure if the test substance does not produce a clear-cut sigmoid curves. For these reasons, a large database of substances that are negative or elicit weak responses needs to be established. This database could then be used to build the appropriate statistical models for the various measurements and endpoints. Prevalidation studies, or studies before entering pre-validation, can be used to generate this needed data.

When undertaking a receptor binding assay it is important that the $K_d$ and $B_{\text{max}}$ be determined. There was an unresolved question whether $B_{\text{max}}$ should be determined every time a binding assay is performed, or just for every lot of receptor. This determination ensures that the reference preparation of receptor is performing properly and that values can be compared across laboratories. Titration assays are justified because chemicals may interact with, and damage, the receptor in a non-ligand-binding manner. The $K_i$ should also be calculated, and this can easily be done using commercial software packages. Such calculations show good agreement with the values obtained using a Scatchard plot. The statisticians noted that it is also important to calculate standard errors or other confidence levels associated with the $K_i$, and that these calculations may not be trivial. It was proposed that the $K_d$ and $B_{\text{max}}$ values for a number of model chemicals be established as part of pre-validation studies.

3. \textbf{Recommendations for In Vitro ER Binding Test Method Protocols for Validation Studies}

The Panel reiterated its recommendation that an assay using a recombinant ER protein, preferably the human ER$\alpha$, should be developed. The assay protocol could be modeled on the BRD recommended RUC assay protocol, which is similar to the U.S. EPA protocol currently being used to measure ER binding of 21 substances by three laboratories. The cytosol-based assay can be refined to accommodate a purified protein instead of a cytosolic preparation. The Panel proposed
that a concurrent positive control be included in the protocol as:

- It is a hallmark of *in vitro* tests used in the regulatory arena worldwide.
- It is a stated “requirement” in protocols submitted to ICCVAM.
- It measures the assay’s performance and stability over time.
- It provides the basis for assessing the acceptability of the assay trial and thus the use of data from “unknowns” tested concurrently.
- It provides a basis for comparison of assay performance across laboratories.

4. **Recommended List of Substances to be Used for Validation of In Vitro ER Binding Assays**

The Panel expressed concerns regarding the composition of the list of substances for validation as to whether it included the kinds of substances that the U.S. EPA is interested in screening. They stated that an adequate representation of substances across chemical classes and across the range of potencies must be considered. Also, there should be an appropriate ratio of estrogens to non-estrogens in the list. The Panel made specific recommendations as follows:

- The number of negative substances must be increased from the current 9% to at least 25% in order to determine the specificity of an assay. Ideally, at least half the test substances should be non-estrogens, with about 60 compounds included in a validation set. A wider range of negative substances that belong to a wider range of chemical classes is needed so that appropriate criteria for negative results can be developed. This is especially important since many of the positive chemicals that will be encountered in the testing of industrial or environmental chemicals are likely to be weak, and the test needs to be sufficiently sensitive to detect these substances. Presently, there is insufficient information available to evaluate the utility of the binding assays at low potency ranges.
- An underrepresented class, the phthalates, was recommended by a number of Panel members as a group of substances that should be added to the list of negative substances, although no specific phthalates were identified.
- It was recommended that the EPA should maintain a repository of the chemicals to be used in the validation studies. A suggestion was made that there be two lists of substances, one set of substances would be used to test the protocol (which includes the pre-validation studies), and a second, more extensive set of substances for use in the validation studies.
- There was limited discussion regarding quality assurance issues. It was recommended by one Panel member that entry and exit assays be incorporated into the testing. This refers to the analysis of test chemical stock solutions before and after the assay is performed to assure the identity and purity of the chemical, and its stability in solution. This recommendation was not generally acceptable to the Panel. It was noted that where many diverse chemicals are being screened, the analytical chemistry could be more complex and more expensive than the biological tests.

**Public Comments Session (Morning, May 21)**

Dr. Richard Becker (American Chemistry Council) commented that patent and proprietary issues, and restrictions on the use of certain methods, were often stumbling blocks to international
acceptance of methods. The Panel was requested to give consideration to these concerns and to the availability of methods and materials.

B. **Estrogen Receptor (ER) Transcriptional Activation (TA) Assays**

*Primary reviewers:* J. Stegeman, Group Chair (Woods Hole Oceanographic Institute); G. Charles (Dow Chemical Co.); E. Mihaich (Rhodia, Inc.); T. Weise (Tulane and Xavier Universities; not present at meeting); J. Yager (Johns Hopkins School of Public Health); T. Zacharewski (Michigan State University); S. Peddada (National Institute of Environmental Health Sciences).

**Summary of the ER Transcriptional Activation Background Review Document**

Dr. Shane briefly summarized the assays described in the BRD. The same on-line databases, as searched for the ER binding reports, were searched for relevant publications for the ER Transcriptional Activation BRD. Key words included *screen*, *tests*, *batteries*, *bind*, *ligand*, *agoni*, *antagoni*, *transcription*, *estrogen*, and *receptor*. The search yielded 258 records; data were available from 86 for inclusion in the BRD. For the agonism assays, the qualitative positive or negative response, a measure of relative activity, $EC_{50}$, ($\mu$M), and cell growth information were extracted. For the antagonism assays, the qualitative response, relative activity, and the $IC_{50}$ (M) were extracted.

The BRD database contains 95 assays, and data on 703 unique chemicals. Of these chemicals, 634 were tested for agonism; 228 (36%) were tested in $\geq 2$ assays and 51 (8%) were tested in $\geq 5$ assays. Of the 255 chemicals tested for antagonism, 94 (37%) were tested in $\geq 2$ assays and 8 (3%) were tested in $\geq 5$ assays. The database of chemicals was comprised of 15 chemical classes and 11 product classes, for which there were 10 or more entries. The most frequently tested substances in the chemical and product classes were polychlorinated biphenyls and pesticides (including metabolites), respectively.

The 95 assays included 63 permutations of 9 human cell lines: BG-1, HEC-1, HEK293, HeLa, HepG2, MCF-7, MDA-MB-231, T47D, and Ishikawa cell lines and three other mammalian cell lines: CHO-K1, COS-1, and ELT-3. The ER$\alpha$ and ER$\beta$ proteins were purified from human, mouse, and rat ER (unspecified). The activity of luciferase or chloramphenicol acetyltransferase reporter genes were used as a measure of TA. There were 10 mammalian cell proliferation assays that used Ishikawa, MCF-7, T47D, and ZR-75 cell lines. In addition, there were 22 yeast assays involving 13 *S. cerevisiae* strains with the hER, hER$\alpha$, hER$\beta$, mER, and rtER receptors, and a $\beta$-galactosidase reporter gene.

Comparative performance and reliability analyses of $EC_{50}$ or $IC_{50}$ values were not conducted because the numbers of substances tested in multiple assays, or multiple times using the same assay in the same or different labs, were too limited for an adequate comparison.

Based on these considerations of the available data, recommendations for minimum procedural standards were prepared for the BRD. In addition 31 chemicals were recommended for use in
future validation studies in agonism assays, and 21 were recommended for use in future validation studies in antagonism assays.

1. Recommendations and Prioritization of ER TA Assays for Validation Studies

The Panel agreed that there was too little information to recommend one assay over another. No specific cell line could be preferentially recommended for ER TA because there was not enough data presented in the BRD from the different mammalian cell lines. One concern was that the activity of a chemical will probably be species-, tissue-, cell-, and promoter-specific, and therefore its response can not be generalized based on results from any single assay. Also, potential differences in co-activator populations, cross talk with other receptors, and other signal transduction pathways between different cell types, etc., could alter the response in a cell. As a result of this complexity, there are a number of aspects of the various cell lines that will have to be investigated further before any decision can be made on the most appropriate cell line for an assay.

Discussion then ensued on whether a stably transfected or transiently transfected ER cell line or a cell line with an endogenous ER should be recommended. A stably transfected cell line would seem preferable but no conclusion can be drawn until appropriate comparative data are collected on cell lines with each of these different types of receptors.

The difficulties with stably transfected cell lines are the instability of the constructs, frequent problems encountered in maintaining highly responsive lines, and the limited availability of these lines. Since transiently transfected cell lines have more flexibility, they may be more appropriate for screening. The Panel suggested that an important part of the validation process would be a study to determine if stably transfected lines perform better or are more sensitive than transiently transfected cell lines. It was recommended, therefore, that before any test validation is begun, a research and development effort is implemented to compare the responses of stably and transiently transfected cell lines to the same small group of chemicals. This would involve a comparison of the response of a mammalian line stably transfected with receptor and reporter constructs with one transiently transfected with the same ER and reporter plasmids. In addition, the response of a cell line with an endogenous receptor needs to be evaluated alongside these transfected cell lines. If stable cell lines are selected for validation, there should be a standard procedure for evaluating their performance and the stability of the constructs. Stability can be monitored by antibiotic selection.

The Panel agreed that in the development of an assay, a number of different constructs with different components transfected into different cell lines need to be evaluated, optimized, and at a minimum, clearly defined for each assay. These include the components of the reporter construct, the number of EREs, the presence of other enhancers in the construct, the types of promoters, as well as the co-activators and co-repressors in the cell line. As a beginning to pre-validation, it was recommended that a series of transient transfection assays for individual receptor subtypes be developed and evaluated.
The Panel agreed that the ERα and ERβ are the most appropriate receptor types, but if patent issues arise with the use of human ER, the rat ERs would be an acceptable alternative. Supporting data needs to be obtained to determine whether the use of ERα alone, would be sufficient.

The Panel was of the opinion that although the vitellogenin response element (vitERE) responds to substances that bind to the progesterone or corticosterone receptors found in some cell lines, this estrogen response element should be used due to its sensitivity. For optimized sensitivity, multiple vitERE constructs were recommended. Chimeric ligand binding domain ER’s should also be considered for these preliminary studies due to their mechanistic specificity.

Although the metabolic activities of the various cell lines need to be considered, most cell lines used in these assays have not been characterized with regard to their metabolism of xenobiotics. To characterize the metabolism for a range of chemical structures is an enormous undertaking although it can be done with a few model chemicals. It is also possible that certain test substances can induce metabolism. Therefore, the metabolic characterization of untreated cells may not be relevant. A caution was presented regarding the exogenous metabolic activation systems, and those inherently present in the cell lines being used, that they may not mimic those found in the relevant in vivo target tissues.

2. **Minimum Procedural Standards for In Vitro ER TA Assays**

The Panel agreed with the recommended minimum procedural standards in the BRD, with the following additions:

**Concentration Range of Test Substances:**
- The Panel agreed that the limit concentrations could be 1mM as long as the solubility characteristics and cytotoxicity of the test substance is taken into consideration. There was a consensus, however, that in general, concentrations of the test substances above 10 µM should not be used because this concentration is excessive and often problematic due to solubility issues in aqueous media. A concentration range from 1 nM to 10 µM should be sufficient for a screening study. The Panel recommended that since certain chemicals (such as tamoxifen) can be estrogenic at low doses and anti-estrogenic at high doses, tests should be performed over a wide dose range, and single-dose experiments be avoided. Incorporation of a measure of cellular cytotoxicity into the assay could help define the upper limit for test material concentrations, similar to the Maximum Tolerated Dose (MTD) approach used in in vivo studies. This measure of potential cell cytotoxicity/cell proliferation should be a part of the data collected to ensure non-toxic doses are being used.
- Since solubility could affect absorption of the test substance by the cell, it might be necessary, to evaluate the uptake of the substance using isotope-labeled substances.

**Solvent and Positive Controls:**
- The Panel suggested that guidelines be provided with regard to the concentration of solvent in the stock solution. Whether ethanol or DMSO is used, compounds to be tested could be prepared in stock solutions so that the test substance concentration approaches the solubility limits. However, this approach could introduce variation from laboratory to laboratory and...
thus should be standardized. In addition, controls need to be in the same carrier solvent as the test substances. A pre-validation of the TA assay should be performed with the reference estrogen, to assess the level of solvent that does not adversely affect assay response.

• The Panel agreed that ICI 182,780 appears to completely block 17β-estradiol at 0.1 µM and thus, it should be used as the positive antagonist. However, availability of ICI 182,780 may be limited. Clear guidelines should be given for the positive antagonist and the expected extent of antagonism when testing the compound.

• Each test substance that is positive in the agonist assay could also be tested with ICI 182,780 to confirm a receptor-mediated activity.

Within-Test Replicates:
• The test must be run in triplicate at each concentration.

Data Analysis: (for more details see Expert Panel Report)
• The Panel recommended that preliminary studies be performed with multiple transactivation assays to statistically define assay performance expectations for 17β-estradiol dose response curves (i.e., maximum fold induction, EC₅₀ values, confidence limits).

• The biostatisticians on the Panel stated that currently, insufficient data are available to address all the statistical or data evaluation issues that would enable them to recommend specific statistical analyses. Before specific statistical procedures and action levels can be identified, more details are needed about the methods and their performance criteria. It will be necessary to evaluate confidence limits, standard errors etc., to better understand the data. Different data and statistical analyses will be required depending on whether the test will be used simply as a yes/no indicator, than if the results will be used in a quantitative manner.

Assay Acceptance Criteria:
• The transcriptional activation-inducing ability of 17β-estradiol must be demonstrated. A consistent minimum response would be an appropriate criterion for assay acceptability.

• Reference compounds for agonism and antagonism should give responses within appropriate confidence limits. These confidence limits should be determined in preliminary studies. Guidelines should be provided for a certain expected range in response for the reference standards in agonism and antagonism assays, and responses in these ranges should be required if the assay is to be accepted.

Evaluation and Interpretation of Results:
• The interpretation of positive results for a compound as an agonist or antagonist should incorporate some elements of dose-response in comparison to the reference standards. Simply classifying a substance as an ER agonist based on a significant response above the concurrent control without consideration of a dose-response is not sufficient.

Test Report:
• The complete DNA sequence of constructs and vectors used for receptor and reporter genes should be identified.

• All assay parameters regarding cells, plasmids, culture methods, transfection methods, and a method for measuring luciferase activity must also be reported.
- For a transfection assay, a constitutive reporter gene assay must be included to control for transfection efficiency between wells.
- The passage number of cells should be tracked.
- The % CO₂ in the incubator must be monitored.
- EC₅₀/IC₅₀ values, fold change, and confidence limits must be reported.
- Solubility information should be included in the test report.
- A description of the justification for the chemical concentrations used must be included in the report.

**Additional Minimal Procedural Standards**

- **Cell Toxicity:** The Panel discussed what level of toxicity would be acceptable for inclusion of the data if cell toxicity was observed. No agreement was reached on the definition of toxicity, nor how it should be measured. Two suggested endpoints were overt cell death or decreased expression of a specific marker product. The measurement used may be dependent on the test system. Although some Panel members proposed a 10% killing as a cut off value, no consensus was reached regarding this value or any other specific value. It was agreed, however, that some value(s) would have to be defined. Methods for quantifying cytotoxicity in the TA assays included measurement of the activity of the gene product of a co-transfected β-galactosidase or luciferase gene that fluoresces at a different wavelength than the luc reporter gene used in the same cell. CMV-driven luc plasmids were suggested as the carrier of the co-transfected gene, although these plasmids might be affected by some test substances and therefore respond to non-endocrine transcription signals.

- **Corrections to the BRD:** There were two observations in the BRD that require clarification. Firstly, there is an inconsistency in the statements on pages 12-1 and 12-11 [in the BRD] concerning stable vs. transiently transfected cells. Secondly, there was no discussion of individual assays for ERα and ERβ.

Discussion ensued as to whether a tiered strategy should be adopted for the TA assays. For example, if the compound is positive for agonist activity in the TA assay, is there any value in testing it for ER antagonist activity or AR-mediated activities? It was pointed out that a positive result in any of these assays will likely warrant further examination in tests other than transactivation assays. However, other Panel members disagreed with such a tiered strategy because the assays will be used as part of an integrated test battery and, thus, the elimination of one of the endpoints (agonism or antagonism) would be equivalent to losing part of the data. No consensus on a tiered approach was reached.

A discussion then followed as to whether data in the *in vitro* assays would trigger the testing of a substance in an *in vivo* assay. The Chair then asked Gary Timm (U.S. EPA) to clarify the roles of these tests.

According to Mr. Timm, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) report, and the U.S. EPA Endocrine Disruptor Testing Program (EDSP) proposals view all the Tier 1 tests as an integrated battery, and no single test result will trigger Tier 2 testing, or the designation of a chemical as a potential endocrine disrupter. The EDSTAC report...
had a preference for TA assays for mechanistic reasons, but binding assays were regarded as equally acceptable. The in vitro Tier 1 assays are not considered a sub-tier for the in vivo assays because the EPA proposes to evaluate the results from the entire Tier 1 battery in a weight-of-evidence approach. The composition of the specific Tier 1 battery to be used has not yet been determined. This determination will be based on the outcomes of the validation studies for each test method proposed for the battery. EDSTAC proposed that the Tier 1 in vitro and in vivo tests be run simultaneously, but recognized the role of in vitro tests in identifying chemicals for in vivo testing. Mr. Timm stated that the EPA does not contemplate running thousands of chemicals through the entire Tier 1 battery. Mr. Timm stated that the in vitro assays would not be used for priority setting.

Mr. Timm went on to say that the data that are presently being generated in Tier 1 testing by contractual arrangements will be publicly available; but when, and in what form the data will be released has not yet been determined. The EPA is sensitive to the potential problems associated with piece-meal release of the data and will probably release the data on a chemical-by-chemical basis, not by test. The EPA is also concerned with potential confidential business information issues that may attach to some of the data and is working to resolve this issue.

Comments were made that, in the future, gene expression profile patterns may be used to identify endocrine-active substances, and to distinguish estrogens from androgens, and agonists from antagonists. Gene panels can be developed for different tissues. Binding assays, as they are currently performed, may be considered relatively “old science.”

3. Recommendations for In Vitro ER TA Test Method Protocols for Validation Studies

The Panel agreed that the protocols are described adequately but the details of the protocols are contingent on the incorporation of the minimum procedural standards. The Panel was of the opinion that any laboratory with cell/yeast culture and basic molecular experience should be able to produce dependable results. Some interlaboratory variability may be expected due to laboratory specific techniques (e.g., cell counting). Acceptance standards should be specified for culture techniques such as cell counting, determination of % confluency, ability to seed plates evenly, etc. to limit interlab variability. Additional procedure details should be added if volatile chemicals are tested.

The following topics need to be added or expanded in the protocols:
• Standards for uniform counting and plating of cells in wells between experiments.
• Review of methods for making DCC stripped sera or a recommendation for commercial sources of this serum.
• Review of known sources of estrogen contamination in the lab.
• Discussion regarding the culturing of cells in estrogen rich media and withdrawal to an estrogen-free medium.
• Discussion of washing techniques and number of days for withdrawal.
• Discussion of procedures to demonstrate that the lab and each particular experiment is performed under estrogen-free conditions (e.g., ICI 182,780 vs. blank reporter activity).
Other Available Standardized Protocols

Dr. Thomas Weise in a written contribution (Dr. Weise was not able to attend in person) suggested that the MVLN Assay, that uses MCF-7 cells stably transfected with the vitellogenin-luciferase reporter plasmid, is among those that should be considered further. [Copies of the procedure were made available to the Panel members]. The Panel agreed that it should be one of the assays validated with the other proposed assays.

The Panel emphasized that standardization and validation of assays across laboratories is critical and must occur before these assays are used for regulatory purposes. A formal validation process is needed in order to establish a “gold standard” study for use and not just to have personal variants of similar assays.

4.  **Recommended List of Substances to be Used for Validation of In Vitro ER TA Assays**

The Panel was of the opinion that the distribution of the recommended substances seemed appropriate, but more thought should go into the final compilation of the list that is used for validation. The following criteria should be considered:

- Inclusion of more chemicals expected to be negative.
- More overlap of chemicals used for validation of the ER binding assays and the ER TA agonist and antagonist assays.
- Close collaboration and cooperation is encouraged regarding chemical selection with the *in vivo* test validation studies being reviewed by the EPA’s Endocrine Disrupter Methods Validation Subcommittee.
- Possible inclusion of phthalates, polychlorinated biphenyls, and additional polycyclic aromatic hydrocarbons. These classes were originally omitted from the list of substances due to their limited availability from a commercial source and difficulties with their disposal.
- All substances for validation should come from one EPA repository.
- Chiral compounds (i.e., compounds that cannot be superimposed upon their mirror images and are thus asymmetrical) need to be included in the validation list as different components of a racemic mixture may elicit different responses. There is a possibility that one enantiomer could be an agonist while the other is an antagonist with the racemate being neutral. These substances should be included in the validation list, but be omitted from the pre-validation list.

**Public Comments Session (Afternoon, May 19)**

Dr. George Clark (Xenobiotic Detection Systems, Inc.) presented information describing the construction and performance of his company’s chemical-activated luciferase expression (CALUX) screening system for ER transcriptional activation. This assay uses a stably transfected cell line, BG1, which contains a luciferase reporter gene. Information on this assay was submitted to NICEATM for inclusion in the ER TA BRD. Based on the information presented, the test system is amenable to high-throughput screening and is highly reproducible. The cells express predominantly ERα (95%) with low amounts of ERβ (5%). This test system is available
commercially and the company can supply the cells, or multiwell plates that are coated with the cells, for use by the customer. Alternatively, the company also provides testing services.

A Panel member asked what approach will be used by regulatory agencies in identifying companies such as Xenobiotic Detection Systems, Inc., that might be developing or have developed an *in vitro* method useful for screening. Dr. Stokes responded from an NIEHS and ICCVAM perspective and stated that Dr. Clark’s report would be made available to the public and forwarded to Federal Agencies so that it can be considered by individuals or organizations that wish to support validation. Once methods have gone through validation, the data can then be submitted to EPA and/or ICCVAM for further evaluation.

**C. Androgen Receptor (AR) Binding Assays**

*Primary reviewers: T. Brown, Group Chair (Johns Hopkins University); T. Gasiewicz (University of Rochester Medical Center); T. Inoue (National Institute of Health Sciences, Japan); B. Robaire (McGill University, Canada); A.M. Vinggaard (Danish Veterinary and Food Administration, Denmark); W. Piegorsch (University of S. Carolina).*

**Summary of the AR Binding BRD**

Dr. Shane provided an overview of the AR binding BRD. The same on-line databases were searched for relevant publications for inclusion in the AR binding BRD, but using the following key words, *screen, tests, batteries, bind, ligand, androgen, and receptor.* The search yielded 108 records; data from 23 were included in the BRD. The same data as abstracted in the ER BRD were collected from the records.

The BRD database contains 11 assays, and data on 108 unique chemicals from AR binding. Of these chemicals, 33 (31 %) were tested in ≥2 assays, and 11 (10%) were tested in ≥6 assays. The chemicals were assigned to chemical and product classes with nonphenolic steroids and pharmaceuticals being the most frequent chemical and product classes, respectively.

The 11 assays included: calf uterine cytosol, rat prostate cytosol, and rat epididymal cytosol and the nuclear fraction from rat epididymal cells, MCF-7 cell cytosol; COS-1 cells transfected with the hAR (COS-1+hAR); LNCaP cells; intact human genital fibroblast (HGF) cells; and purified recombinant human AR (rhAR).

Comparative performance and reliability analyses of IC$_{50}$ or RBA values were not conducted because the numbers of substances tested in multiple assays, or multiple times using the same assay in the same or different laboratories, was too limited for an adequate comparison. Thirty-one chemicals were suggested for validation; three (10%) of which were negative.

1. **Recommendations and Prioritization of Assays for Validation Studies**

The Panel discussed the advantages and disadvantages of the three different assay systems used to measure AR binding namely, the rat prostate cytosol assay, the cell-based assay using COS-1
cells transfected with a human AR, and the assay using purified human AR (hAR). The Panel recommended that an assay using purified recombinant hAR or rat AR (rAR) (or other species) be developed. The Panel did not recommend that a metabolic activation system be incorporated into the assay system at this time.

The Panel was concerned that a potential difficulty in using purified AR is that the human AR cDNA sequence is protected by patent, and commercial use of the hAR in functional assays is restricted by a license. It is not known whether the rat AR cDNA sequence is also protected by patent restrictions. As a result, the full-length recombinant AR is not presently available for use in an AR binding assay. A recombinant human AR protein is available, but it only contains the ligand binding domain of the protein. The reliability of this protein in the binding assay has not been established.

The Panel noted that an assay with whole cells that contains an endogenous AR is unlikely to be restricted by patents, and that some of these cell lines express significant amounts of AR. However, they noted that cells containing endogenous receptors do not always express levels of AR that are as high as transduced or transfected cells, and they may have other inherent disadvantages such as stability. The relative simplicity of the transfected cell assay (e.g. COS + hAR/rAR) is amenable to high throughput screening and requires simple methods, minimal volumes of reagents, and few variations in buffers and solutions. One possible source of recombinant AR might be derived from nonhuman primates.

Dr. Hattan wondered what the implications would be if a substance was positive for binding or TA using the human receptor in vitro, but was negative in the in vivo rodent tests. Could such a response be based solely on the different sources of the receptor? The Panel thought that the similarities in receptors between humans and rodents are such that it would be the rare exception where differences between the in vitro and in vivo responses were based solely on the composition and responsiveness of the receptor. Because of their homologies, the receptors are expected to have similar binding characteristics, although the binding kinetics could be affected by the contribution of other parts of the receptor molecule besides the binding domain. In the situation that was described, the activity of the substance in vitro can be examined further using the rodent receptor. This may be an important consideration because of possible post-translational changes to the receptor that does not occur in vitro. It was noted that it is not unusual to get positive in vitro and negative in vivo test results because of the differences in their sensitivities. The problem is not so much one of biology as it is of public perception of the relevance of the in vitro test.

Following this discussion, the Panel recommended Government agencies should, in light of the status of the patents and licenses, provide guidance for the development and use of AR assays in the public and private domains.

The Panel unanimously agreed that rat prostate cytosol (RPC) was not the best source of the AR for these assays because:

- The RPC contains other steroid receptors that may interfere with the assay for AR binding.
- Some metabolism of the test substance may occur even in cytosol preparations.
• RPC cannot substitute for hAR, or AR in those wildlife species where significant exposure to androgenic chemicals may occur.
• The AR is extremely unstable in cytosolic preparations and in fact, the protein is usually degraded so that only the AR binding domain remains intact.
• Although the RPC has been the most utilized assay to measure AR binding, this is the more difficult of the assays to perform in a standardized format.

The Panel recommended that the simplest and most consistent assay would be one in which the AR protein would be fixed in multiwell plates and tracer and test ligands added in appropriate amounts to develop data for a Scatchard (or equivalent) analysis. They also recommended that there should be a move away from radioactive tracer ligands toward more environmentally friendly and safer fluorescent ligands.

The Panel recommended that irrespective of which assay was developed and validated, that it should be acceptable at the international level (e.g., It should not have to comply with patent regulations and regulations regarding the use of radionuclides).

2. Minimum Procedural Standards for In Vitro AR Binding Assays

The Panel agreed with the AR binding BRD regarding minimum procedural standards, with the following additions and revisions:

Dissociation Constant:
• The $B_{\text{max}}$ and $K_d$ of the reference androgen should be determined in each assay and all laboratories should be able to generate comparable values within accepted limits. These values are a critical measure of the robustness of the procedure and the abilities of the laboratory.
• The minimum number of concentrations used to obtain the $K_d$ should be stated.
• Straightforward procedures, such as ligand titration arrays for determining the $K_d$ value of the radiolabeled reference ligand and the unlabeled test substance should be considered.

Reference Androgen:
• 5α-Dihydrotestosterone (DHT) is recommended as the reference androgen for an assay based on a purified receptor while methyltrienolone (R1881) or mibolerone is recommended for an assay based on cytosol or cells.
• Triamcinolone acetonide or a synthetic progesterone receptor (PR) agonist to block binding to the PR should be used in assays where PR is present and R1881 is used in the assay. Alternatively mibolerone could be used.

Preparation of Test Substances
• Preparation of stock solutions should be performed under rigorous quality control. The stability of stock solutions must be established.
Concentration Range of Test Substances:
- At least 5 concentrations of the test substance should be examined to increase the likelihood of obtaining a satisfactory competition curve for estimation of the IC$_{50}$.
- The limit dose should be 1 mM, taking into consideration the solubility characteristics of the compound.

Solvent and Positive Controls:
- As discussed for the ER binding assays, preference should be given to the solvent that allows testing of the maximal concentration of the test substance without exceeding the limit dose.
- A set of solvent-only controls (with solvent concentrations identical to those used with reactions containing test substances) must be included in each set of assays.
- The solvent volumes must remain constant throughout the concentration range tested.
- The positive control compound should have a binding affinity within two orders of magnitude of the limit of sensitivity of the assay. A second positive control within 1-10% of the RBA of the reference androgen should be included.
- One minimum procedural standard that was discussed at some length was the use of a positive control that is close to the level of detection of the assay. There was no clear consensus as to whether this is necessary. It would depend on whether one wants to categorize a substance as binding to the AR or whether one wants to determine an IC$_{50}$ value. The routine use of a weakly positive control would establish the lower level of sensitivity of the assay, and confidence in low-level responses.

Within-Test Replicates:
- Triplicate measurements should be performed at each dose level.

Data Analysis:
- More details are needed on statistical models for non-linear regression to assess $K_d$, $K_r$, and IC$_{50}$ values.
- Mode of calculation and assumptions for the statistical methods must be justified.
- The designation of “equivocal” for compounds that do not bring about a 50% reduction in specific androgen binding is acceptable.
- The classification of a test substance as “positive for binding” requires the use of statistical methods.
- The biostatisticians on the Panel stated that currently insufficient data are available to address all the statistical or data evaluation issues that would enable them to recommend specific statistical analyses. Before specific statistical procedures and action levels can be identified, more details are needed about the methods and their performance criteria. It will be necessary to evaluate confidence limits, standard errors etc., to better understand the data. Different data and statistical analyses will be required depending on whether the test will be used simply as a yes/no indicator, than if the results will be used in a quantitative manner.
- It may be useful to determine whether binding is through a non-competitive, competitive, or uncompetitive mechanism for substances that demonstrate an unusual binding curve. This determination is most easily accomplished by adding different concentrations of the test substance to different concentrations of radiolabeled hormone to generate a number of
curves as proposed in the ligand binding array. The slopes of the lines are then plotted and the intercept of the line with the X axis is the $K_i$.

**Assay Acceptance**
- The Panel recommends that the assays be performed in compliance with Good Laboratory Practice (GLPs).

**Additional Minimum Procedural Standards**
- The assay used for protein determination should be specified and the concentration of protein used in the reactions reported.
- The chemical and radiochemical purity and the supplier of the radiolabeled androgen should be stated.
- A new range of reference IC$_{50}$, $K_d$, and $K_i$ values with a standardized AR preparation using a set of test compounds should be established.

**3. Recommendations for In Vitro AR Binding Test Method Protocols for Validation Studies**

The Panel concluded that there is no existing, standardized, acceptable protocol for an AR binding assay. However, the RPC protocol, which was well written, could be used as a model for the development of a protocol using a purified AR (either the entire protein or the binding domain if the entire protein cannot be used). The protocol described for the COS cell binding assay did not have the necessary details that are required for future testing of AR binding substances. In addition to the minimum procedural standards recommended by the Panel, the following considerations should be taken into account before a final protocol is developed.
- If a transfected cell line is adopted, a standard transfection protocol based on commercially available transfection agents and a standardized cell line would be necessary.
- The production of a stable cell line expressing the AR would avoid the problems inherent in transient transfection assays.

**Additional Protocol Elements**

The Panel agreed that the following details should be included in the RPC protocol:
- The maximal time of storage at -80°C/-20°C of cytosol, cells, or other material used as the source of AR should be indicated.
- The type of tubes/culture dish for homogenization and storage of cytosol or of cells should be indicated.
- Information on preparation and purity of the AR vector should be provided.
- Protocol elements for the COS cell binding assay (e.g., preparation and stability of the vector, detailed timing on cell transfections, confluency of cells, transfection efficiencies, rationale for the choice of timing, incubation conditions, etc.) should be provided.
- If a cytosolic protein preparation is to be used, a cocktail of protease inhibitors, must be included to increase stability of the AR.
Other Available Standardized Protocols

The Panel suggested that if PanVera® is developing an AR binding assay using the AR ligand binding domain (LBD), this assay should be considered for validation. However, since only the LBD is being used, it is not apparent what the sensitivity and reliability of this assay will be. Use of only the ligand binding domain recombinant protein is much less desirable than use of full-length AR in either an \textit{in vitro} or \textit{in vivo} assay because there is scientific evidence that the LBD interacts with other domains of the AR protein during the binding process.

There is no indication that a full-length recombinant AR will be available in the near future. Competitive binding assays for ER, PR, and GR that are available from PanVera® are based upon full-length recombinant proteins and do not use radioactivity.

The Panel is not aware of any other assay under development that would meet the desired criteria described in C.1 in the BRDs.

4. \textbf{Recommended List of Substances to be Used for Validation of In Vitro AR Binding Assays}

The Panel was in agreement with the list of chemicals proposed in the BRD with the following additions and considerations:

- The same range and types of substances should be used for validation of both AR binding and AR TA assays.
- Anti-androgenic chemicals flutamide (or hydroxyflutamide, if used \textit{in vitro}) and bicalutamide that bind to AR but do not initiate transcriptional activity, should be included in the list.
- Finasteride (the commercially available 5'-reductase inhibitor which does not bind to AR) should be added as a negative control.
- One or more of the estrogens (ethinyl estradiol, estrone or DES) can be omitted from the list, as 17β-estradiol is included.
- A number of negative substances should be added to the list. For example, phthalates, which can be activated \textit{in vivo}, but do not bind to the AR should be added.
- A few substances that have been tested \textit{in vivo} for which the \textit{in vitro} database is extremely small or non-existent should be considered for testing.
- Additional non-binding chemicals need to be included in the recommended list of chemicals for validation studies. Androgen antagonists that do not have high binding activities should be included.

Public Comments Session (Morning, May 20)

Dr. Yoji Ikawa (Otsuka Pharmaceutical Co., Japan) presented information describing the construction of cell lines and performance of the company’s EcoScreen Transfection Assay (transiently transfected) and ER/AR-EcoScreen (stably transfected) assay systems for AR transcriptional activation. This information had previously been submitted to NICEATM for inclusion in the relevant BRD. The EcoScreen Transfection Assay is designed for high throughput screening, but ER/AR-EcoScreen cannot be used for high throughput screening. Testing was
successfully performed using a liver cytosolic fraction from homogenized cells that had been centrifuged at 9,000x gravity (S9 preparations) for metabolic activation. This test system is available commercially from Otsuka Pharmaceutical Co.

D. Androgen Receptor (AR) Transcriptional Activation (TA) Assays

Primary reviewers: E. Wilson, Group Chair (University of North Carolina); K. Gaido (CIIT Centers for Health Research); W. Kelce (Pharmacia Corp.); S. Peddada (National Institute of Environmental Health Sciences).

Summary of the AR TA BRD

Dr. Barbara Shane summarized the information that had been included in the AR TA BRD. The same on-line databases were searched to retrieve publications with data on AR TA. The following key words, screen, tests, batteries, bind, ligand, agonist, antagonist, transcription, androgen, and receptor were included in the search, which yielded 108 records; data from 27 records were available for inclusion in the BRD. Similar data as described for the ER TA BRD were abstracted from all the records.

The BRD database contains data on 146 unique chemicals from 17 assays. Of these, 109 were tested for agonism; 49 (45%) were tested in ≥2 assays and 17 (16%) were tested in ≥4 assays. Of the 87 chemicals tested for antagonism, 22 (26%) were tested in ≥2 assays and 6 (7%) were tested in ≥4 assays. The most frequent chemical and product classes were nonphenolic steroids (35 substances) and pharmaceuticals (55 substances), respectively.

The 17 assays were comprised of 15 mammalian cell-based assays using six human cell lines: (HeLa, HepG2, MDA-MB-453, MDA-MB-453-kb2, PC-3, and PALM), two mammalian cell lines (CHO and CV-1), and one carp cell line (EPC). The ARs were derived from human, mouse, and rainbow trout. The luciferase and chloramphenicol acetyltransferase reporter genes were used. There was one mammalian cell proliferation assay that used the LNCaP-FGC cell line. In addition, there was one yeast assay using S. cerevisiae YPH500 with the hAR receptor and a β-galactosidase reporter gene.

Comparative performance and reliability analyses of EC50 or IC50 values were not conducted because the numbers of substances tested in multiple assays, or multiple times using the same assay in the same or different laboratories, were too limited for an adequate comparison.

Based on these considerations of the available data, recommendations for minimum procedural standards were prepared for the BRD. In addition, 28 chemicals were recommended for use in future validation studies of agonism assays, and 25 were recommended for use in future validation studies of antagonism assays.
AR TA Group Presentation and Discussion

The discussion of the AR TA BRD was led by Dr. George Daston because Dr. Elizabeth Wilson recused herself from participation in decisions regarding the AR methods due to her potential conflict of interest. Dr. William Kelce presented the draft conclusions and recommendations for the AR TA assays.

1. Recommendations and Prioritization of Assays for Validation Studies

The Panel decided that they could not recommend a specific assay at this time because the available assays are not yet ready for standardization nor validation. There is a need for further methods development and standardization before a specific assay can be recommended for validation.

The Panel agreed with the BRD recommendation that a stable cell line be used for testing. The Panel proposed that the MDA-MB-453-kb2 cell line, which harbors an endogenous AR and which has been transduced with an adenovirus carrying the reporter gene, be developed further. This cell line has a high sensitivity with a 24-fold induction of luciferase in the presence of DHT. However, this cell line is deficient in that:

- It lacks specificity for the AR (activated by glucocorticoid (GR) and progesterone receptors (PR).
- A 248-fold induction with dexamethasone has been reported due to the presence of GR. The presence of AR can be overcome by adding hydroxyflutamide that blocks its activity. This would entail the use of an additional set of reagents for each substance being tested to distinguish AR activity from GR activity.
- The AR in this MDA-MB-453-kb2 cell line has not been sequenced to confirm that it is intact and has no mutation.
- A central source of adenovirus, for transduction purposes, will be required by the testing laboratories because propagation of adenovirus is technically challenging.

A discussion ensued on the difficulties of recommending any of the cell lines discussed in the BRD because of their lack of sensitivity (less than 10-fold induction), lack of specificity due to the activation of the endogenous GR by the MMTV ERE (HepG2, HeLa, CHO cells), and the activation of the AR by 17β-estradiol. The LNCaP cells contain a mutant AR that does not discriminate agonists from antagonists and yeasts have different metabolic and cell wall transport proteins potentially limiting “exposure.” The stably transfected cell lines are unstable and require continuous selection with an antibiotic, which is costly, and by the 40th passage their sensitivity has dropped to a 5 to 6-fold induction.

Since all of the cell lines discussed in the BRD have drawbacks, the Panel recommended that ideally the chosen cell line should have the following characteristics:

- Little metabolic activity.
- An endogenous wild-type hAR (little or no PR protein; cells apparently require some low level of GR for survival).
• Adenovirus infected or stable expression of a specific ARE-Luc reporter (Use of the promoter from the C3 prostate binding protein, sex-limited protein, and probasin genes have an advantage over the MMTV promoter because of their specificity, but they are not ideal because they are less sensitive than the MMTV). At least a 20-fold induction with 0.1-1 nM R1881/DHT is needed for maximum sensitivity. Minimal agonist activity with estrogens and glucocorticoids.
• Large scale screening capability (multi-well format).
• No patent restrictions.
• Use of a constitutively active luciferase reporter (CMV-Luc, pSG5-Luc) to monitor cytotoxicity.
• Control to measure any direct inhibition of luciferase activity.
• A 20% inter-and intra-assay coefficient of variation.
• A cell line in which weak agonists increase induction of luciferase activity by at least two to three fold and antagonists decrease induction of the enzyme by at least 25%.

Discussion ensued regarding the potential problem associated with the presence of GR and the MMTV promoter. It was pointed out that it is unlikely that many cells would survive without glucocorticoids, so the solution to this problem would be the use of a cell with a different promoter. Discussion also ensued about the difficulties of using a yeast cell line due to the different metabolic pathways in these cells compared to mammalian cells and the transport of substances into the cells. The latter could be overcome by manipulating the permeability of the cell wall through mutagenesis of the genes coding for cell wall proteins. It was the consensus of the Panel that yeast should not be used for the assay.

2. **Minimum Procedural Standards for In Vitro AR TA Assays**

The Panel was in agreement with the minimal procedural standards outlined in the BRD but added that the following standards must be included for future assays.

**Reference Androgens:**
• R1881 should be the reference agonist because it is not metabolized.
• 5α-DHT should be included as one of the positive controls in all tests. Maximal transcriptional activity of R1881 should be obtained with a concentration of ~0.1-1 nM.
• Hydroxyflutamide should be used as the reference antagonist.
• The IC\(_{50}\) should be ~ 500 nM with a ~70-90% inhibition occurring with 1-5 µM hydroxyflutamide.

**Concentration Range of Test Substances:**
• For both agonism and antagonism the limit dose should be 1nM but the solubility characteristics and potential cytotoxicity must be taken into consideration.
• Seven concentrations at log intervals should be tested.
• A measure of cell toxicity will help define the upper limit for test material concentration similar to the Maximum Tolerated Dose approach.
• Data should be expressed in relative light units (RLU) or fold induction relative to the background control (RLU for background control must be stated).
A suitable nonlinear regression model such as the Hill equation must be used to estimate the potency ($EC_{50}$ or $IC_{50}$) and slope of the dose-response curve with the calculation of a 95% confidence interval.

Diagnostics need to be performed on the model by checking for suitability and normality of the curve. If necessary, suitable transformations need to be performed.

For agonist or antagonist activity that does not exhibit a full dose-response, (e.g., partial agonist) a trend analysis to detect a dose-response must be used. This can be followed up with confidence interval estimation at each dose level if the trend is significant. If the trend is not significant, then no further action is necessary. Significant trends imply potential activity and may be examined further.

The biostatisticians on the Panel stated that currently insufficient data are available to address all the statistical or data evaluation issues that would enable them to recommend specific statistical analyses. Before specific statistical procedures and action levels can be identified, more details are needed about the methods and their performance criteria. It will be necessary to evaluate confidence limits, standard errors etc., to better understand the data. Different data and statistical analyses will be required depending on whether the test will be used simply as a yes/no indicator, than if the results will be used in a quantitative manner.

**Assay Acceptance Criteria:**
- At least a 10-fold induction with the control androgen is required to ensure sensitivity to detect weakly active substances.
- The concentration of R1881 used in the antagonist assays should induce transcriptional activity ~75% of the maximal response using a concentration of ~0.1-1.0 nM R1881.
- For a substance to be classified as a positive agonist it must induce at least a 2-3 fold increase in transcriptional activity over background levels.
- For a positive antagonist response, a substance must inhibit at least 25-50% agonist-induced transcriptional activity (using concentrations of R1881 that are ~75% maximal activity).
- The inter- and intra-assay % coefficients of variation should not exceed ~20%.

**Evaluation and Interpretation of Results:**
- There should be no activation with other steroid hormones ($17\beta$-estradiol, glucocorticoids cortisol, corticosterone, progesterone) due to the presence of other receptors (GR or PR) in the cell line.
- The assays should be performed under GLPs.

**Test Report:**
- Information on controls for the activity of other steroid receptors and controls for cytotoxicity.
- Source of supplies (e.g., plasticware used in the assays).
- Cell passage number.
- IUPAC chemical names are sufficient (structures not required).
- Solvent justification, if other than ethanol or DMSO.
- DNA isolation method (not detailed procedure).
- Name and reference for reporter vector (structure not needed).
- Justification for reference androgen, only if is not R1881 or DHT.
• Statistical analysis (e.g., Hill Equation) for potency and steepness of the dose-response curve.
• Solubility information should be included in the test report.
• A description of the justification for the chemical concentrations used must be included in the report.

Additional Minimal Procedural Standards
• Serum free and phenol red free media should be used rather than charcoal stripped serum when possible based on cell viability.
• The stability of the stable cell lines must be monitored using selection media.
• The cell doubling time must be monitored.
• Cytotoxicity controls using one of the following plasmids (CMV-Luc, pSG5-Luc) must be included up to the highest dose. Cytotoxicity above 10% is not acceptable.
• Controls for direct inhibition of luciferase activity must be included.

The Panel discussed the possible methods for quantifying cytotoxicity in the assay. The approach that seemed to have the greatest promise included the measurement of the activity of the gene product of a co-transfected luciferase gene that fluoresces at a different wavelength than the luc reporter gene used in the same cell. CMV-driven luc plasmids were suggested as the carrier of the co-transfected gene, although these plasmids may be affected by some test substances, and therefore respond to non-endocrine transcription signals.

The issue of entry and exit assays was discussed. This is a measure of the concentration of the chemical in the stock solution before and after the binding or TA assay is performed. There was also the question of whether this analysis should be performed after the test chemical is added to the cells and media. The analysis would then be performed before the binding or TA assay was run and then again after the assay was run. This analysis would indicate whether the test substance was degraded during the assay’s incubation, whether it was absorbed to the glassware or plasticware, and also whether the substance was metabolized during the course of the incubation. This latter point is very important for many of the substances that are AR antagonists, because the parent compound is inactive but the metabolite is active. These entry and exit assays would increase the cost of performing the ER and AR binding and TA assays particularly if they were performed on the substance after it was dissolved in the media used in the assays. The Panel thought that this additional analysis and expense would place too large a burden on the laboratories running the assays.

3. Recommendations for In Vitro AR TA Test Method Protocols for Validation Studies

The Panel was of the opinion that the three test method protocols lacked sufficient detail.

The yeast-based assays are not appropriate because they:
• Cannot distinguish an agonist from an antagonist.
• Have a cell wall that affects active transport.
Transfection-based assays may not be appropriate due to patent restrictions. The adenovirus assay may be appropriate but it needs to be improved.

Other Protocol Elements

Additional information that needs to be included in the protocols are:
- Fold induction by the control androgen.
- Intra- and inter-assay coefficients of variability.
- Stability of cell responsiveness over time and passage number.
- A standardized method for comparing potencies of agonists and antagonists in the different assays.

Other Available Standardized Protocols

The Panel pointed out that the N/C interaction assay had not been mentioned in the BRD. In this assay expression vectors are made of the GAL4 and VP16 genes with the N terminal end of the AR and AR ligand binding domain. These vectors are transfected into HeLa cells which can then be used to measure TA. The advantages of this assay are that the HeLa cell line is conducive to a multi-well format, both 17β-estradiol and cortisol are negative in the assay, its sensitivity is significantly greater than that achieved with stable transfected cell lines, with a 20 fold induction in response with 0.1 nM DHT, and it has a GAL-Luc reporter constant with which no other steroid receptors are active. The disadvantage of the assay is that it is subject to the same patent restrictions that apply to other transient co-transfection assays that use the AR expression vector and that apply to stable cell lines with an integrated AR plasmid.

4. Recommended List of Substances to be Used for Validation in In Vitro AR TA Assays

The Panel recommended that the following substances be included in the list for validation of the AR TA assay:

**Agonists:** R1881, DHT, testosterone, androstenedione, fluoxymesterone

**Antagonists:** hydroxyflutamide, casodex (bicalutamide), cyproterone acetate, p,p’-DDE, linuron

**Mixed activity:** progesterone (PR agonist), medroxyprogesterone acetate (GR and PR agonist)

**No activity:** dexamethasone (GR agonist), cortisol (GR agonist), 17β-estradiol (ER agonist)\(^1\)

\(^1\)17β-Estradiol is listed here as having no activity in AR-TA assays despite the many reports of positive responses in the literature reviewed for the BRD. The reason for this listing, according to the Work Group members, is that it does not induce transcriptional activation \textit{in vivo}, and the positive responses seen in the \textit{in vitro} systems are artifacts of the recombinant systems used.
**Negative controls:** cycloheximide (*protein synthesis inhibitor*), actinomycin D (*RNA synthesis inhibitor*), sodium azide (*cytotoxicant*), specific inhibitors of luciferase activity, TPA (*ligand independent activation*)

The Panel recommended that heavy metals, acids and bases, insoluble solids or reactive agents, liquid and gaseous volatiles were not required for validation. However, there was a question as to whether organotins are positive in the assay. A concern with testing metals is the concentration of EDTA in the assay system. There was a consensus that as long as the concentration of EDTA is kept at 1.5mM or lower there would be no problem in testing metals.

More weak compounds could be included but inactive parent compounds such as flutamide, methoxychlor, vinclozolin, and DDT should be deleted. Although the respective active intermediates of the above mentioned compounds, namely hydroxyflutamide, HPTE, the major metabolite of methoxychlor, M2, a metabolite of vinclozolin, and $p,p'$-DDE are active in the assay, only hydroxyflutamide and $p,p'$-DDE were recommended for testing because HPTE and M2 are difficult to obtain.

As mentioned previously, the working group suggested that the U.S. EPA should provide a standard set of chemicals for validation purposes.

**Public Comments Session (Afternoon, May 20)**

Dr. Daston asked if there were any public comments before adjournment of the meeting.

Dr. Gray (U.S. EPA). When developing or recommending an “ideal” protocol, it is important to distinguish between required and desirable features. There is a need to challenge the assays with weak agonists and antagonists. However, there are no known, weakly acting non-steroidal androgen agonists. With respect to measuring fold-induction during the TA assays, it is important to examine the variability of the response. He also requested that the Panel expand on the list of negative compounds that they would recommend for testing in the AR TA assays so that he could challenge the two assays that he was presently evaluating in his laboratory.

Dr. Richard Becker (American Chemical Council) echoed the request of Dr. Gray that the Panel attempt to determine which of the procedural standards were desirable and which ones were necessary in the development of these assays.

Dr. Becker also stated that the recommendations and report by the Panel is critical. It is clear that there are no validated assays and research will be needed to develop such assays. The Panel is asked to include practical recommendations to help identify valid assays for screening.

Dr. Stokes thanked the Panel on behalf of the NTP and ICCVAM for their thoughtful deliberations and careful evaluation of the test methods and background review documents. The Chair adjourned the Panel Meeting at 2:15 pm.
May 21-22, 2002
Expert Panel Evaluation of the Validation Status of In Vitro Test Methods for Detecting Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays

“These Summary Minutes have been read and approved by the Chair of the Expert Panel Meeting on the Evaluation of the Validation Status of In Vitro Test Methods for Detecting Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays, as certified below.”

______________________________  _________________________
Dr. George Daston                  Date
Panel Chair

______________________________  _________________________
Dr. William Stokes                Date
Panel Executive Secretary
APPENDIX E

ICCVAM Expert Panel Evaluation
Federal Register Notices

Federal Register notices are available at https://www.federalregister.gov/

Request for Data and Nominations of Expert Scientists

E-2 Vol. 67, No. 66, pp. 16415-16416, April 5, 2002
Notice of an Expert Panel Meeting and Request for Comments

E-3 Vol. 67, No. 204, pp. 64902-3, October 22, 2002
Announcing Availability of Expert Panel Report, Proposed List of Substances and Final Background Review Documents
APPENDIX F

Public Comments in Response to the Federal Register Request for Comments

Public comments are available on request from NICEATM.

Dr. Charles B. Breckenridge, Syngenta Crop Protection, Inc
Mr. Mike Scully, Amersham Biosciences
Ms. Barbara S. Losey, APE Research Council
Dr. Richard A. Becker, American Chemistry Council
Dr. Mitsuru Iida, Otsuka Pharmaceutical Co., Ltd.