

# Background Review Document

## Current Status of Test Methods for Detecting Endocrine Disruptors: *In Vitro* Androgen Receptor Transcriptional Activation Assays



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

**THE INTERAGENCY COORDINATING COMMITTEE  
ON THE VALIDATION OF ALTERNATIVE METHODS  
and  
THE NTP INTERAGENCY CENTER FOR THE  
EVALUATION OF ALTERNATIVE TOXICOLOGICAL METHODS**

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**

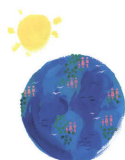
Additional information can be found at the ICCVAM/NICEATM Website: <http://iccvam.niehs.nih.gov> and in the publication: *Validation and Regulatory Acceptance of Toxicological Test Methods, a Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods* (NIH Publication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

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**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.

## **NOTICE TO READER**

This Background Review Document contains data, a proposed list of substances, and minimum procedural standards that were reviewed by an independent Expert Panel in May 2002.

The reader is referred to the final report entitled, "ICCVAM Evaluation of *In Vitro* Test Methods for Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays," (NIH Publication No. 03-4503) for the final ICCVAM recommended substances and minimum procedural standards.

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for Detecting Endocrine Disruptors:  
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Transcriptional Activation Assays**

**Background Review Document**

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

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## LIST OF ACRONYMS AND ABBREVIATIONS

AF	Transactivation function
ANOVA	Analysis of variance
AR	Androgen receptor
AR	Androgen receptor alpha
ARE	Androgen response elements
ATP	Adenosine triphosphate
$\beta$ -gal	Bacterial gene for $\beta$ -galactosidase
BRD	Background Review Document
CASRN	Chemical Abstracts Service Registry Number
CAT	Chloramphenicol acetyl transferase
cDNA	Complementary DNA
CDTA	<i>trans</i> -1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid
CHO	Chinese hamster ovary
CMA	Chemical Manufacturers Association
CMV	Cytomegalovirus
CUP	Copper responsive yeast metallothionein promoter
DDT	Dichlorodiphenyltrichloroethane
DEAE	2-(Diethylamino)ethyl
DHT	5 $\alpha$ -Dihydrotestosterone
DMSO	Dimethyl sulfoxide
EC <sub>50</sub>	Half-maximal effective concentration
EDSP	Endocrine Disruptor Screening Program
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
EPA	Environmental Protection Agency
ER	Estrogen receptor
ER	Estrogen receptor alpha
ER	Estrogen receptor beta
FFDCA	Federal Food, Drug, and Cosmetic Act
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act

fmol	Femtomole
FQPA	Food Quality Protection Act
GLP	Good Laboratory Practices
GR	Glucocorticoid receptor
hAR	Human AR
HRE	Hormone responsive elements
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IC <sub>50</sub>	Concentration of test substance causing a 50% reduction in the reporter gene product induced by a reference androgen
kDa	Kilodalton
<i>luc</i>	Gene coding for the production of luciferase
μL	Microliter
μM	Micromolar
mAR	Mouse androgen receptor
mM	Millimolar
MMTV	Mouse Mammary Tumor Virus
mRNA	Messenger RNA
NAS	National Academy of Sciences
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
ng	Nanogram
nm	Nanometer
nM	Nanomolar
OD	Optical density
OECD	Organisation for Economic Co-operation and Development
ONPG	<i>o</i> -Nitrophenyl-β-galactoside
pM	Picomolar
QC	Quality control
RPP	Relative proliferative potency
rtAR	Rainbow trout androgen receptor

SAB	Science Advisory Board
SAP	Scientific Advisory Panel
SAR	Structure-activity relationship
SDS	Sodium dodecyl sulfate
SDWA	Safe Drinking Water Act
SV-40	Simian virus 40
TA	Transcriptional activation
TeBG	Testosterone estradiol binding globulin
Tris	Tris(hydroxymethyl)aminomethane
TSCA	Toxic Substances Control Act
WWF	World Wildlife Fund



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## PREFACE

The Food Quality Protection Act and Amendments to the Safe Drinking Water Act in 1996 directed the U.S. Environmental Protection Agency (U.S. EPA) to develop and validate a screening program to determine whether certain substances may have hormonal effects in humans. In response, the U.S. EPA developed an Endocrine Disruptor Screening Program (EDSP), and is currently evaluating the scientific validity of screening and testing methods proposed for incorporation into the EDSP. *In vitro* estrogen receptor (ER) and androgen receptor (AR) assays have been proposed as possible components of the EDSP Tier 1 screening battery. The U.S. EPA asked the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) to evaluate the validation status of these *in vitro* assays. ICCVAM, which is charged with coordinating the technical evaluations of new, revised, and alternative test methods, agreed to evaluate the assays based on their potential interagency applicability and public health significance.

In order to assess the current validation status of these *in vitro* methods, it was first necessary to compile all of the available data and information for existing assays. The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), which provides operational support for the ICCVAM, subsequently arranged for preparation of this Background Review Document (BRD) by its support contractor, Integrated Laboratory Systems, Inc. (ILS) with financial support from the U.S. EPA. This BRD reviews available data and procedures for existing *in vitro* AR transcriptional activation (TA) assays and is organized according to published guidelines for submission of test methods to ICCVAM (ICCVAM, 1999). Separate BRDs have also been prepared for *in vitro* ER binding assays, *in vitro* AR binding assays, and *in vitro* ER TA assays.

As part of the ICCVAM evaluation, the U.S. EPA also asked for development of minimum performance criteria that could be used to define an acceptable *in vitro* AR TA assay. It was envisioned that these criteria would be based on the performance of existing standardized *in vitro* AR TA assays. The minimum performance criteria could then be used to assess the acceptability of new or revised assays proposed in the future. However, a comprehensive review determined that there were no standardized *in vitro* AR TA assays with adequate validation data that could serve as the basis for establishing these performance criteria. An independent Expert Panel

(Panel) was therefore convened to assess the status of existing *in vitro* AR TA assays and to develop recommendations for standardized assays and validation studies that should be conducted. After adequate validation studies have been completed on one or more standardized AR TA assays, an independent Peer Review Panel will be convened to evaluate the validated assay(s) and to recommend minimum performance criteria for *in vitro* AR TA assays.

This BRD reviews available *in vitro* AR TA assays and presents the data available for substances evaluated in these assays. The relative performance of various types of *in vitro* AR TA assays is compared using this existing data, which was very limited for some of the assays. Based on the comparative performance and advantages and disadvantages of each type of assay, several assays are proposed as priority candidates for standardization and future validation. In addition, minimum procedural standards that should be used for *in vitro* AR TA assays are proposed. These standards include elements such as dose selection criteria, minimum number of replicates, appropriate positive and negative controls, criteria for an acceptable test run, and proficiency standards for participating laboratories. Finally, the BRD proposes a list of substances recommended for the validation of *in vitro* AR TA screening assays.

An Expert Panel was convened in a public meeting on May 21-22, 2002, to review the information and proposals provided in this BRD, and to develop 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 proposed minimum procedural standards.
- The adequacy of protocols for specific assays recommended for validation studies.
- The adequacy and appropriateness of substances proposed for validation studies.

The Expert Panel meeting was announced to the public in a *Federal Register* notice (Vol. 67, No. 66, pp. 16415-16416, Apr. 5, 2002; also available on the internet at: <http://iccvam.niehs.nih.gov/docs/FR/6716415.pdf>)

An ICCVAM Endocrine Disruptor Working Group (EDWG) was organized to coordinate the technical evaluation of *in vitro* endocrine disruptor screening methods. The EDWG is co-chaired

by Drs. David Hattan and Marilyn Wind, and consists of knowledgeable scientists from ICCVAM agencies. The EDWG functions include identification and recommendation of experts for the Expert and Peer Review Panels, the review of test method BRDs for completeness, preparation of questions for the Expert and Peer Review Panels, and development of draft ICCVAM test recommendations based on Panel evaluations. Final ICCVAM test recommendations will be forwarded from the ICCVAM to Federal agencies for their consideration.

In July 2002, the draft of this BRD was revised to address corrections and omissions noted by the Expert Panel and published as a final version. The final report of the Expert Panel and a proposed list of substances for validation studies of *in vitro* ER and AR methods was published and made available to the public for comment as announced in a *Federal Register* notice (Vol. 67, No. 204, pp. 64902-64903, October 22, 2002; available at <http://iccvam.niehs.nih.gov/docs/FR/6764902.htm>). A final ICCVAM Test Method Evaluation report will be published in early 2003. This report will include ICCVAM recommendations, the final Expert Panel report, a recommended list of substances for validation studies, and public comments. The report will be forwarded to federal agencies for their consideration and made available to the public.

The efforts of the many individuals who contributed to the preparation, review, and revision of this BRD are gratefully acknowledged. These include Barbara Shane, Christina Inhof, Errol Zeiger, Raymond Tice, Bradley Blackard, Steven Myers, and Linda Litchfield, from ILS, Inc. who prepared the BRD. The suggestions and advice from the ICCVAM EDWG members and co-chairs on early drafts and subsequent versions were invaluable, as were the comments from *ad hoc* reviewers on the final draft. Additional comments and suggestions for improvement of this and future test method documents are welcome at any time.

William S. Stokes, D.V.M., Diplomate, ACLAM  
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July 31, 2002

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## EXECUTIVE SUMMARY

The objectives of this BRD are to: (1) provide comprehensive summaries of the published and publicly available unpublished data on the scientific basis and performance of *in vitro* assays used to test substances for their ability to initiate transcriptional activation of the androgen receptor (*in vitro* AR TA assays); (2) assess the *in vitro* AR TA assays considered for their effectiveness in identifying endocrine-active substances; (3) identify and prioritize *in vitro* AR TA assays that might be considered for incorporation into future testing programs for validation; 4) develop minimum performance criteria by which to judge the effectiveness of proposed *in vitro* AR TA assays; and (5) generate a list of recommended substances to be used in validation efforts.

The data summarized in this BRD are based primarily on information obtained from the peer-reviewed scientific literature. An online literature search identified 105 records related to androgen binding and TA assays with 26 publications containing relevant data on *in vitro* AR TA assays for inclusion in this BRD. Some of the peer-reviewed publications that contained *in vitro* AR TA assay data were not abstracted for inclusion in this BRD because the studies lacked the appropriate details or contained data from unique procedures or substances that were not clearly identified. In addition to the published data, the BRD includes *in vitro* AR TA data from one submitted report containing unpublished information.

In *in vitro* AR TA assays, the cell lines used include those that have been transfected with foreign DNA consisting of an AR and a reporter gene (luciferase, chloramphenicol acetyltransferase, or -galactosidase) that is transcribed when an androgen or test substance binds to the AR, and mammalian cells harboring an endogenous AR in which a reporter gene is added. In these tests, one of four reference androgens (5 -dihydrotestosterone, methyltrienolone, testosterone, or mibolerone) was used. Studies that evaluated the potential AR agonism of a test substance used enzyme activity as an indirect measure of AR-induced transcriptional activation, while AR antagonism studies measured the ability of the test substance to inhibit reporter gene enzyme activation induced by the reference androgen.

Data were abstracted for 18 different *in vitro* AR TA assays. These assays used either CHO (Chinese hamster ovary cells), CV-1 (monkey kidney cell line), HeLa (human cervical cancer cell line), HepG2 (human liver tumor cell line), MDA-MB-453 (human breast carcinoma cell line), PC-3 (human prostate tumor cell line), and EPC (carp skin tumor cell line) cells, or yeast (*S. cerevisiae*) transfected predominantly with the human (h) AR, although mouse (m) and rainbow trout (rt) AR have been used also. Some cell lines were manipulated so that the foreign DNA was incorporated permanently into cellular DNA. However, many of the assays described in the BRD used cells transiently transfected with the AR and the reporter gene. Under these conditions, the transfected DNA remains intact in the cell for a few days.

*In vitro* AR TA assay data were collected for a total of 145 substances, of which 68 were tested for both agonism and antagonism activity, 51 for agonism activity only, and 20 for antagonism activity only. The chemical classes tested most extensively have been nonphenolic steroids, organochlorines, phenolic steroids, and polycyclic aromatic hydrocarbons, while the most common product classes tested have been pharmaceuticals and pesticides.

More substances (65; 44.5%) were tested in the CHO-K1 hAR(T)+Luc(T)+EGFP(T) than in any other assay. The next most frequently used assay was the PALM hAR(S)+Luc(S) (43 or 29.5% of substances tested). Thirty-two substances (21.9%) were tested in the Yeast (*S.cer*) AR + gal assay and 27 (18.5%) were tested in the CV-1+hAR(T)+Luc(T) assay.

The quantitative results of the *in vitro* AR TA studies for agonism were most commonly presented in terms of relative activity expressed as the fold induction of enzyme activity produced by the test substance relative to the activity in the untreated controls, as the ratio of the response of the test substance to that of the reference androgen, or as the concentration of the test substance that produced a certain percent response relative to the reference androgen. An EC<sub>50</sub> value (the half-maximal concentration) was provided infrequently. For antagonism studies, the inhibition of reference androgen-induced enzyme activity by the test substance was measured and sometimes expressed as an IC<sub>50</sub> value (i.e., the concentration that inhibited the reference androgen-induced AR transcriptional activation by 50%).

Relatively few of the substances had been tested by more than one investigator in the same *in vitro* AR TA assay or in multiple assays in the same or different laboratories. Furthermore, because the primary focus of many of the studies reviewed in this BRD was on understanding the mechanisms of AR-induced transcriptional activation and not at identifying substances with AR agonist or antagonist activity, much of the published data are of limited value for the analysis of assay performance or reliability.

Based on the limited data available, there is no single *in vitro* AR TA assay that can be concluded to perform better or to be more reliable than any other assay. However, it might be anticipated that mammalian cell assays would be preferred over yeast assays, simply because of the increased ability of test substances to cross the mammalian cell membrane compared to the yeast cell wall. Although the transiently transfected cell lines have some advantages over the stably transfected cell lines in that the level of the AR is higher in the former, the ability to reproducibly transfect the same amount of DNA on a routine basis is difficult. Not all of the laboratories using this technique monitored the transfection efficiency. Patent issues are another disadvantage of the transiently transfected cell lines. Taking these factors into consideration, it would seem that a cell line that contains endogenous AR and is stably transfected with a luciferase reporter plasmid (e.g., MDA-MB-453-kb2) would offer the greatest utility by eliminating the need to continuously prepare multiple batches of transiently transfected cells. This cell line would also eliminate concerns regarding patents on the transient transfection of cell lines with the AR.

Formal validation studies should be conducted using appropriate substances covering the range of expected  $EC_{50}/IC_{50}$  values to adequately demonstrate the performance characteristics of any *in vitro* AR TA assay recommended as a possible screening test method for AR agonists and antagonists. The role of metabolic systems in activating some substances to an AR agonist or antagonist needs to be considered prior to the implementation of future validation studies.

An important step towards acceptance of an *in vitro* AR TA assay into a regulatory screening program is production of high quality data. To achieve this goal, it is recommended that any

future prevalidation and validation studies on *in vitro* AR TA assays be conducted with coded substances and in compliance with GLP guidelines. Ideally, if multiple laboratories are involved in the validation study, the substances should be obtained from a common source and distributed from a central location.

The facilities needed to conduct *in vitro* AR TA assays are widely available, as is the necessary equipment from major suppliers. Although information on the commercial cost of these assays was not available, it can be assumed that the costs for most if not all of the assays are roughly equivalent.

Since there are no published guidelines for conducting *in vitro* AR TA studies, and no formal validation studies have been performed to assess the reliability or performance of such assays, the U.S. EPA requested that minimum procedural standards based on a comparative evaluation of *in vitro* AR TA assays be developed. The minimum procedural standards provided include methods for determining the ability of the reference androgen to induce transcriptional activation, methods for establishing a stable cell line, the concentration range of the test substance (including the limit dose) to test for agonists and antagonists, the use of negative and positive controls, the number of replicates to use, dose spacing, data analysis, assay acceptance criteria, evaluation and interpretation of results, minimal information to include in the test report, and the potential need for replicate studies. These minimum procedural standards are provided to ensure that *in vitro* AR TA studies will be conducted in such a manner as to allow the results to be understandable and comparable among procedures.

Six *in vitro* AR TA assay protocols developed by experts in the field are provided in **Appendix B**. Inspection of these protocols provides a perspective on how various *in vitro* AR TA assays are conducted by different investigators, and for developing a more general protocol, one that takes into account the recommended minimum procedural standards. Prior to developing that protocol, the submitted protocols need to be evaluated for completeness and adequacy for their intended purpose.

The U.S. EPA requested that a list of recommended test substances be provided for use in validation studies. Testing of substances encompassing a wide range of agonist/antagonist responses are needed to adequately demonstrate the performance characteristics of *in vitro* AR TA test methods recommended as screening assays. A number of factors were considered in developing this list of substances, including the EC<sub>50</sub> and IC<sub>50</sub> value of the substance in all the assays in which it had been tested. Because the number of substances with replicate quantitative agonist or antagonist data was insufficient to generate the desired number of substances for consideration, selection of most substances was based on results obtained in a single assay by a single investigator. The selected substances were sorted according to whether they were positive, weak positive, or negative in at least one *in vitro* AR TA assay.

It is anticipated that this BRD and the guidance it provides will help to stimulate validation efforts for *in vitro* AR binding assays.

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## **1.0 INTRODUCTION AND RATIONALE FOR THE USE OF *IN VITRO* AR TA ASSAYS**

### **1.1 Introduction**

#### **1.1.1 Historical Background of *In Vitro* Endocrine Disruptor Assays and Rationale for Their Development**

It is well known that small disturbances in endocrine function, especially during highly sensitive stages of the life cycle (e.g., fetal and prepubertal development), can lead to significant and lasting effects on the exposed organism (Kavlock et al., 1996; U.S. EPA, 1997; NAS, 1999). In recent years, evidence has been accumulating to suggest that exposure to natural and anthropogenic substances in the environment may adversely affect the endocrine and reproductive systems of mammals, fish, reptiles, amphibians, and birds. Substances that cause such effects are classified as “endocrine disruptors.” Disruption of the endocrine system has been demonstrated in laboratory animals and documented in wildlife (Ankley et al., 1998). For example, male fish in rivers in many regions of the United States have high levels of vitellogenin, a female-specific protein (Purdom et al., 1994; Folmar et al., 1996), and female mosquitofish living in streams in which pulp mill effluents containing steroidal substances have been discharged possess male gonadal structures (Bortone et al., 1989). The degree to which humans are affected by endocrine disruptors is unknown, although there are reports that these substances might be contributing to increasing incidences of breast, prostate, and testicular cancers (Glass and Hoover, 1990; Adami et al., 1994; Toppari et al., 1996), precocious puberty and hypospadias, and decreased sperm counts (Carlsen et al., 1992; Sharpe and Skakkabaek, 1993). However, other investigators have concluded that there is no evidence for endocrine disrupting effects in humans (Barlow et al, 1999; Safe, 2000)

In 1996, the U.S. Congress responded to societal concerns by passing legislation requiring the U.S. EPA to develop a screening and testing program, using appropriately validated test methods, to detect potential endocrine disruptors in pesticide formulations (the Food Quality Protection Act; FQPA) (P.L. 104-170), and drinking water (the 1996 amendments to the Safe Drinking Water Act; SDWA) (P.L. 104-182). As a result of these mandates, the U.S. EPA formed the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) to provide advice on how to best design a screening and testing program for identifying endocrine

disruptors. In August 1998, EDSTAC issued a report recommending that the U. S. EPA evaluate both human and ecological (wildlife) effects; examine effects to estrogen, androgen, and thyroid hormone-related processes; and test both individual substances and common mixtures (U.S. EPA, 1998a). In December 1998, based on these recommendations, the U.S. EPA proposed the EDSP (U.S. EPA, 1998b). In 1999, the EDSP and its proposed approach to screening for endocrine disruptors were endorsed by the U.S. EPA Science Advisory Board (SAB) and the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP), which also made a number of recommendations concerning the proposed approach (U.S. EPA, 1999).

The EDSP proposes a two-tiered approach for screening and testing. Tier 1 is comprised of *in vitro* and *in vivo* assays and is designed as a screening battery to detect substances capable of interacting with the estrogen, androgen, or thyroid hormone systems. Tier 2 is comprised of *in vivo* assays and is designed as a testing battery to (1) determine whether an endocrine-active substance (identified in Tier 1 or through other processes) causes adverse effects in animals; (2) identify the adverse effects; and (3) establish a quantitative relationship between the dose and the adverse effect (U.S. EPA, 2000).

The EDSP's proposed Tier 1 screening battery includes the following assays:

*In vitro* assays:

- ER binding/TA assays
- AR binding/TA assays
- Steroidogenesis assay with minced testis

*In vivo* assays:

- Rodent 3-day uterotrophic assay (subcutaneous dosing)
- Rodent 20-day pubertal female assay with enhanced thyroid endpoints
- Rodent 5-7 day Hershberger assay
- Frog metamorphosis assay
- Fish gonadal recrudescence assay



The alternative Tier 1 assays include:

- Placental aromatase assay (*in vitro*)
- Modified rodent 3-day uterotrophic assay with intraperitoneal dosing (*in vivo*)
- Rodent 14-day intact adult male assay with thyroid endpoints (*in vivo*)
- Rodent 20-day thyroid/pubertal male assay (*in vivo*)

According to the EDSP, the Tier 1 assays should:

- Detect all known modes of action for the endocrine endpoints of concern;
- Maximize sensitivity to minimize false negatives, while permitting a to-be-determined level of false positives;
- Include a sufficient range of taxonomic groups among the test organisms to reduce the likelihood that important pathways for metabolic activation or detoxification of the test substances are not overlooked; and
- Incorporate sufficient diversity among the endpoints and assays to permit conclusions based on weight-of-evidence considerations.

The proposed Tier 2 testing battery includes the following *in vivo* assays:

- Two-generation mammalian reproductive toxicity assay
- Avian reproduction assay
- Fish reproduction assay
- Amphibian reproduction and developmental toxicity assays
- Invertebrate reproduction assay

The alternative Tier 2 assays include:

- Alternative mammalian reproductive test
- One-generation mammalian reproduction toxicity test

According to the EDSP, the Tier 2 assays should:

- Encompass critical life stages and processes in mammals (equivalent to humans), fish, and wildlife;

- Encompass a broad range of doses and the administration of the test substance by a relevant route of exposure; and
- Provide a comprehensive profile of biological consequences of substance exposure and relate such results to the causal dose and exposure.

Two proposed *in vitro* components of the Tier 1 screening battery are ER binding/TA assays, and AR binding/TA assays. The primary rationale for inclusion of *in vitro* assays in the EDSP Tier 1 screen is that they:

- Are suitable for large-scale screening;
- Are based on well-elucidated mechanisms of action; and
- Measure specific endpoints.

The Tier 1 assays are informative with regard to the mechanism of action of the presumptive endocrine disruptor and provide guidance for prioritization for further testing. Due to their sensitivity, these *in vitro* tests should permit the identification of an active substance(s) within a complex mixture. TA assays have an advantage over binding assays because they measure the biological response to receptor binding (i.e., RNA transcription) and thus, unlike binding assays, can distinguish between an agonist (i.e., a substance that mimics the action of endogenous androgens) and an antagonist (a substance that binds to a receptor without initiating a biological response, blocking the action of endogenous hormones) (U.S. EPA, 1998b). However, it needs to be emphasized that these *in vitro* assays cannot be used to predict the risk of an adverse health effect in humans or wildlife.

As part of the validation process for the proposed EDSP assays, the U.S. EPA is supporting an effort by NICEATM to prepare BRDs on the Tier 1 *in vitro* ER binding, AR binding, ER TA, and AR TA screening assays. Other EDSP-proposed assays will be validated through other organizations (e.g., the U.S. EPA and the Organisation for Economic Co-operation and Development [OECD]). The objectives of each BRD are to:

- Provide a comprehensive summary of the available published and submitted unpublished data on the scientific basis and performance of the identified assays;
- Identify available assays that might be considered for incorporation into the EDSP;

- Assess the effectiveness of the assays for identifying endocrine-active substances;
- Develop minimum procedural standards for acceptable ER and AR binding and TA assays; and
- Generate a list of substances suitable for use in future validation studies.

### **1.1.2 Prior or Proposed Peer Reviews of *In Vitro* AR TA Assays**

Although there has been some research conducted in the past few years to develop new or improved *in vitro* assays to identify substances with AR TA activity, there have been no formal peer reviews of the validation status of such assays. This BRD has been prepared for an anticipated ICCVAM expert review of *in vitro* AR TA assays, in concert with reviews of *in vitro* AR binding assays and *in vitro* ER binding and TA assays.

## **1.2 Scientific Basis for the Proposed Tier 1 *In Vitro* AR TA Assays**

### **1.2.1 Purpose for Using *In Vitro* AR TA Assays**

The *in vitro* AR TA assays are designed to identify substances that might interfere with normal androgen activity *in vivo* by acting as an androgen agonist or antagonist. Unlike receptor binding assays, TA assays can distinguish between these two types of activity. *In vitro* AR TA assays used to evaluate agonism are generally performed by quantifying the induction of a reporter gene product or the stimulation of cell growth in response to activation of the AR by the test substance. *In vitro* AR TA assays that evaluate antagonism measure the ability of a test substance to inhibit the induction of the reporter gene product or the stimulation of cell growth by a reference androgen, such as 5 $\alpha$ -dihydrotestosterone (DHT) or 17 $\beta$ -hydroxy-estra-4,9,11-trien-3-one (methyltrienolone or R1881). However, a positive response in an *in vitro* AR TA agonist or antagonist assay is not sufficient to predict *in vivo* effects. For this reason, results of the *in vitro* AR TA assays will be used in conjunction with Tier 1 *in vivo* screening assays in a weight-of-evidence approach to prioritize substances for Tier 2 testing.

### **1.2.2 Development of *In Vitro* AR TA Assays: Historical Background**

Reporter gene assays provide a relatively simple way to measure whether substances can activate or inhibit the TA of androgen-regulated genes. The accurate quantitation of the AR-dependent TA of endogenous, hormone-dependent genes has been difficult, due largely to the complex

signaling networks and transcriptional controls that are involved in the process. An AR reporter gene assay eliminates these complexities by creating an artificial gene expression system in a host cell. These assays use cellular processes that have been genetically manipulated to allow for the measurement of one specific gene product, typically an enzyme, the production of which is under AR control. Since most cultured cells lack the AR and some of the necessary components of the pathway for AR TA, these genes must be inserted into each cell. This is accomplished by transfecting a plasmid containing AR complementary DNA (cDNA) and androgen-responsive promoters into the host cell, along with the cDNA for a reporter gene, which is linked to an androgen response element (ARE).

The technology for reporter gene assays was well established by the time the AR was cloned, having been developed for TA studies with the glucocorticoid, progesterone, and estrogen receptors (DeFranco and Yamamoto, 1986). The cloning of human AR (hAR) cDNA by Lubahn et al. (1988 a, b) and Chang et al. (1988) facilitated the development of AR reporter gene assays. At that time, Lubahn et al. (1988a) also constructed a plasmid containing hAR cDNA and transfected it into monkey kidney cells (COS M6). This approach was possible due to the technology already developed for producing recombinant DNA and introducing plasmids into cells. The transfection procedure used by Lubahn was a modification of a DEAE-dextran procedure developed in 1983 for introducing the polyoma virus shuttle vector into mouse lymphoid cells (Deans et al., 1983). Modifications of these recombinant DNA and plasmid transfection techniques are currently used in *in vitro* AR TA reporter gene assays.

Yarbrough et al. (1990) was one of the first investigators to demonstrate the use of a reporter gene system to measure AR functional activity. Two vectors were simultaneously introduced into monkey kidney CV-1 cells, which lack an endogenous AR. The first vector was an AR expression vector containing either wild-type or mutant hAR cDNA, and the second a reporter vector containing a gene for chloramphenicol acetyltransferase (CAT) linked to the mouse mammary tumor virus (MMTV) promoter. CAT was originally chosen as the reporter gene because it was absent in mammalian cells and because the assay was considered relatively sensitive (Gorman et al., 1982). The AR expression vector contained the cDNA for wild-type or mutant hAR, which had been inserted in the pCMV1 eukaryotic expression vector containing the

cytomegalovirus promoter (CMV) and the simian virus 40 (SV-40) origin of replication. The plasmids were introduced into the CV-1 cells using a calcium phosphate procedure. The synthetic androgen, R1881, induced less CAT activity in CV-1 cells transfected with mutant AR than in cells transfected with wild-type AR.

In a series of deletion mutagenesis experiments, Simental et al. (1991) used the same gene expression system described above to demonstrate that a domain in the NH<sub>2</sub> region of hAR was necessary for full transcriptional activity, while a domain in the ligand-binding site served an inhibitory function.

Cell lines other than CV-1 have been used also in *in vitro* AR TA assays. Deslypere et al. (1992) investigated the mechanisms of DHT and testosterone induced AR activity in a reporter gene assay using Chinese hamster ovary (CHO) cells transfected with plasmids containing AR cDNA and a reporter encoding MMTV-CAT. This study demonstrated that DHT (0.1 nM) and testosterone (1 nM) cause maximal activation of the CAT reporter gene at different concentrations.

Genetically engineered yeast cells have been used by some investigators. The technology for *in vitro* AR TA assays using yeast cells was adopted from systems originally developed for the ER. In 1988, it was demonstrated that the recombinant human ER produced in yeasts can bind estrogen and that this interaction of hormone with receptor is capable of directing hormone-dependent activation of genes containing estrogen response elements (Metzger et al, 1988). With these characteristics in mind, researchers began to engineer yeast cells by reconstituting a hormone responsive transcription unit in the cells and by using novel gene fusion technology to produce an active human steroid receptor (McDonnell et al., 1989). Two expression vectors were constructed, one vector used the copper responsive yeast metallothionein promoter (CUP) to drive the synthesis of receptor messenger RNA (mRNA), when the cDNA for the human steroid receptor was inserted into the cell. Initiation in this vector was from the natural start codon of the receptor. The second vector, using the same promoter, consisted of the fusion of the cDNA for the receptor to the carboxyl terminus of a synthetic cassette-adapted ubiquitin molecule. Initiation was from the start codon of the ubiquitin DNA producing a fusion protein.

Soon after translation of this fusion protein, the yeast enzymes remove the fused ubiquitin part of the molecule, leaving the natural receptor molecule in the cell. Having the ubiquitin molecule in the system enhanced the production and stability of the receptor protein. The CUP 1 promoter is tightly regulated by copper ions, thus permitting controlled expression of the receptor in the yeast cell. The reporter plasmid contained a responsive element fused to the proximal promoter elements of the enhancerless iso-1-cytochrome c that was fused to the  $\beta$ -galactosidase gene.

Utilizing this technology, plasmids with any steroid receptor, including the AR, could be constructed. The yeast system has been used to measure AR-induced TA by only a few investigators, possibly due to the limited ability of some substances to penetrate the cell wall (Gaido et al., 1997). Another limitation of the stably transfected yeast system is the lower AR induction response compared to mammalian cells (i.e., for the same androgen, the maximal fold-increase in reporter gene response is less than that detected in mammalian cells). To enhance the reporter gene response in yeast, Gaido et al. (1997) transfected the yeast with a plasmid encoding the SPT3 protein. The rationale for this approach is that the mammalian counterpart of this yeast gene, namely the TAF18 gene, enhanced the efficiency of AR-induced TA when it was coexpressed in yeast cells (Imhof and McDonnell, 1996).

The three reporter enzymes used in *in vitro* AR TA assays include CAT and luciferase in the mammalian cell-based systems and  $\beta$ -galactosidase in the yeast-based systems. While *in vitro* AR TA assays were first developed with the *CAT* reporter gene, in the mid-1990s researchers began to use a luciferase (*Luc*) reporter. Zhou et al. (1994) was the first to report measurement of AR-induced transcriptional activity in a system using a *Luc* reporter in CV-1 cells. Wong et al. (1995) used this same system to investigate the agonist and antagonist activities of the fungicide vinclozolin, relative to that of hydroxyflutamide.

Cell proliferation has been used also as an indicator of androgen-induced TA. Sonnenschein et al. (1989) measured cell proliferation in a mammalian cell line, LnCaP-FGC, containing an endogenous AR, as a measure of AR-induced TA. LnCaP-FGC was established from a supraclavicular lymph node removed from a patient with metastatic prostatic adenocarcinoma. Although cell proliferation was stimulated by certain androgens and progesterone, estrogen-

related compounds were relatively weak in stimulating cell proliferation. Sonnenschein et al. (1989) came to the conclusion that, while an AR was present in this cell line, the proliferative response may actually have been due to the presence of plasma-borne trypsin sensitive inhibitors of cell proliferation that were eliminated by the addition of androgens to the cell medium. The cell line was subsequently discovered to have an important mutation in the ligand-binding domain of the AR (Veldscholte et al., 1990), which would preclude its use in *in vitro* AR TA screening assays.

Several procedures have been used to introduce the AR and reporter gene cDNA into the host cells used for *in vitro* AR TA assays. These procedures include viral transduction, electroporation of cells, a calcium phosphate precipitation procedure, and the use of commercial transfection reagents such as FuGene™ and LipofectAMINE™.

Data analysis approaches have varied from a visual inspection of the data to more formal statistical approaches using either one- or two-way analysis of variance (ANOVA) (with main effects being treatment or replicates and treatment, respectively) using a general linearized model. For agonist assays, responses are compared to the concurrent solvent control while for antagonist assays, the response elicited by the test substance in combination with a reference androgen is compared to the response induced by the reference androgen alone. In some studies, the induced reporter gene response for each replicate has been converted to a fold induction above the concurrent control level, and means and variances of these data used as the basis for analysis. Other measures of potency include the EC<sub>50</sub>, the concentration of an agonist that produces 50% of the maximal reporter gene response, and the IC<sub>50</sub>, the concentration of an antagonist that produces a 50% reduction in the maximal reporter gene response produced by an agonist. EC<sub>50</sub> values (for agonist assays) or IC<sub>50</sub> values (for antagonist assays) have been calculated using various curve-fitting programs. One curve-fitting approach was 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 mid-point is determined (see Deslypere et al. 1992; Gaido et al., 1997). Asymptotic standard errors of the parameter estimates are employed to perform two-sided “t” tests.

The *in vitro* AR TA assays produce measures of enzyme activity. The values obtained depend on a number of factors, such as the specific assay system used, the binding affinity of the test substance for the AR, the AR concentration, and the experimental conditions (e.g., pH, exposure duration). Because different investigators have reported their data in many different formats, comparison of data between studies has proven to be difficult. The EC<sub>50</sub> values that have been reported cover approximately seven orders of magnitude. However, there is no current guidance as to which levels of activity are biologically meaningful.

The primary focus of *in vitro* AR TA studies conducted prior to the mid-1990s was on mechanisms. However, by the year 2000, the majority of *in vitro* AR TA assay-related publications focused on the testing of industrial chemicals and environmental contaminants. Currently, there are no standardized *in vitro* AR TA assays for the routine testing of substances for AR agonist or antagonist activity. The *in vitro* AR TA assays, as currently performed, are described in detail in **Section 2.0**.

### **1.2.3 Mechanistic Basis of *In Vitro* AR TA Assays**

Transcriptional activation is one step in a series of events that is used to control gene expression in an androgen responsive cell. The AR is the primary receptor for endogenous androgens that enter the cell from the bloodstream to initiate the transcription of mRNA and ultimately protein synthesis. The interaction of androgens with the AR in a cell initiates a cascade of events. Upon ligand binding, the AR undergoes a conformational change that allows the recruitment of co-activator proteins. The ligand-bound AR complex dimerizes and binds, to an ARE located upstream from the genes under androgen control or within intron regions. Coactivator molecules also participate in the transcriptional activation of the responsive genes but whether these co-activators bind to the AR before or after the dimer has bound to DNA is not known with certainty. This binding initiates or inhibits the transcription of androgen-controlled genes, which leads to the initiation or inhibition of cellular processes, respectively, including those necessary for cell proliferation or adult homeostasis.



The AR, a transcriptional regulatory protein belonging to the nuclear hormone receptor superfamily, is involved in steroid hormone signaling, functioning as a ligand-dependent transcriptional activator. The AR protein plays a major role in controlling the TA and/or repression of androgen-responsive genes (Culig et al., 2000). The human AR gene was cloned and sequenced by Lubahn et al. (1988a, b) and Chang et al. (1988). It is located on the long arm of the X-chromosome as a single copy and encodes a protein of 110-114 kD (Lubahn et al. 1998a; Brown et al., 1989; Tilley et al., 1989). The human AR contains 919 amino acids and is localized in the soluble nuclear fraction of androgen target cells. The AR contains two discrete domains that are necessary for its role as a transcription factor -- a DNA-binding domain in the center of the protein, and a ligand-binding domain in the C-terminal region of the protein (Lamb et al, 2001). The DNA-binding domain contains two zinc finger motifs, which are associated with DNA-binding activity. AR isolated from different rat tissues is identical in structure and function (Wilson and French, 1976).

Three major areas of the AR are involved in the receptor's TA function, and are considered essential for this biological activity. Two transactivation domains (AF-1 and AF-5) are located in the NH<sub>2</sub>-terminal domain (Brinkmann et al., 1999; Zhou et al., 1994; Simental et al., 1991) and one (AF-2) is located in the COOH-domain of the receptor in the ligand-binding domain. The AF-1 and AF-2 functions are ligand dependent.

The precise mechanism underlying the action of AR antagonists, which bind to the AR without initiating transcription, is not known, but is under investigation. Androgen antagonists can induce inactive allosteric conformations of the AR that are different from the conformation induced by agonists. These conformational changes prevent activation of the transactivation function (AF-2) in the ligand-binding domain. Mutations in the AF-2 activation domain have been shown to reduce the activation function without affecting the capacity for ligand binding (Brinkmann et al., 1999; Kempainen and Wilson, 1996).

Some AR ligands display agonist and antagonist activity depending on the concentration of the test substance and the presence of the reference androgen competitor. For example, in one particular AR TA assay, hydroxyflutamide acts as an agonist at higher concentrations (10  $\mu$ M)

but as an antagonist at lower concentrations (1  $\mu\text{M}$ ). Similarly, the vinclozolin metabolite, M2, is an agonist at 10  $\mu\text{M}$  and an antagonist at 0.2  $\mu\text{M}$  (Wilson et al., 2002). Wong et al. (1995) proposed that mixed-ligand dimers of DHT-AR and test substance-AR could explain the dual agonist/antagonist activity of some AR ligands.

The current hypothesis for AR-mediated endocrine disruption is that certain xenobiotic substances that are similar in structure or conformation to DHT, the highest affinity natural ligand for the AR, may mimic or block its activity. The former action would produce an androgen-like effect while the latter would interfere with normal, physiological, androgen-mediated processes. In some cases, antagonists might not bind directly to the AR but rather inhibit the interaction of an activated receptor with coactivators required for transcriptional activation.

Agonist or antagonist activity may be inferred for a substance by its ability to activate or inhibit AR transcriptional activation *in vitro*. *In vitro* AR TA assays have been proposed as predictors of androgen disruption in intact organisms (U.S. EPA 1997; 1998a,b; 1999). The validity of the TA assay results for this purpose will require a determination that the substance also elicits similar responses *in vivo*. Kelce et al. (1995) and Lambright et al. (2000) have reported such concordance for a few chemicals.

Since transcriptional activation cannot occur unless an agonist first binds to the AR, factors that affect binding also have an impact on this process. These factors include:

- *Affinity for the AR*. The affinity depends on the rates of the association and disassociation of the ligand with the receptor. Although the association and disassociation rates of a natural ligand, DHT, have been studied in rat prostate cytosol (Wilson and French, 1976), little is known of these rates in the artificial mammalian cell systems used to study AR-induced transcriptional activation.
- *Half-life of the ligand*. The *in vivo* half-life will depend on the rate of metabolism of the substance or to an inactive product, and to the clearance of the ligand and its metabolites from the organism. *In vitro*, the half-life will depend on the metabolic capacity of the

different cell lines used for *in vitro* AR TA assays. The half-life of the test substance can also be altered by components in the cell culture medium.

#### **1.2.4 Relationship of Mechanisms of Action in the *In Vitro* AR TA Assay Compared to the Species of Interest**

The AR ligand binding domain is highly conserved among vertebrate species; thus, substances that activate or inhibit AR-induced transcriptional activation in one species are expected to have the same activity in other vertebrate species. However, because of differences in the types and rates of the associated substances that interact with the receptor-ligand complex, the relative activity of a substance may vary in different tissues of the same animal, and among different species.

Due to a lack of information on interspecies comparisons, the present working hypothesis is that androgen-induced biological effects in one vertebrate species are expected to occur in other species. This hypothesis is the basis for the use of *in vitro* AR TA assays as a general screen for androgenic effects. The most widely used *in vitro* assay systems use human or primate cells, with AR derived from humans. Substances that bind the AR in these cells and initiate or inhibit transcriptional activation of AR responsive genes are presumed to be capable of producing androgenic effects in multiple species. However, studies to support this working hypothesis are yet to be conducted.

### **1.3 Intended Uses of the Proposed *In Vitro* AR TA Assays**

*In vitro* AR TA assays are proposed as components of the EDSP Tier 1 screening battery. The Tier 1 screening battery is comprised of multiple *in vitro* and *in vivo* assays designed to assess both receptor- and non-receptor-mediated mechanisms of action and endpoints. This battery is designed to detect substances that might affect estrogen, androgen, and thyroid hormone systems in multiple species, including human.

#### **1.3.1 Validation of *In Vitro* AR TA Assays**

The FQPA requires the U.S. EPA to develop its endocrine screening program using validated test systems, and that the assays selected for inclusion in endocrine screening be standardized prior to

their adoption. The ICCVAM Authorization Act (Sec. 4(c)) mandates that “[e]ach Federal Agency ... shall ensure that any new or revised ... test method ... is determined to be valid for its proposed use prior to requiring, recommending, or encouraging [its use]” (P.L. 106-545, 2000). The validation process will provide data and information that will allow the U.S. EPA to develop guidance on the development and use of functionally equivalent assays and endpoints prior to the implementation of the screening program.

Validation is the process by which the reliability and relevance of an assay for a specific purpose are established (ICCVAM, 1997). Relevance is defined as the extent to which an assay will correctly predict or measure the biological effect of interest (ICCVAM, 1997). For *in vitro* AR TA assays, relevance is restricted to how well an assay identifies substances that are capable of activating or inhibiting transcription of androgen-inducible genes. The reliability of an assay is defined as its reproducibility within and among laboratories and should be based on a diverse set of substances representative of the types and range of responses expected to be identified.

The first stage in assessing the validation status of an assay is the preparation of a BRD that presents and evaluates the relevant data and information about the assay, including its mechanistic basis, proposed uses, reliability, and performance characteristics (ICCVAM, 1997). This BRD summarizes the available information on the various types of *in vitro* AR TA assays that have been commonly used to characterize substances as potential endocrine disruptors. Where appropriate data are available, the qualitative and quantitative performance of the assays are evaluated and the reliability of each assay is compared with the reliability of the other assays. These evaluations are used to determine whether a specific assay or assay type (e.g., mammalian cell-based assay or yeast-based assay using stably or transiently transfected AR and reporter genes) have been validated sufficiently to allow its recommendation for adoption by the U.S. EPA as an EDSP Tier 1 assay. If there are insufficient data to support the recommendation of an assay, this BRD will aid in identifying which specific assays should undergo further development or validation. The analyses can also be used to identify minimum procedural standards for current and future *in vitro* AR TA assays.

### **1.3.2 Where Can *In Vitro* AR TA Assays Substitute, Replace, or Complement Existing Methods?**

There are no *in vitro* assays for AR binding or TA that are currently accepted by regulatory agencies as validated assays. The *in vitro* AR TA assays are intended, along with other *in vitro* and *in vivo* tests, to be a component of the proposed EDSP Tier 1 screening battery for identifying endocrine disruptors.

### **1.3.3 Similarities and Differences with Currently Used Methods**

The measurement of AR TA activity *in vitro* is not currently required for regulatory decision-making. However, there are a number of *in vitro* assays available for assessing the ability of test substances to induce AR-dependent TA. These assays are based on the same general principles, but often use different cell lines, AR sources, and protocols.

The most frequently used *in vitro* AR TA assays use mammalian cell lines that are transiently or stably transfected with vectors encoding hAR and a reporter enzyme, typically luciferase. To test the potential agonism of a substance, TA is measured as the amount of reporter gene product (e.g., luciferase activity) induced by the test substance. Antagonism of a test substance is quantified by measuring the reduction of enzyme activity that occurs when the test substance and reference androgen are incubated together.

### **1.3.4 Role of *In Vitro* AR TA Assays in Hazard Assessment**

The *in vitro* AR TA assays are proposed as a component of the EDSP Tier 1 screening battery that also includes androgen, estrogen and thyroid receptor binding assays, *in vitro* ER TA assays, and *in vivo* assays for endocrine effects in rodents, amphibians, and fish. EDSTAC recognized that TA assays provide more information than binding assays because they also measure the consequences of binding. However, the limited databases at that time did not allow a determination of whether assays that measured binding or TA or both were preferred for screening (U.S. EPA, 1998a). Subsequently, the EDSP expressed a preference for TA assays over receptor binding assays because these assays can distinguish agonists from antagonists, and can be conducted with and without exogenous metabolic activation (U.S. EPA, 1999).

The assays in the Tier 1 screening battery have been combined in a manner such that limitations of one assay are complemented by strengths of another. The *in vitro* assays measure the interactions between the test substance and binding and/or TA process, and might produce results that are not biologically meaningful *in vivo* as a result of limited absorption and distribution, or rapid metabolism and excretion of the substance. The *in vitro* assays may also produce false negative results due to the absence of active metabolites that are formed *in vivo*, or to endocrine-related effects that are mediated by mechanisms not addressed by the *in vitro* assays.

A positive result in an *in vitro* AR TA assay (or in any Tier 1 screening assay) is not, in itself, sufficient to make the determination that a substance would produce a hormone-related adverse health effect in humans or other species. A weight-of-evidence approach will be used to evaluate the battery of Tier 1 results and to make decisions about whether or not a test substance would be subject to Tier 2 testing (U.S. EPA, 1998b). The Tier 2 assays are all performed *in vivo* and were selected to determine if a substance identified in Tier 1 as a potential endocrine disruptor exhibits endocrine-mediated adverse effects in animals and to identify, characterize, and quantify these effects.

### **1.3.5 Intended Range of Substances Amenable to the *In Vitro* AR TA Assay and/or Limits of the *In Vitro* AR TA Assay**

The range of substances amenable to testing in *in vitro* AR TA assays has yet to be determined and will depend on the outcome of an independent peer review of the assays considered in this BRD and any future validation studies. The *in vitro* AR TA assay is intended to be used to test food components and contaminants, as described in the FQPA (P.L. 104-170), and water contaminants, as described in the 1996 Amendments to the SDWA (P.L. 104-182). In addition, the U.S. EPA has authority to test commercial substances regulated by the Toxic Substances Control Act (TSCA, 1976) under the following three circumstances: 1) the SDWA provides for testing of TSCA substances present in drinking water; 2) the FQPA amendments and the Federal Food Drug and Cosmetic Act (FFDCA; P.L. 105-115, 1997) provide for testing of “inerts” in pesticide formulations; and 3) the FQPA and FFDCA provide for testing of substances that “act cumulative to a pesticide.”

#### 1.4 Search Strategy and Selection of Citations for the *In Vitro* AR TA BRD

The *in vitro* AR TA assay data summarized in this BRD are based on information found in the peer-reviewed scientific literature. An online literature search was conducted for entries in MEDLINE, CANCERLIT, TOXLINE, AGRICOLA, NIOSHTIC, EMBASE, CABA, BIOSIS, and LifeSci that reported on the *in vitro* testing of substances for endocrine disrupting effects. The search was conducted in the database basic index, which includes words in the title and abstract, and indexing words. Specifically, records on androgen TA assays were sought. The search strategy involved the combining of “*vitro*” with alternative terms for estrogens, androgens, receptors, binding, transcription, activation, and testing. Each database record included authors, bibliographic citation, and indexing terms. Most records also included abstracts.

The initial search identified 105 records related to androgen binding and TA assays. These abstracts were reviewed and full text copies of articles judged to be relevant were obtained and a database of the literature citations was established. Since the initial search, additional articles with relevant information have been found and retrieved, many of which were identified from the bibliographies of the previously selected articles. Scanning of the literature using *Current Contents* and the British Lending Library’s *Table of Contents* continued through the writing of the BRD, and recently published articles were added to the database as they became available. Identification of AR TA-related publications for data extraction ended on January 25, 2002.

The most relevant reports were those containing data on substances that have been tested in more than one laboratory using identical or related protocols. Every effort was made to include data from these publications because they provided information that could contribute to the assessment of the performance and reliability of the different assays. Because relatively few test substances have been evaluated in *in vitro* AR TA assays, data were extracted from some reports of studies that tested obscure compounds, such as structural or positional isomers of known binding agents, if the compounds had been tested in a commonly used protocol. In addition, data were extracted from some reports of studies using unique procedures if the study included substances that had been tested in one of the more commonly used assays. Of the publications identified, 26 contained data that have been abstracted and included in this BRD. In addition, the

BRD contains data from one unpublished report that was submitted to NICEATM for consideration in this review of *in vitro* AR TA assays.



## **2.0 METHODS FOR *IN VITRO* AR TA ASSAYS**

### **2.1 Introduction**

There are no standardized methods for performing AR TA assays. The majority of published AR TA studies were conducted to investigate the process of AR-induced transcriptional activation or to identify structure-activity relationships; relatively few studies were designed to assess the ability of a test substance to act as an AR agonist or antagonist. Furthermore, very few studies have been conducted using the same cell line, AR construct, and reporter gene construct (**Table 2-1**). The 27 reports reviewed in this BRD (26 peer-reviewed publications and one submitted report containing unpublished data) described studies using yeast (*Saccharomyces cerevisiae*), nine different mammalian cell lines, and one fish cell line (EPC). The mammalian cell lines used included six human (HepG2, HeLa, LnCaP-FGC, MDA-MB-453 and its derivative MDA-MB-453-kb2, PC-3 and its derivative PALM), two monkey (CV-1, COS-1), and one using Chinese hamster ovary (CHO) cells. The majority of published AR TA studies used cells that were transiently transfected with the AR. Thus, new transiently transfected cells were produced for each experiment, and the sensitivity and/or responsiveness of each batch of transfected cells were determined by the characteristics of the cell line and the constructs used, and by the efficiency of transfection. Less frequently used were cells stably transfected with a plasmid containing the gene coding for the AR (HeLa, PALM, CHO, yeast), or those containing an endogenous AR (LnCaP-FGC, MDA-MB-453). The human AR (hAR) was used in all but two of the studies included in this BRD; these two studies used cells transfected with trout and mouse AR.

All but one of the AR TA assays considered in this BRD used a reporter gene to assess TA. The non-reporter gene based test method used the induction of cell proliferation as an indicator of transcriptional activation (Sonnenschein et al., 1989). In assays that use transiently transfected cells, the cells are transfected with an expression plasmid and/or a reporter plasmid. The plasmid known as the expression construct contains the AR that is under the control of a viral promoter gene (often from SV-40).

**Table 2-1 Cell Lines, Plasmids, and Reference Androgens Used in *In Vitro* AR TA Assays**

Cell Line	Species	Tissue or Strain	AR*	AR Plasmid <sup>a</sup>	Reporter Plasmid <sup>a</sup>	Other Plasmids	Reporter gene	# Sub. Tested	Ref. Agonist	Ref. Antag.	Reference
CHO	Chinese hamster	Ovary	h	hAR(stable)	MMTV- <i>Luc</i> (stable)		luciferase	9	DHT		Deckers et al. (2000)
CHO	Chinese hamster	Ovary	h	pZeoSV2AR (stable)	pIND ARE B10- <i>Luc</i> (stable)		luciferase	12	DHT	DHT	Otsuka Pharmaceutical Co. (2001)
CHO	Chinese hamster	Ovary	h	pCMV3.1.hAR (transient)	MMTV-CAT (transient)	SV40-pCH110 (-gal)	CAT	9	T		Deslypere et al. (1992)
CHO	Chinese hamster	Ovary	h	pSVAR0 (transient)	MMTV- <i>Luc</i> (transient)		luciferase	11	R1881	R1881	Vinggaard et al. (1999)
CHO	Chinese hamster	Ovary	h	pSVAR0 (transient)	MMTV- <i>Luc</i> (transient)		luciferase	10	R1881	R1881	Vinggaard et al. (2000)
CHO	Chinese hamster	Ovary	h	pSVAR0 (transient)	MMTV- <i>Luc</i> (transient)		luciferase	4	R1881	R1881	Bonefeld-Jorgenson et al. (2001)
CHO	Chinese hamster	Ovary	h	pZeoSV2AR (transient)	pIND ARE B10- <i>Luc</i> (transient)	p-EGFP	luciferase	65		DHT	Otsuka Pharmaceutical Co. (2001)
CV-1	Monkey	Kidney	h	Ad5 hAR (transduced)	MMTV- <i>Luc</i> (transduced)		luciferase	9	DHT	DHT	Hartig et al. (2002)
CV-1	Monkey	Kidney	h	pCMVhAR (transient)	MMTV- <i>Luc</i> (transient)		luciferase	9		DHT	Kelce et al. (1995)
CV-1	Monkey	Kidney	h	pCMVhAR (transient)	MMTV- <i>Luc</i> (transient)		luciferase	5	DHT	DHT	Kemppainen and Wilson (1996)
CV-1	Monkey	Kidney	h	pCMVhAR (transient)	pMTV29VTM (transient)		CAT	9	R1881	R1881	Kemppainen et al. (1992)
CV-1	Monkey	Kidney	h	pCMVhAR (transient)	MMTV- <i>Luc</i> (transient)		luciferase	12	DHT	DHT	Kemppainen et al. (1999)
CV-1	Monkey	Kidney	h	pCMVhAR (transient)	MMTV- <i>Luc</i> (transient)		luciferase	2		DHT	Lambright et al. (2000)

Cell Line	Species	Tissue or Strain	AR*	AR Plasmid <sup>a</sup>	Reporter Plasmid <sup>a</sup>	Other Plasmids	Reporter gene	# Sub. Tested	Ref. Agonist	Ref. Antag.	Reference
CV-1	Monkey	Kidney	mo	Not provided (transient)	pSV2-CAT (transient)		CAT	3	DHT		Van Dort et al. (2000)
EPC	Carp	Skin tumor	tr	pCMV-rtAR- (transient)	pARE3TK-CAT (transient)		CAT	8			Takeo and Yamashita (2000)
HeLa (E19)	Human	Cervical cancer	h	pTetCMV-F0(S)-AR (stable)	MMTV- <i>Luc</i> (transient)		luciferase	4	T		Wang and Fondell (2001)
HeLa (E19)	Human	Cervical cancer	h	pTetCMV-F0(S)-AR (stable)	ARE2-DS- <i>Luc</i> (transient)		luciferase	4	T		Wang and Fondell (2001)
HeLa (E19)	Human	Cervical cancer	h	pTetCMV-F0(S)-AR (stable)	PB(-285/+32) <i>Luc</i> (transient)		luciferase	1			Wang and Fondell (2001)
HepG2	Human	Hepatoma	h	pRSAR (transient)	MMTV- <i>Luc</i> (transient)	pCMV-gal	luciferase	15		DHT	Gaido et al. (2000)
HepG2	Human	Hepatoma	h	pRSAR (transient)	MMTV- <i>Luc</i> (transient)	pCMV-gal	luciferase	14	DHT	DHT	Maness et al. (1998)
HepG2	Human	Hepatoma	h	pRSAR (transient)	MMTV- <i>Luc</i> (transient)	pCMV-gal	luciferase	2	DHT	DHT	Tamura et al. (2001)
LnCaP-FGC	Human	Metastacized prostate adeno-carcinoma	h	Endogenous AR	NA**		Cell growth	16	T		Sonnenschein et al. (1989)
MDA-MB-453	Human	Breast carcinoma	h	Endogenous AR	Ad/m <i>Luc7</i> (transduced)		luciferase	8	DHT	DHT	Hartig et al. (2002)
MDA-MB-453-kb2	Human	Breast carcinoma	h	Endogenous AR	MMTV- <i>Luc</i> (stable)		luciferase	2		DHT	Lambright et al. (2000)
MDA-MB-453-kb2	Human	Breast carcinoma	h	Endogenous AR	MMTV- <i>neo-Luc</i> (stable)		luciferase	13		DHT	Wilson et al. (2002)

Cell Line	Species	Tissue or Strain	AR*	AR Plasmid <sup>a</sup>	Reporter Plasmid <sup>a</sup>	Other Plasmids	Reporter gene	# Sub. Tested	Ref. Agonist	Ref. Antag.	Reference
PALM (PC-3)	Human	Prostate adenocarcinoma	h	PSG <sub>5</sub> -puro-hAR (stable)	MMTV- <i>neo-Luc</i> (stable)		luciferase	12		R1881	Sultan et al. (2001)
PALM (PC-3)	Human	Prostate adenocarcinoma	h	PSG <sub>5</sub> -puro-hAR (stable)	pMMTV- <i>neo-Luc</i> (stable)		luciferase	17	M	M	Terouanne et al. (2000)
PALM (PC-3)	Human	Prostate adenocarcinoma	h	pCMV5-hAR (stable)	MMTV. pMAM. <i>neo.Luc</i> (stable)		luciferase	21	DHT	DHT	Schrader and Cooke (2000)
PC-3	Human	Prostate adenocarcinoma	h	PSG <sub>5</sub> -puro-hAR (transient)	MAM- <i>neo-Luc</i> (transient)	pCMV- <i>-gal</i>	luciferase	4			Terouanne et al. (2000)
Yeast	<i>S. cerevisiae</i>	Not provided	h	Not provided (stable)	LacZ (stable)		-gal	8	DHT	DHT	Moffat et al. (2001)
Yeast	<i>S. cerevisiae</i>	YPH500	h	CUP1-Met hAR (stable)	Not provided (stable)		-gal	7	DHT	DHT	O'Connor et al. (1998)
Yeast	<i>S. cerevisiae</i>	YPH500	h	CUP1-Met hAR (stable)	Not provided (stable)		-gal	4	DHT	DHT	O'Connor et al. (1999)
Yeast	<i>S. cerevisiae</i>	YPH500	h	CUP1-Met hAR (stable)	Not provided (stable)		-gal	5	DHT	DHT	O'Connor et al. (2000)
Yeast	<i>S. cerevisiae</i>	YPH500	h	CUP1-Met hAR (stable)	YRPGALE1 (stable)		-gal	22	DHT		Gaido et al. (1997)

Abbreviations: Antag. = Antagonist, DHT = 5 $\alpha$ -Dihydrotestosterone, -gal = beta-Galactosidase, M = Mibolerone, Ref. = Reference, R1881 = Methyltrienolone, Sub. = Substances, T = Testosterone.

<sup>a</sup>Stable, transient, and transduced in parenthesis indicate whether the plasmid was stably or transiently integrated, or transduced with adenovirus, respectively, into the cell.

\*AR refers to the source of the androgen receptor; h = human, mo = mouse, and tr = trout.

\*\*NA refers to the fact that cell proliferation was measured in this assay, and thus no reporter construct was required.

\*\*\* Not all publications included information on the precise composition of the various vectors.

The reporter plasmid contains the hormone responsive elements (HRE) controlling the expression of a reporter gene, usually luciferase (*Luc*), chloramphenicol acetyltransferase (*CAT*) or, in yeast,  $\beta$ -galactosidase ( $\beta$ -*gal*). Since the HRE sequence is contained within the mouse mammary tumor virus long terminal repeat, this DNA sequence is frequently used as the source of the HRE. In AR TA assays that use stably transfected cells, the cells can contain the stably integrated expression plasmid only or both the expression and the reporter plasmids. In the former case, such cells are transiently transfected with the reporter plasmid. Using either transient or stably transfected cells, when the transfected cells are exposed to a substance that interacts with the AR, the AR becomes activated by a change in its conformation. The activated AR binds with soluble cell factors, and then the complex binds to the AR response elements on the second plasmid. This binding initiates the expression of the reporter gene and the production of its associated enzyme. An appropriate substrate added to the incubation mixture is metabolized by the enzyme resulting in the production of an easily detected product. The majority of AR TA studies use luciferase to assess transcriptional activation because its use makes the assay more rapid, more sensitive, and easier to perform than CAT-based assays. Also, in contrast to luciferase-based assays, CAT-based assays require a radiolabeled substrate (either chloramphenicol or acetyl-CoA).

Cytotoxicity can be a complicating factor in AR TA assays, particularly when antagonism is being assessed. The absence of or a decrease in the AR transcriptional activation response might be the result of cell toxicity rather than reflecting the ability of the test substance to interact with the AR. Cell toxicity can be corrected for by performing a parallel cytotoxicity experiment or by measuring the product of a constitutively active gene transfected into the cell on a separate plasmid. Some of the mammalian cell lines transfected with *Luc* or *CAT* reporter constructs have also been transfected with a plasmid coding for the  $\beta$ -*gal* gene. The synthesis of  $\beta$ -galactosidase is independent of a receptor-mediated effect, and a comparison of its level in treated versus control cells can be used as a measure of treatment-related cell toxicity.

In studies to measure agonism, the cells are treated with a test substance and the induction of luciferase, CAT, or  $\beta$ -galactosidase measured. To assess relative potency, the response obtained with the test substance can be compared with the response obtained when the cells are treated

with a reference androgen (e.g., DHT, R1881). In studies to determine antagonism, the cells are treated simultaneously with the test substance and the reference androgen and the ability of the test substance to inhibit transcriptional activation is measured.

Because there are no “consensus” cell lines, vectors, or specific treatment protocols for AR TA studies, the following sections describe general protocols for agonism and antagonism studies using mammalian or yeast cells transfected with a reporter gene, and mammalian cells using growth as an endpoint.

## **2.2 Mammalian Cell AR TA Reporter Gene Assays**

### **2.2.1 Expression and Reporter Gene Constructs**

For transfection into mammalian cells, the recombinant plasmid is constructed by ligating the cDNA of the AR gene into a eukaryotic expression vector that contains the viral early gene promoter SV-40, the human growth hormone transcription termination and polyadenylation signals, the SV-40 origin of replication, and an antibiotic resistance gene for selection. An alternative to the SV-40 gene used in some studies is the CMV early gene promoter. Also, a number of genes with different termination and polyadenylation signals have been used in the various expression constructs used in AR TA studies.

The *Luc* reporter plasmid contains the *Luc* gene regulated by the glucocorticoid-inducible HRE found in the mammary mouse tumor virus long terminal repeat. The *CAT* reporter plasmid pMTV29VTM contains two glucocorticoid response elements separated by 29 base pairs and positioned 5' to the *CAT* gene. It is important to note that the MMTV promoter sometimes used in the reporter plasmid, can be regulated by the GR and progesterone receptor (PR) and that certain compounds may interact with the AR and also with either the PR or GR. Unless the investigator is cognizant of this possible cross reactivity, the data obtained with certain substances may not truly reflect AR-induced transcriptional activation. These potential interferences can be compensated for by adding a specific chemical that blocks the activation of the GR or PR.

### 2.2.2 Stably and Transiently Transfected Cell Lines

The majority of AR TA studies considered for this BRD used transiently transfected cells, despite the fact that a new batch of transfected cells must be produced for each new experiment. Transfection is performed by exposing the cells to both plasmids in the presence of, for example, calcium phosphate, DEAE dextran, or a lipofection agent such as FuGene<sup>TM</sup>. These substances increase cell membrane permeability, allowing for the passive uptake of the plasmids by the cells. These foreign DNAs are typically rejected by the cell within three to seven days after transfection. In cells that harbor an endogenous or stably transfected AR, only the reporter construct and perhaps the construct to assess cytotoxicity is transfected. The transfected cell lines that have both constructs either stably incorporated into their genome or as stable plasmids in the cell are easier to use since they do not require genetic manipulation before performing the assay.

### 2.2.3 *In Vitro* Mammalian Cell AR TA Assays with a Reporter Gene

Mammalian cells at the recommended density for the particular cell line are seeded into culture dishes or wells of microtiter plates and cultured for 18 to 24 hours at 37°C. The cells are transfected with the appropriate plasmids using either calcium phosphate, DEAE dextran, or a lipofection agent. After attachment for 4-24 hours at 37°C to express the AR, the cells are treated with the test substance dissolved in the culture medium or other appropriate solvent, such as absolute ethanol or dimethyl sulfoxide (DMSO). The cells are incubated for 24 to 48 hours at 37°C. The medium is aspirated, the cells are washed with an appropriate buffer and then lysed with the same buffer containing MgCl<sub>2</sub>, Triton X 100, and dithiothreitol, or other agents appropriate to the reporter construct used. After 15 minutes at room temperature and centrifugation, if necessary, for a short time to sediment cell debris, an aliquot of the supernatant is removed to measure induction of the reporter gene product. For the induction of luciferase, adenosine triphosphate (ATP) and coenzyme A are added in glycylglycine buffer to the cell lysate in a microtiter plate. Luciferin is added to start the reaction and the luminescence is measured using a microtiter plate luminometer. The data are expressed in relative light units. For the induction of CAT, an aliquot of the lysed cells is incubated with radiolabeled chloramphenicol and acetyl coenzyme A (Gorman et al., 1982). The extracts are incubated for 30 minutes at 37°C with samples removed at various time points. The reaction is stopped with

ethyl acetate, which is used to extract the acetylated chloramphenicol. The organic phase is dried, redissolved in ethyl acetate, and spotted on silica gel plates. The radioactive acetylated product is separated from the parent chloramphenicol using thin layer chromatography. The radioactive spots are located following autoradiography of the plates for 18 hours, cut out, and counted in a scintillation counter. When  $\beta$ -galactosidase is used as a measure of toxicity, the enzyme activity is measured using *o*-nitrophenyl-  $\beta$ -galactoside (ONPG) as the substrate. Following hydrolysis of ONPG by  $\beta$ -galactosidase, the intensity of the yellow product is measured using a spectrophotometer.

In agonism studies, the cells are treated with a test substance and the induction of the reporter gene and its associated product are used to indicate a positive response. To assess relative potency, the maximal fold-increase induced by the test substance can be compared with that induced by the reference androgen or, where dose-response data are generated, EC<sub>50</sub> for the test substance and the reference androgen can each be calculated and compared. A reference androgen (e.g., DHT, R1881) is included not only for an assessment of relative potency but also to demonstrate the adequacy of the test system. For antagonism studies, the cells are exposed simultaneously to the reference androgen and the test substance while control cells are exposed to the reference androgen only. The difference in induction of the reporter gene product in the presence and absence of the test substance is used as a measure of antagonism.

### **2.3 Yeast Cell AR TA Reporter Gene Assays**

#### **2.3.1 Expression and Reporter Gene Constructs**

The yeast expression plasmid contains the CUP metallothionein promoter fused to the cDNA of hAR. The reporter plasmid carries two copies of the androgen response element upstream of  $\beta$ -gal.

#### **2.3.2 Yeast Cell AR TA Assays with a Reporter Gene**

In the yeast assay, various strains of *S. cerevisiae* with a stably transfected hAR and a construct containing the  $\beta$ -gal reporter gene are grown overnight at 30°C in an orbital shaker in a selective medium containing a yeast nitrogen base and ammonium sulfate. The next day, an aliquot of the overnight culture is grown to mid-log phase. This suspension is diluted and the test substance



dissolved in medium, ethanol, or DMSO is added. As the hAR in these cells is linked to a copper metallothionein promoter, copper sulfate (CuSO<sub>4</sub>) is added to the yeast to induce receptor production. The cells are incubated overnight at 30°C with vigorous shaking and the optical density (OD) is read at 600 nm to assess cell growth or toxicity. A diluted aliquot of the cells is pipetted into a microtiter plate. Assay buffer containing OPNG and a lysing solution containing sodium dodecyl sulfate (SDS), mercaptoethanol, and oxalyticase is added to the cells. The increase in production of *o*-nitrophenol by the induced  $\beta$ -galactosidase is measured at 420 nm using a microtiter plate reader. The OD is also measured at 550 nm to correct for colorimetric distortion due to debris.  $\beta$ -Galactosidase activity is calculated according to the Miller equation,

$$\text{Miller Units (A}_{420}\text{/min/mL cells/OD}_{600}) = 1000 \times \frac{\text{OD}_{420} - (1.75 \times \text{OD}_{550})}{T \times V \times \text{OD}_{600}}$$

where T = minutes of reaction time and V = volume of assay in mL.

In agonism studies, the yeast cells are treated with the test substances and the induction of  $\beta$ -galactosidase is measured. A positive response is indicated by a dose-related increase in the induction of  $\beta$ -galactosidase. For an assessment of relative potency, the induction may be compared to the results from a reference androgen. For antagonism studies, the cells are exposed simultaneously to the reference androgen and the test substance; control cells are exposed to the reference androgen only. The difference in  $\beta$ -galactosidase activity in the presence and absence of the test substance is used as a measure of androgen antagonism.

#### **2.4 *In Vitro* Mammalian Cell AR TA Assays using Growth as an Endpoint**

Mammalian cells (LnCaP) containing an endogenous hAR are seeded in 12-well plates in the presence of 5% fetal bovine serum and grown for 48 to 72 hours at 37°C to allow the cells to attach to the plastic surface (Sonnenschein et al., 1989). The medium is removed and replaced with fresh medium containing human serum that has been charcoal stripped to remove contaminating hormones. Various concentrations of the test substance are added and the cells are grown for seven days at 37°C. A number of procedures can be used to quantitate total cell

growth. For example, cell lysing solution is added to the wells and the cell nuclei are counted using a Coulter counter. The parameter of growth generally considered is relative proliferative potency (RPP). This parameter is calculated as the ratio (x100) between the concentration of the reference androgen and the test substance that was required to elicit a maximal cell yield after seeding 10,000 cells/well.

## **2.5 Reference Androgens**

The majority (21 of 31, 68%) of AR TA studies considered in this BRD used DHT as the reference androgen for agonist/antagonist studies. R1881 was used as the reference androgen in five AR TA studies (16%), testosterone in four studies (13%), and mibolerone in one study (3%).

### 3.0 CHARACTERIZATION OF SUBSTANCES TESTED IN *IN VITRO* AR TA ASSAYS

#### 3.1 Introduction

*In vitro* AR TA assay data were collected for a total of 145 substances that had been evaluated in assays that measured the ability of a substance to activate or inhibit transcription of androgen-inducible genes. As shown in **Table 3-1**, 68 of these substances had been tested for both agonism and antagonism, while 51 had been tested for agonism only, and 20 for antagonism only. Seventeen substances had been tested in a cell proliferation assay, and of these, two had also been tested for agonism, eight for both agonism and antagonism, one for antagonism and six for neither agonism nor antagonism.

**Table 3-1 Distribution of Substances by Type of *In Vitro* AR TA Assay**

Type of TA Assay	Number of Substances
Agonism and Antagonism	68
Agonism	51
Antagonism	20
Agonism and Cell Proliferation	2
Agonism, Antagonism and Cell Proliferation	8
Antagonism and Cell Proliferation	1
Cell proliferation	6

The data were obtained from 26 peer-reviewed, scientific journal articles and one report containing unpublished data. The majority of these studies used DHT as the reference androgen; however, other reference androgens including R1881 (five publications), testosterone (four publications), and mibolerone (one publication) were also used.

Relevant information on the substances tested (i.e., chemical name, Chemical Abstract Service Registry Number [CASRN], chemical supplier or source, and purity) was extracted from the publications and entered into a database. Some publications did not include all of this information. For publications in which only chemical structures were provided, every effort was made to identify the names and CASRN of the substances tested. CASRNs were obtained from various sources, including the National Library of Medicine's ChemID database and *The Merck*

*Index.* However, no attempt was made to determine the source and purity of test substances if this information was not provided in the publication. Different publications often used a unique chemical name for the same substance. When this occurred, the most commonly used chemical name was chosen and assigned to the substance, regardless of the chemical name used in a particular publication, and the unique chemical nomenclature was entered into the database as a synonym (**Appendix C**).

### **3.2 Rationale for Selection of Substances/Products Tested in *In Vitro* AR TA Assays**

Many of the substances tested in *in vitro* AR TA assays were selected to address basic research questions regarding the nature of AR binding and transcriptional activation processes. Mechanistic studies investigating the steps involved in AR activation or inhibition of target genes used both naturally-occurring steroids (e.g., DHT, testosterone, androstenedione, and 17 $\beta$ -estradiol) and synthetic AR agonists and antagonists (e.g., oxandrolone, fluoxymesterone, hydroxyflutamide, and cyproterone acetate). Some of these substances, particularly the natural androgens and synthetic anti-androgens, were studied to obtain a better understanding of their different potencies and biological activities. Some of the synthetic anti-androgens (e.g., hydroxyflutamide and bicalutamide) have been investigated in AR TA studies to evaluate their mechanisms of action as therapeutic agents, and to determine why some of these substances have both agonist and antagonist activities. In addition, some substances were investigated to determine which derivative (e.g., norethisterone and 11-ketonorethisterone) or metabolites of a specific substance (e.g., DDE and dihydroxy-DDE) enhanced or inhibited AR-induced transcriptional activation.

During the last decade, with the growing concern about endocrine disruptors, some of these substances (e.g., vinclozolin and its major metabolites, *o,p'*-DDT and its major metabolites, atrazine, kepone, and linuron) were tested in AR TA assays to identify substances that may act as androgen agonists or antagonists in humans and wildlife. Most of the publications that reported AR TA assay data on industrial chemicals, pesticides, and environmental contaminants tested these substances to evaluate their potential to disrupt the endocrine system. Typically, these publications also reported on the utility of a particular AR TA assay as a screen for endocrine disruptor activity.

### 3.3 Chemical and Product Classes Tested

Chemical and product class information for the substances tested in *in vitro* AR TA assays is provided in **Appendix C**. Substances were assigned to chemical classes based on available information from standardized references (e.g., *The Merck Index*) and from an assessment of chemical structure. As shown in **Table 3-2**, the chemical classes that have been tested most extensively in *in vitro* AR TA assays are nonphenolic steroids, organochlorines, phenolic steroids, and polycyclic aromatic hydrocarbons. Of the 145 substances included in this BRD, 17 could be assigned to two chemical classes.

**Table 3-2 Chemical Classes Tested in *In Vitro* AR TA Assays**

Chemical Classes	Number of Substances
Alcohol	1
Alkyl sulfonate	1
Alkylphenol	4
Anilide	3
Aromatic amine	1
Azole	1
Benzophenone	1
Biphenyl	1
Bisphenol	2
Carboxylic acid	1
Coumarin	1
Dioxin	1
Diphenolalkane	2
Ether	3
Glucuronic acid	2
Imidazole	5
Imide	1
Indene	1
Isoflavone	1
Lactone	2
Nitrile	5

Chemical Classes	Number of Substances
Organochlorine	30
Organothiophosphate	2
Phenol	6
Phenyl ether	1
Phthalate	4
Polychlorinated biphenyl	3
Polycyclic aromatic hydrocarbon	10
Pyrethrin	4
Pyrimidine	2
Resorcylic acid lactone	1
Steroid, nonphenolic	35
Steroid, phenolic	12
Stilbene	4
Sulfonylurea	1
Triazine	1
Triphenylethylene	3
Urea	3

Product classes were assigned based on information from *The Merck Index* and the National Library of Medicine's ChemFinder. Only a few product classes are represented, as shown in **Table 3-3**. The most common product classes tested in *in vitro* AR TA assays are pharmaceuticals and pesticides. Of the substances included in this BRD, 21 had no known commercial use, so were not classified within a product class.

**Table 3-3 Product Classes Tested in *In Vitro* AR TA Assays**

<b>Product Classes</b>	<b>Number of Substances</b>
Adhesive	1
Buffer	1
Chemical intermediate	12
Coating	1
Dielectric fluid	3
Dye	1
Natural product	6
Pesticide (includes metabolites, derivatives, and degradation products)	45
Pharmaceutical (includes metabolites)	60
Plasticizer	3
Preservative	2
Unclassified	21

#### 4.0 REFERENCE DATA

AR TA assays measure the ability of a test substance to initiate or block transcription of a reporter gene or cell proliferation in an appropriate cell line. The ability of a test substance to activate or inhibit androgen-induced TA *in vitro* suggests, but does not demonstrate, the ability of the substance to act as an androgen agonist or antagonist *in vivo*.

The purpose of this BRD is to assess the performance of various *in vitro* AR TA assays with regard to their sensitivity for detecting weak AR agonists and antagonists and their reliability within and among laboratories and across procedures. No attempt is made to evaluate their performance with respect to other biological effects *in vivo*, such as growth promotion of male reproductive tissues. Such comparisons will be addressed elsewhere. Therefore, no reference data are included for measuring the biological relevance of AR TA assays.

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## 5.0 DATA ON AR TA ASSAYS

### 5.1 Introduction

*In vitro* AR TA assay data and methodology information were collected from 26 publications and one submitted unpublished report on substances that had been evaluated *in vitro* for their ability to act as an AR agonist and/or antagonist. Where provided, the specific information extracted for each tested substance includes its name, source, purity, methodological details, and relevant data. If available, a CASRN was identified for each substance. This identifier was obtained from various sources, including the source publication, the National Library of Medicine's ChemID database, and *The Merck Index*. Chemical name synonyms were collected for substances that were identified in the literature by more than one name, and for substances where the name used in the publication may have been different from the generic name. All substances with the same CASRN were listed under the same name, usually the common name, regardless of the name that was used in the original publication. No attempt was made to identify the source and purity of a substance if the investigators did not provide such information. **Appendix C** provides information on the names, synonyms, CASRN, and chemical/product class, if identified, for each substance. **Appendix D** contains the *in vitro* AR TA assay data, organized by substance name, CASRN, and assay.

### 5.2 Availability of Detailed *In Vitro* AR TA Protocols

The Methods sections in the *in vitro* AR TA publications and the unpublished report provided various levels of detail. To the extent possible, the most relevant method parameters were extracted from each source and summarized in **Appendix A**. Details about the following method parameters are included in the Appendix to the extent this information was available:

- *Characteristics of the cell line* (e.g., name of cell line, its source).
- *Transfection of cells with plasmids* (e.g., identify whether transfections were stable or transient, AR expression vector, AR source, reporter vector, endpoint measured, plasmid transfected for cell toxicity measurements, endpoint measured for cell toxicity).
- *Preparation of cells for assay* (e.g., growth of cells before transient transfection, plating time prior to treatment of cells with a test substance).

- *TA assay* (e.g., identify whether assay evaluated agonism and/or antagonism, test substance solvent, test substance exposure duration, reference androgen, number of replicates per experiment, and number of times assay was repeated).

### 5.3 Availability of *In Vitro* AR TA Assay Data

*In vitro* AR TA assay data were collected on a total of 145 substances tested in the following AR reporter gene and cell proliferation assay systems:

- CHO cells stably transfected with an expression vector encoding hAR, and a reporter vector encoding luciferase linked to the mouse mammary virus tumor promoter (CHO hAR(S)+Luc(S));
- CHO-K1 cells transiently transfected with an expression vector encoding hAR, and a reporter vector encoding luciferase linked to the mouse mammary virus tumor promoter and a third plasmid encoding (CHO-K1 hAR(T)+Luc(T)+EGFP(T));
- CHO-K1 cells stably transfected with an expression vector encoding hAR, and a reporter vector encoding luciferase linked to the mouse mammary virus tumor promoter (CHO-K1 hAR(S)+Luc(S));
- CHO cells transiently transfected with an expression vector encoding hAR, and a reporter vector encoding CAT linked to the mouse mammary virus tumor promoter and a third plasmid encoding -gal (CHO hAR(T)+CAT(T)+ -gal(T));
- CHO cells transiently transfected with an expression vector encoding hAR, and a reporter vector encoding luciferase linked to the mouse mammary virus tumor promoter (CHO hAR(T)+Luc(T));
- CV-1 monkey kidney cells transiently transfected with vectors containing hAR cDNA and luciferase cDNA linked to MMTV (CV-1 hAR(T)+Luc(T));
- CV-1 monkey kidney cells transiently transfected with vectors containing hAR cDNA and CAT cDNA linked to MMTV (CV-1 hAR(T)+CAT(T));
- CV-1 monkey kidney cells transiently transduced with vectors containing hAR cDNA and luciferase cDNA linked to MMTV (CV-1 hAR(T)+Luc(T)\*) (\* refers to being transduced);
- HeLa human tumor cells stably transfected with the vector containing hAR cDNA and transiently transfected with luciferase cDNA (HeLa hAR(S)+Luc(T));

- HepG2 human hepatoma cells transiently transfected with vectors containing hAR cDNA and luciferase cDNA and -gal (HepG2 hAR(T)+Luc(T)+ -gal(T));
- Human supraclavicular lymph node cells from prostate adenocarcinoma (LnCaP-FGC) containing an endogenous AR (LnCaP-FGC hAR(E)+CP);
- Human breast carcinoma cells containing endogenous AR and stably transfected with luciferase cDNA (MDA-MB-453-kb2 hAR(E)+Luc(S));
- Human breast carcinoma cells containing endogenous AR and transiently transduced with luciferase cDNA (MDA-MB-453 hAR(E)+Luc(T)\*) (\* refers to being transduced);
- Human prostate adenocarcinoma cells transiently transfected with vectors containing hAR cDNA and luciferase cDNA (PC-3 hAR(T)+Luc(T));
- Human prostate adenocarcinoma cells stably transfected with vectors containing hAR cDNA and luciferase cDNA (PALM hAR(S)+Luc(S));
- CV-1 monkey kidney cells transiently transfected with vectors encoding mouse AR and CAT (CV-1 mAR(T)+CAT(T));
- Carp skin tumor cells transiently transfected with vectors encoding rainbow trout AR and CAT (EPC rtAR ( ) +CAT(T)); and
- Yeast cells (*S. cerevisiae* or *S. cerevisiae YPH500*) stably transfected with hAR linked to a copper metallothionein promoter and a -galactosidase expression vector (Yeast (*S. cer*) hAR(S)+ -gal(S)) or (Yeast(*S. cer YPH500*) hAR(S)+ -gal(S)).

In studies that evaluated the potential agonism of a substance in an *in vitro* AR reporter gene assay, enzyme (i.e., luciferase; CAT; -galactosidase) activity was used as an indirect measure of AR-induced transcriptional activation. To assess potency, enzyme levels induced by the test substance were typically compared to that produced by a reference androgen (DHT, R1881, testosterone, or mibolerone). The quantitative results of these *in vitro* AR TA studies were most commonly presented in terms of relative activity. However, the definition of relative activity varied greatly among the reports. Relative activity was expressed as:

- The fold induction of enzyme activity produced by the test substance relative to the activity in the untreated controls;
- The ratio of the response of the test substance to that of the reference androgen (sometimes termed relative potency);

- The concentration of the test substance that produced a certain percent response relative to the reference androgen; and
- The concentration of test substance that produced a specified fold-induction (e.g., 10-fold induction of enzyme activity) over background.

When provided, these various quantitative measures of agonism were extracted from the publications. Such data are provided in the “AGONISM Maximum Fold” column in **Appendix D**. Normalizing these values for comparison across the assays was not attempted. Instead, data from each study was assigned a qualitative response of positive or negative for the particular assay system (shown in the column named “AGONISM Qualitative” in **Appendix D**).

EC<sub>50</sub> values were occasionally reported in the agonism studies. These values were extracted from the reports and are shown in the “AGONISM EC<sub>50</sub>” column in **Appendix D**. The measures of EC<sub>50</sub> were relative to the specific assay system used and were not compared across assays.

The antagonism studies using reporter gene expression systems measured the inhibition of reference androgen-induced enzyme activity by the test substance, and the IC<sub>50</sub> value was often presented as a measure of response. These values are summarized in the “ANTAGONISM IC<sub>50</sub>” column in **Appendix D**. In reports where an IC<sub>50</sub> value was not provided but dose response data were presented, the IC<sub>50</sub> values of the test substance and the reference androgen were estimated. These estimated IC<sub>50</sub> values are italicized in **Appendix D**. For publications in which an IC<sub>50</sub> value was not reported or a dose response curve not presented, test substances were assigned a qualitative response of positive or negative in the assay system used (shown in the “ANTAGONISM Qualitative” column in **Appendix D**).

Sonnenschein et al. (1989) used growth in a cell line that is dependent on androgens for replication as an *in vitro* measure of test substance-induced transcriptional activation. The investigators reported the study results in terms of RPP, which is the ratio (X 100) between the concentrations of the reference androgen (testosterone in this case) and the test substance necessary to induce maximal cell growth, as defined by the investigator. The RPP values for the substances tested were assigned a qualitative value of positive or negative (**Appendix D**).

#### **5.4 In Vitro AR TA Assay Results for Individual Substances**

The numbers of substances tested in each of the *in vitro* AR TA assays considered in this BRD are provided in **Table 5-1**. Of the 145 substances tested, only 23 (15.9%) were tested for agonism in three or more assays, irrespective of the reference androgen used. DHT was the most frequently tested substance in the AR TA agonism assays (15 assays), followed by 17  $\beta$ -estradiol and testosterone, which were tested in 12 and 11 assays respectively. Only 12 (8.3%) substances were tested in three or more antagonism assays, irrespective of the reference androgen used. The greatest number of different *in vitro* AR TA antagonism assays used to test the same substance was nine (for hydroxyflutamide).

A majority of the substances (91; 62.8%) were tested in only one laboratory for either agonism and/or antagonism. Among the *in vitro* AR TA assays included in this BRD, the assays that tested the greatest number of different substances, irrespective of testing for agonism or antagonism, were the CHO-K1 hAR(T)+Luc(T)+EGFP(T) assay (65 substances, 44.8%), the PALM hAR(S)+Luc(S) assay (43 substances, 29.7%), the Yeast (*S.cer*) AR+ -gal assay (32 substances, 22%), and the CV-1+hAR(T)+Luc(T) assay (27 substances, 18.6%).

#### **5.5 Use of Coded Chemicals and Compliance with Good Laboratory Practice (GLP) Guidelines**

Based on the available information, it appears that none of the *in vitro* AR TA studies used coded chemicals or complied with GLP guidelines.

**Table 5-1 Substances Tested in Three or More *In Vitro* AR TA Assays Irrespective of the Reference Androgen Used**

Substance	No. of Assays (agonism <sup>a</sup> )	No. of Assays (antagonism)
DHT*	15	
17 -Estradiol	12	4
Testosterone*	11	
Progesterone	10	3
Hydroxyflutamide	6	9
Cyproterone acetate	6	5
Flutamide	6	5
R1881*	6	
Cortisol	5	
Dexamethasone	5	
Mifepristone	5	
<i>p,p'</i> -DDE	4	6
Diethylstilbestrol	4	
Medroxyprogesterone acetate	4	
2,2-Bis-( <i>p</i> -chlorophenyl)-1,1,1-trichloroethane (HPTE)	3	4
Aldosterone	3	
Estrone	3	
11-Ketotestosterone	3	
Levonorgestrel	3	
Methyltestosterone	3	
Mibolerone*	3	
<i>p</i> -Nonylphenol	3	
Norethisterone	3	
Bicalutamide		4
3',5'-Dichloro-2-hydroxy-2-methylbut-3-enanilide		4
2-[[3,5-(Dichlorophenyl)carbamoyl]oxy]-2-methyl-butenoic acid		4
Spironolactone		3
Vinclozolin		3

Abbreviations: *p,p'*-DDE = 1,1 Dichloro-bis[4-chlorophenyl]ethylene; DHT = 5 - Dihydrotestosterone; R1881 = Methyltrienolone.

<sup>a</sup> Includes the cell proliferation assay performed by Sonnenschein et al. (1989).

\*Includes assays in which these substances were used as the reference ligand.

## 6.0 *IN VITRO* AR TA TEST METHOD PERFORMANCE ASSESSMENT

### 6.1 Introduction

The ICCVAM Submission Guidelines (ICCVAM, 1999) request a description of what is known about the performance (i.e., accuracy, sensitivity, specificity, positive and negative predictivity, and false positive and false negative rates<sup>1</sup>) of the proposed test method. The extent to which the new test method predicts or measures the effect of interest is compared to the reference test method currently accepted by regulatory agencies. Where feasible, an assessment is made of the ability of the new method to predict adverse health outcomes in the species of interest (e.g., humans, wildlife). Currently, there are no methods accepted by regulatory authorities to assess AR-induced transcriptional activation, and data on endocrine disruption in humans or wildlife are too limited to be used for this purpose. The approach taken to evaluate the performance of AR TA assays in this BRD is a comparison of the data from existing *in vitro* AR TA assays against each other with regard to their ability to detect AR agonists and antagonists.

### 6.2 Quantitative Assessment of Assay Performance

For a number of reasons, a quantitative analysis of the relative performance of the 18 *in vitro* AR TA assays considered in this BRD could not be conducted (see *In Vitro ER Binding Assay BRD, Section 6*). The reasons included the limited number of substances tested within and across different assays, the lack of quantitative data for substances that had been tested, and the numerous and varied approaches used by different investigators to express *in vitro* AR TA assay results, particularly from agonism studies. Agonism data was reported as the maximum fold increase compared to the concurrent control, relative activity compared to the reference androgen, or the EC<sub>50</sub> value. Antagonism data was reported as relative activity compared to the reference androgen alone, or as an IC<sub>50</sub> value. The numbers of compounds tested for agonism

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<sup>1</sup> Accuracy is defined as the proportion of correct outcomes of a method, often used interchangeably with concordance; Sensitivity is defined as the proportion of all positive substances that are correctly classified as positive in a test; Specificity is defined as the proportion of all negative substances that are correctly classified as negative in a test; Positive predictivity is defined as the proportion of correct positive responses among substances testing positive; Negative predictivity is defined as the proportion of correct negative responses among substances testing negative; False positive rate is defined as the proportion of all negative substances that are falsely identified as positive; False negative rate is defined as the proportion of all positive substances that are falsely identified as negative (ICCVAM, 1997).

**Table 6-1** Number of Substances Tested in Multiple *In Vitro* AR TA Assays

	Number of Assays										
	1	2	3	4	5	6	7	8	9	10	11
Number of Substances Tested for Agonism Activity <sup>a</sup>	81	20	7	3	3	6	0	0	0	1	1
Percentage of substances	65.3	16.1	5.6	2.4	2.4	4.8	0	0	0	0.8	0.8
Number of Substances Tested for Antagonism Activity	59	16	3	5	2	1	0	0	1	0	0
Percentage of Substances	67.8	18.4	3.4	5.7	2.3	1.1	0	0	1.1	0	0

	Number of Assays								Total
	12	13	14	15	16	17	18		
Number of Substances Tested for Agonism Activity <sup>a</sup>	1	0	0	1*	0	0	0	124	
Percentage of substances	0.8	0	0	0.8	0	0	0	100	
Number of Substances Tested for Antagonism Activity	0	0	0	0	0	0	0	87	
Percentage of Substances	0	0	0	0	0	0	0	100	

\*This substance is DHT.

<sup>a</sup>Includes the cell proliferation assay performed by Sonnenschein et al. (1989).



and antagonism are tabulated in **Table 6-1**. The type of reference androgen (DHT, mibolerone, R1881, testosterone) used for the antagonism assays was not considered in compiling the number of substances tested in each assay.

### 6.3 Qualitative Assessment of Assay Performance

A qualitative comparative assessment of assay performance was conducted that considered the relative ability of the various *in vitro* AR TA assays to identify substances that induced or inhibited transcriptional activation. In conducting this assessment, it was assumed that there were no false positive study results. The qualitative assessment was performed separately for AR TA agonism and antagonism test methods. Inspection of the *in vitro* AR TA database (**Appendix D**) suggests that negative calls for some substances in some assays could be the result of limitations in protocol design (i.e., the highest dose tested might have been inadequate) rather than due to intrinsic differences in assay sensitivity. However, no effort was made to account for this possible limitation in the qualitative assessment of assay performance.

To maximize the numbers of substances available for consideration during the qualitative assessment, data from different studies were combined where possible after taking into account the cell line, the source of the AR, the specific ARE on the reporter construct, and the reporter gene used. The major difference between the various *in vitro* AR TA assays used in the different studies was the cell line, and this criterion was used as the primary basis for combining or not combining data from different laboratories. Within each of the different cell lines, the AR was either transiently or stably transfected or was endogenous. Various cell lines differ in their ability to metabolize hormones and xenobiotics, as well as in their intracellular concentration of other hormone receptors (e.g., glucocorticoid, progesterone) (**Table 6-2**). These receptors can interfere with the binding of certain substances (e.g., medroxyprogesterone acetate) to the AR and subsequent transcriptional activation (Poulin et al., 1991). Differences in the metabolic capabilities of the cell lines are exemplified by the presence of two enzymes, 17 $\alpha$ -oxidase and 5 $\alpha$ -reductase, which metabolize testosterone and testosterone-like compounds, and are present in CV-1 and HeLa cells but not in CHO cells (Deslypere et al., 1992).

**Table 6-2 Characteristics of Cell Lines Used in *In Vitro* AR TA Assays**

Cell Line	Stable Transfection		Transient Transfection		Level of AR (fmol/mg protein)	Steroid Metabolizing Enzymes	Other Receptors
	EXP	REP	EXP	REP			
CHO	No	No	Yes	Yes		Metabolize vinclozolin	
CV-1	No	No	Yes	Yes	30	17 -oxidase 5 -reductase	No GR
HepG2	No	No	Yes	Yes			No ER and ER
MDA-MB-453	Yes*	No	No	Yes**	240		GR present; Very low level of ER
MDA-MB-453-kb2	Yes	Yes	No	No	240		GR present; Very low level of ER
PC-3	No	No	Yes	Yes	1200		
PALM	Yes	Yes	No	No	1200		
Yeast	Yes	Yes	No	No			

Abbreviations: EXP = Expression plasmid; REP = Reporter plasmid; GR = Glucocorticoid receptor; ER = Estrogen receptor.

\*The AR is endogenous in this cell line. \*\*Reporter introduced into cells by viral transduction.

Qualitative analysis was performed on each of the assays described in **Section 5**:

CHO hAR(S)+Luc(S); CHO hAR(T)+Luc(T); CHO-K1 hAR(S)+Luc(S); CHO-K1 hAR(T)+Luc(T)+EGFP(T); CHO hAR(T)+CAT(T)+ -gal(T); CV-1 hAR(T)+Luc(T)\*(transduced); CV-1 hAR(T)+CAT(T); CV-1 hAR(T)+Luc(T); MDA-MB-453 hAR(E)+Luc(T)\*(transduced); MDA-MB-453-kb2 hAR(E)+Luc(S); HepG2hAR(T)+Luc(T)+ -gal(T); PALM hAR(S)+Luc(S); PC-3 hAR(T)+Luc(T); Yeast (*S.cer*) hAR(S) + -gal(S); and LnCaP-FGC hAR(E)+CP. Excluded from the qualitative analysis were the two studies that did not use the hAR (Van Dort et al., 2000; Takeo and Yamashita, 2000), the HeLa cell-based assay used by Wang and Fondell (2001), and any substance not tested in at least two different assays. The HeLa cell-based assay was excluded because only four substances had been tested in one laboratory. The resulting data, separated by agonism and antagonism assays, are provided in **Appendix E**.

A total of 43 substances were tested for agonism activity in at least two of the fifteen *in vitro* AR TA assays considered during the qualitative assessment. In conducting this assessment, it was

assumed that there were no false positive calls in the published literature even in situations where multiple tests were conducted and the number of positive calls was in the minority. The primary limitation associated with this approach is that the substance might truly be negative for AR agonist or antagonist activity (i.e., the positive call was incorrect). Based on this approach, the results obtained using the CHO assays were the most frequently discordant (i.e., a negative response was obtained for nine substances that tested positive in another assay(s), which is 25.7% of the 35 substances tested in this assay). The LnCaP-FGC hAR(E)+CP cell proliferation assay was discordant for one of 10 substances (10%) that tested positive in at least one other assay, and the yeast-based assays were discordant for one of 17 substances (5.8%) that tested positive in at least one other assay. There was no discordance among the responses obtained for substances tested in common among the CV-1, HepG2, MDA-MB-453, and PC-3 cell-based assays.

A total of 28 substances were tested for antagonism in at least two of the 11 *in vitro* AR TA assays considered for the qualitative assessment. The yeast-based assay was discordant for one of three substances (33%) that tested positive in at least one other assay, the CHO-K1 hAR(T)+Luc(T)+EGFP(T) was discordant for one of three substances (33%), the HepG2 assay was discordant for one of 12 substances (8%), and the CV-1 hAR(T)+Luc(T) assay was discordant for one of 17 substances (6%).

This qualitative assessment is confounded by a number of limitations, including:

- The very limited size of the database;
- The lack of replicate test data for most of the substances considered;
- The lack of a common set of substance tested in multiple assays; and
- The assumption that positive results were more accurate than negative results.

#### **6.4 Performance of *In Vitro* AR TA Assays**

The *in vitro* AR TA assays that would be the most useful as screening tests for endocrine disrupting substances are those that are the most sensitive (i.e., have the ability to detect weak acting agonists and antagonists) and the most reliable (i.e., exhibit the lowest variance) within and across laboratories (see **Section 7**). In addition, it might be anticipated that assays that use

AR derived from the species of interest (e.g., human for predicting human-related effects, wildlife species for predicting effects in wildlife) might be the most informative. Since none of these assays use animals, animal welfare is not a consideration. Finally, when taking human health and safety issues into consideration, assays that do not use radioactivity might have the greatest utility. Only the CAT assay, which was used much less frequently than luciferase-based assays, utilized radioactivity. However, an ELISA assay for this enzyme is now available, eliminating the need for radioactivity if this reporter gene system is used.

Based on the very limited data available, there is no single assay that can be concluded to perform better than any other assay. However, it might be anticipated that mammalian cell-based assays would be preferred over yeast-based assays, simply because of differences in the increased ability of test substances to cross the mammalian cell membrane compared to the yeast cell wall (Gray et al., 1997; Krall and Yamamoto, 1996).

The cell lines used in the various *in vitro* AR TA assays differ from each other in a number of characteristics (**Tables 2-1** and **6-2**). One important difference is whether the cell line contains expression and/or reporter genes that are stable or whether these constructs have to be transfected into the cells prior to each experiment. Except for two of the CHO-based assays, the remaining CHO, CV-1, and HepG2 cell lines used in the majority of *in vitro* AR TA assays were all transiently transfected with expression and reporter plasmids prior to each experiment (**Table 2-1**). The LnCaP-FGC and MDA-MB-453-kb2 cell lines contain a functional endogenous hAR gene (Sonnenschein et al., 1989; Lambright et al., 2000), while a stably transfected cell line (PALM) was developed from the PC-3 cell line (Terouanne et al., 2000; Schrader and Cooke, 2000).

Two different approaches were used to incorporate the reporter construct into the MDA-MB-453 cell line. In one approach, cells were transduced before each experiment with a reporter gene (luciferase) by integrating the reporter and ARE into an infective but nonreplicative adenovirus (Hartig et al., 2002). In a different approach, cells were transfected with a reporter construct and, following antibiotic selection, a clone (MDA-MB-453-kb2) with a stably transfected reporter gene was isolated (Wilson et al., 2002). This cell line has both the expression and reporter

constructs stably integrated into the genome. Selection and expansion of the clone resulted in a cell line that could be used for many passages to measure AR TA. From passages 1-10, luciferase induction by 1 nM of DHT was 10-fold compared to control (Wilson et al., 2002). Over 30 to 40 passages, the fold induction decreased to 5 to 6 fold but then stabilized and remained at this level out to 80 passages.

### **6.5 Strengths and Limitations of *In Vitro* AR TA Assays**

Data from *in vitro* AR TA assays indicate whether a substance can interact with the target receptor which, in turn, binds to responsive elements in the DNA that initiate transcription of genes related to hormone-stimulated events in the cell. In contrast to binding assays, the TA assays provide sufficient evidence to conclude whether a substance is an agonist or an antagonist. However, neither assay takes into consideration other mechanisms of action that may lead to endocrine disruption (Zacharewski, 1998). The TA assays can be important components of a battery of screening tests because they:

- Use eukaryotic cells, many of which are derived from human tissues;
- Are cost-effective;
- Are rapid and relatively easy to perform;
- Are based on an easily quantitated, well-elucidated mechanism of action (i.e., binding to a specific protein and initiating the transcription of AR-responsive genes);
- Can be performed using small amounts of test substances;
- Can be used to test multiple substances simultaneously; and
- Can be easily standardized among laboratories.

The limitations of these assays include:

- The potential generation of false positive and false negative results;
- The efficiency of transfection for transiently transfected cells can vary from assay to assay; and
- The responsiveness of transiently transfected cells lasts for only a few days (Terouanne et al., 2000).

For yeast-based assays, additional limitations include:

- Yeast lines are more prone to genetic drift over time than mammalian cells (Joyeux et al., 1997);
- Transport of test substances through the yeast cell wall might be more difficult than transport through a mammalian cell membrane, increasing the likelihood of false negative results; and
- Yeast cells may have steroid metabolic pathways that differ from mammalian cells (Gaido et al., 1997).

False positive results could occur if the cells are unable to detoxify chemicals that are usually detoxified *in vivo*, or for antagonism studies, by test substance-induced cytotoxicity that is not accounted for. Another reason for false positives is induction of the reporter by a mechanism not involving AR activation. This could occur if the MMTV is used as the promoter in the reporter gene construct and the cells used for the assay contain a glucocorticoid, progesterone, or unknown receptor that can activate the ARE. False negative results could occur if the cell line used lacks the enzymes present *in vivo* that would normally activate the test substance to a reactive intermediate that then binds to the AR. The metabolic competency of the various cell lines (except for HepG2) is not very well characterized. The addition of the enzymes and co-factors required for metabolic activation to the assay can help to eliminate this limitation. This approach has been used in two studies in which ER-induced transcriptional activation was assessed (Charles et al., 2000; Sumida et al., 2001). Another reason for obtaining a false negative response would be incomplete solubility of the test substance in the medium.

## 6.6 Summary and Conclusions and Recommendations

Relatively few substances have been tested in more than one laboratory using the same *in vitro* AR TA assay. Also, few of the same substances have been tested for agonism or antagonism in different *in vitro* AR TA assays. Furthermore, because the primary focus of many of the investigations using *in vitro* AR TA assays has been to understand the process of AR-induced transcriptional activation and not to identify substances that act as AR agonists or antagonists, much of the published data are of limited value in terms of a relative analysis of assay performance. This prevents an accurate assessment of the effectiveness and limitations of *in vitro* AR TA assays.

Based on the limited data available, there is no single *in vitro* AR TA assay that can be concluded to perform better than any other assay. However, it might be anticipated that mammalian cell-based assays would be preferred over yeast-based assays, simply because of differences in the ability of test substances to cross the mammalian cell membrane compared to the yeast cell wall. Taking other factors into consideration, it would seem that a cell line with endogenous hAR and stably transfected with a luciferase reporter plasmid (e.g., MDA-MB-453-kb2 hAR(E)+Luc(S)) would offer the greatest utility in terms of eliminating the need to continuously prepare multiple batches of transiently transfected cells, while being the most relevant and sensitive. Due to patents held by a private company, some of the CV-1 cell lines transfected with the AR as described in this BRD may not be available to testing laboratories and, thus, they cannot be recommended for use in a screening assay.

Formal validation studies should be conducted using appropriate substances, covering the range of expected responses for agonist and antagonist from strong to weak to negative. Testing of substances encompassing a wide range of agonist/antagonist responses are needed to adequately demonstrate the performance characteristics of any *in vitro* AR TA test method recommended as a screening assay. A list of potential test substances for use in validation efforts is provided in **Section 12**.

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## **7.0 IN VITRO AR TA TEST METHOD RELIABILITY ASSESSMENT**

### **7.1 Introduction**

The ICCVAM Submission Guidelines (ICCVAM, 1999) recommend that an assessment of test method reliability<sup>1</sup> be performed. This assessment includes an evaluation of the rationale for selecting the substances used to evaluate intra- and inter-laboratory reproducibility, the extent to which the substances tested represent the range of possible test outcomes, and a quantitative statistical analysis of intra- and inter-laboratory reproducibility. In addition, measures of central tendency and variation for historical negative and positive control data and an assessment of the historical control variability need to be conducted. However, no formal validation studies to assess the reliability of *in vitro* AR TA assays have been conducted and the limited nature of the current database for these assays precludes a formal analysis.

### **7.2 Assessment of *In Vitro* AR TA Assay Reliability**

Although many of the reports indicated that the substances tested in *in vitro* AR TA assays were tested in triplicate or quadruplicate within an experiment and that at least replicate assays were conducted, associated error terms were not always provided and/or could not be estimated or calculated. Also, data analysis and presentation varied considerably among investigators assessing the *in vitro* AR agonist and antagonist activity of test substances. These two factors, combined with the great variability in assay protocols, the few substances tested multiple times within and across laboratories (and assays), and the lack of any validation studies made a formal assessment of assay reliability impractical.

In the only cross-assay evaluation located, Gray and colleagues (Hartig et al., 2002; Wilson et al., 2002) commented on the variability for DHT-induced luciferase activity in assays using stably transfected MDA-MB-453-kb2 cells and those using transiently transfected CV-1 cells. The interassay coefficient of variation for the MDA-MB-453-kb2 cell assays was 52.7% across 28 replicate plates, while that for CV-1 cells was 145% across eight replicates. The increased

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<sup>1</sup> Reliability is a measure of the degree to which a test can be performed reproducibly within and among laboratories over time, where reproducibility is the variability between single test results obtained in a single laboratory (intralaboratory reproducibility) or in different laboratories (interlaboratory reproducibility) using the same protocol.

variability for the CV-1 cell-based assays was attributed to the variability in transfection efficiencies from replicate to replicate, a source of variation that does not exist when stably transfected cells are used.

For *in vitro* AR TA studies conducted to assess agonism activity, quantitative data in the form of EC<sub>50</sub> values were reported for only 24 substance/assay combinations (**Table 7-1**), three of which were for the reference androgen DHT. Of these 24 substance/assay combinations,

**Table 7-1 Available EC<sub>50</sub> Values for Substances Tested for Agonism Activity in *In Vitro* AR TA Assays**

Substance	Assay	No. Times Tested	EC <sub>50</sub> Values (μM) <sup>a</sup>		
4-Androstenedione	CHO	2	0.00065* <sup>6</sup>	0.0024* <sup>6</sup>	
Cortisol	CHO	1	0.0427 <sup>6</sup>		
<i>p,p'</i> -DDE	Yeast	2	350 <sup>3</sup>	8820 <sup>1</sup>	
5 -DHT	PALM	1	0.00004 <sup>8</sup>		
5 -DHT	CHO	1	0.00015 <sup>6</sup>	0.00015 <sup>6</sup>	
5 -DHT	Yeast	1	0.0035 <sup>1</sup>	0.0024 <sup>5</sup>	0.002 <sup>2</sup>
17 -Estradiol	Yeast	1	0.0861 <sup>1</sup>		
Estrone	CHO	1	0.0551 <sup>6</sup>		
Hydroxyflutamide	Yeast	2	8.21* <sup>1</sup>	82.0* <sup>3</sup>	
11-Ketotestosterone	CHO	2	0.0015* <sup>6</sup>	0.0058* <sup>6</sup>	
Levonorgestrel	CHO	2	0.00037* <sup>6</sup>	0.0016* <sup>6</sup>	
Methyltestosterone	CHO	2	0.000027* <sup>6</sup>	0.00014* <sup>6</sup>	
Mibolerone	PALM	1	0.00003 <sup>8</sup>		
Mifepristone	CHO	1	0.0136 <sup>6</sup>		
Mifepristone	Yeast	1	2100 <sup>5</sup>		
Norethisterone	CHO	2	0.0037* <sup>6</sup>	0.0072* <sup>6</sup>	
Norgestrel	CHO	2	0.00040* <sup>6</sup>	0.0010* <sup>6</sup>	
19-Nortestosterone	CHO	2	0.000092* <sup>6</sup>	0.00022* <sup>6</sup>	
<i>p</i> -Nonylphenol	Yeast	1	2 <sup>2</sup>		
Progesterone	Yeast	2	0.0089 <sup>1</sup>	5.2 <sup>5</sup>	
Testosterone	CHO	2	0.000053* <sup>6</sup>	0.00011* <sup>6</sup>	
Testosterone	PALM	1	0.0002 <sup>8</sup>		
Testosterone	Yeast	3	0.0047 <sup>1</sup>	0.012* <sup>5</sup>	0.0099 <sup>4*</sup>
Toxaphene	PC-3	1	10 <sup>7</sup>		

DDE = 1,1 Dichloro-bis[4-chlorophenyl]ethylene; DHT = 5 -Dihydrotestosterone.

<sup>a</sup>EC<sub>50</sub> values in italics were estimated from a graphical representation of the data.

\*Values obtained in the same laboratory for that substance/assay combination.

<sup>1</sup>Gaido et al. (1997); <sup>2</sup>Moffat et al. (2001); <sup>3</sup>O'Connor et al. (1998); <sup>4</sup>O'Connor et al. (1999); <sup>5</sup>O'Connor et al. (2000); <sup>6</sup>Otsuka Pharmaceutical Co. (2001); <sup>7</sup>Schrader and Cooke (2000); <sup>8</sup>Terouanne et al. (2000).

only nine sets of EC<sub>50</sub> values were for substances tested twice in the same laboratory using the same assay, and only two sets of EC<sub>50</sub> values were for the same substance tested using the same assay in more than one laboratory. For the same substance/assay/laboratory combination, the least difference in EC<sub>50</sub> values (about a 2-fold difference) was for norethisterone tested twice using CHO cells, while the greatest difference in EC<sub>50</sub> values (about 10-fold) was for hydroxyflutamide tested twice using yeast cells. There was about a 25-fold difference in EC<sub>50</sub> values for *p,p'*-DDE tested using yeast cells in two laboratories.

For *in vitro* AR TA studies conducted to assess antagonism activity, quantitative data in the form of IC<sub>50</sub> values were reported for 63 substance/assay combinations (**Table 7-2**), two of which were reference androgens. Of these 63 substance/assay combinations, only seven sets of IC<sub>50</sub> values were for substances tested at least twice (three of these substances were tested three times) in the same laboratory using the same assay, and only one set of IC<sub>50</sub> values were for the same substance tested using the same assay in more than one laboratory. For the same substance/assay/laboratory combination, there was no difference in IC<sub>50</sub> values for linuron tested twice using MDA-MB-453-kb2 cells, while the greatest difference in IC<sub>50</sub> values (about 4500-fold) was for cyproterone acetate tested three times using PALM cells. There was about a 10-fold difference in IC<sub>50</sub> values for flutamide tested in two laboratories using yeast cells.

Based on the inadequate database available, no conclusions can be made about the relative reliability of the 18 different *in vitro* AR TA assays considered in this BRD. However, these data do indicate the need for future validation studies to adequately evaluate this issue.

### 7.3 Conclusions and Recommendations

The *in vitro* AR TA assays that are the most useful as a screen for endocrine disruptors are those that are the most sensitive (i.e., have the greatest ability to detect weak acting AR agonists and antagonists (see **Section 6**), and the most reliable (i.e., exhibit the least variability within and across laboratories). Based on the available data, no valid assessment of assay reliability was possible.

Taking into account the available *in vitro* AR TA assay database, and the inability to adequately assess the reliability of the *in vitro* AR TA assays considered in this BRD, formal validation

studies should be conducted using appropriate substances covering the range of expected EC<sub>50</sub> values (for agonism) and IC<sub>50</sub> values (for antagonism). These substances should elicit a range of responses ranging from strong to weak to inactive to demonstrate the reliability characteristics of the *in vitro* AR TA assays considered as possible screening assays. A list of potential test substances for use in such validation efforts is provided in **Section 12**.

**Table 7-2 Available IC<sub>50</sub> Values for Substances Tested for Antagonism Activity in *In Vitro* AR TA Assays**

Substance	Assay	No. Times Tested	IC <sub>50</sub> Values (μM) <sup>a</sup>		
Benzo[ <i>a</i> ]pyrene	CHO	1	3.9 <sup>10</sup>		
Benz[ <i>a</i> ]anthracene	CHO	1	3.2 <sup>10</sup>		
Bicalutamide	CHO	1	0.5 <sup>10</sup>		
Bicalutamide	PC-3	1	0.5 <sup>11</sup>		
Bicalutamide	PALM	2	0.75 <sup>9*</sup>	18 <sup>11*</sup>	
2,2 Bis-( <i>p</i> -hydroxyphenyl)-1,1,1-trichloroethane	MDA-MB-453	1	0.1 <sup>1</sup>		
2,2 Bis-( <i>p</i> -hydroxyphenyl)-1,1,1-trichloroethane	MDA-MB-453-kb2	1	10 <sup>11</sup>		
Bisphenol A	PC-3	1	1 <sup>12</sup>		
Butylated hydroxyanisole	PALM	1	7.6 <sup>7</sup>		
Butylated hydroxytoluene	PALM	1	5.7 <sup>7</sup>		
Chrysene	CHO	1	10.3 <sup>10</sup>		
Cyproterone acetate	PALM	2	0.01 <sup>11*</sup>	45.0 <sup>11*</sup>	
Cyproterone acetate	PC-3	1	0.01 <sup>11</sup>		
Cyproterone acetate	CV-1	1	0.1 <sup>2</sup>		
Cyproterone acetate	CHO	1	0.5 <sup>10</sup>		
<i>o,p'</i> -DDE	PALM	1	1.5 <sup>8</sup>		
<i>p,p'</i> -DDE	PALM	2	0.75 <sup>8*</sup>	15.2 <sup>7*</sup>	
<i>p,p'</i> -DDE	CHO	1	1 <sup>10</sup>		
<i>p,p'</i> -DDE	MDA-MB-453-kb2	1	5 <sup>11</sup>		
3',5'-Dichloro-2-hydroxy-2-methylbut-3-enanilide	PALM	1	0.02 <sup>8</sup>		
3',5'-Dichloro-2-hydroxy-2-methylbut-3-enanilide	MDA-MB-453	1	0.1 <sup>1</sup>		
3',5'-Dichloro-2-hydroxy-2-methylbut-3-enanilide	MDA-MB-453-kb2	1	0.2 <sup>11</sup>		
(4-[2,2-Dichloro-1-(4-hydroxyphenyl)vinyl]phenol)	MDA-MB-453	1	0.1 <sup>1</sup>		
(4-[2,2-Dichloro-1-(4-hydroxyphenyl)vinyl]phenol)	MDA-MB-453-kb2	1	5 <sup>11</sup>		
2-[[3,5-Dichlorophenyl)carbamoyl]oxy]-2-methyl-butenoic acid	MDA-MB-453	1	0.1 <sup>1</sup>		
2-[[3,5-Dichlorophenyl)carbamoyl]oxy]-2-methyl-butenoic acid	MDA-MB-453-kb2	1	0.2 <sup>11</sup>		
2-[[3,5-Dichlorophenyl)carbamoyl]oxy]-2-methyl-butenoic acid	PALM	1	0.5 <sup>8</sup>		
Diethylstilbesterol	PALM	1	0.36 <sup>7</sup>		
Dimethylbenz[ <i>a</i> ]anthracene	CHO	1	10.4 <sup>10</sup>		
17 -Estradiol	MDA-MB-453-kb2	1	0.05 <sup>11</sup>		

Substance	Assay	No. Times Tested	IC <sub>50</sub> Values (µM) <sup>a</sup>		
17 -Estradiol	CV-1	1	0.5 <sup>2</sup>		
17 -Estradiol	CHO	1	1 <sup>10</sup>		
Fluoranthene	CHO	1	4.6 <sup>10</sup>		
Flutamide	Yeast	2	22 <sup>5</sup>	220 <sup>6</sup>	
2,2',3,4,4',5-Hexachlorobiphenyl	CHO	1	1 <sup>10</sup>		
-Hexachlorocyclohexane	PALM	1	8.2 <sup>7</sup>		
-Hexachlorocyclohexane	PALM	1	17.9 <sup>7</sup>		
Hydroxyflutamide	CHO	1	0.01 <sup>10</sup>		
Hydroxyflutamide	PALM	3	0.02 <sup>11*</sup>	0.1 <sup>11*</sup>	10 <sup>11*</sup>
Hydroxyflutamide	CV-1	1	0.1 <sup>2</sup>		
Inocterone	PALM	1	30 <sup>11</sup>		
Kepone	PALM	1	6.9 <sup>7</sup>		
Linuron	MDA-MB-453-kb2	2	5 <sup>3*</sup>	5 <sup>11*</sup>	
Linuron	CV-1	1	10 <sup>4</sup>		
Methyltrienolone	CHO	1	0.0001 <sup>10*</sup>		
Mifepristone	PALM	1	0.05 <sup>11</sup>		
Neburon	MDA-MB-453-kb2	1	10 <sup>11</sup>		
Nilutamide	PALM	2	10 <sup>11*</sup>	0.3 <sup>11*</sup>	
Nilutamide	PC-3	1	0.15 <sup>11</sup>		
<i>p</i> -Nonylphenol	Yeast	1	0.001 <sup>4</sup>		
Procymidone	CHO	1	5 <sup>10</sup>		
Procymidone	MDA-MB-453-kb2	1	10 <sup>11</sup>		
Progesterone	CHO	1	0.1 <sup>10</sup>		
Progesterone	CV-1	1	0.5 <sup>2</sup>		
Promegestone	PALM	1	0.09 <sup>11</sup>		
RU2956	PALM	1	45 <sup>11</sup>		
RU56187	CV-1	1	0.0001 <sup>2</sup>		
Spirolactone	PALM	1	0.09 <sup>11</sup>		
Spirolactone	CHO	1	0.5 <sup>10</sup>		
2,3,7,8-Tetrachlorodibenzo- <i>p</i> -dioxin	PALM	1	6.5 <sup>7</sup>		
Toxaphene	PALM	1	1935 <sup>7</sup>		
Vinclozolin	MDA-MB-453-kb2	1	0.05 <sup>11</sup>		
Vinclozolin	CHO	1	0.5 <sup>10</sup>		

DDE = 1,1 Dichloro-bis[4-chlorophenyl]ethylene.

<sup>a</sup>IC<sub>50</sub> values in italics were estimated from a graphical representation of the data.

\*Values obtained in the same laboratory for that substance/assay combination.

<sup>1</sup>Hartig et al. (2002); <sup>2</sup>Kemppainen et al. (1999); <sup>3</sup>Lambright et al. (2000); <sup>4</sup>Moffat et al. (2001);

<sup>5</sup>O'Connor et al. (1998); <sup>6</sup>O'Connor et al. (1999); <sup>7</sup>Schrader and Cooke (2000); <sup>8</sup>Sultan et al. (2001);

<sup>9</sup>Terouanne et al. (2000); <sup>10</sup>Vinggaard et al. (2000); <sup>11</sup>Wilson et al. (2002).

## 8.0 QUALITY OF DATA REVIEWED

### 8.1 Extent of Adherence to GLP Guidelines

Ideally, all data supporting the validity of a test method should be obtained and reported in accordance with GLP guidelines, which are nationally and internationally recognized rules designed to produce high-quality laboratory records. GLPs provide a standardized approach to the reporting and archiving of laboratory data and records, and information about the test protocol, to ensure the integrity, reliability, and accountability of a study (U.S. EPA, 2001, 2002; FDA, 2002).

Based on the information provided in the reports included in this BRD, none of the *in vitro* AR TA studies were conducted in compliance with national or international GLP guidelines.

### 8.2 Assessment of Data Quality

Formal assessments of data quality, such as quality assurance audits, generally involve a systematic and critical comparison of the data provided in a study report or published paper to the laboratory records generated during a study. No attempt was made to formally assess the quality of the *in vitro* AR TA data included in this document. The published and submitted data on the TA of AR-inducible genes were limited, in most reports, to the response of the test substance relative to a reference androgen and, to a lesser extent, EC<sub>50</sub> and IC<sub>50</sub> values, and rates of enzyme activity. Auditing these reported data and values would require obtaining the original data for each study, which is not readily available.

An informal assessment of the *in vitro* AR TA publications revealed certain limitations that complicate interpretation of the reported AR TA data (**Appendix D**):

- *Various formats used to present the data*: The data were reported in a variety of formats (e.g., fold induction or increase, relative potency ratios, relative agonistic activity, EC<sub>50</sub> and IC<sub>50</sub> values, and rates of enzyme activity). The values reported were, as a rule, obtained from different protocols, against different standards. These factors precluded a quantitative analysis of results obtained by different laboratories for the same test substance.

- *Large number of substances tested in only one laboratory:* Less than half of the substances included in this BRD have been tested in more than one laboratory. Therefore, the inter-laboratory reproducibility of the results for these substances is not known.
- *Large number of substances without information regarding within-laboratory reproducibility:* There is often no information in the publications as to the number of replicates or repeat experiments performed. Therefore, the within-laboratory repeatability of many of the test results is not known.
- *Insufficient methodology information:* A number of publications contained limited details about the test methods, cells, and vectors used. In some cases, publications reported that the methods were “performed as previously described,” and in many of these cases the cited publication either referenced another publication for experimental details, or was not relevant to the particular protocol. At times, following this trail of references made it difficult to determine the actual protocol used to produce the data reported in the specific publication being abstracted.
- *Inconsistent nomenclature of test substances:* Most publications did not provide CASRNs for the substances tested, or used a unique chemical nomenclature, which in some cases made unequivocal identification of the test chemical difficult.

### **8.3 Quality Control Audit**

A quality control (QC) audit was conducted of the *in vitro* AR TA database provided in **Appendix D**. In conducting this audit, data input into the database was checked against the original sources and corrected if an entry error had been made.



## **9.0 OTHER SCIENTIFIC REPORTS AND REVIEWS**

### **9.1 Availability of Other *In Vitro* AR TA Data**

Some of the peer-reviewed publications identified during the initial literature search for AR TA studies were not abstracted for inclusion in this BRD. The reasons for not abstracting these publications include:

- The studies lacked appropriate qualitative and/or quantitative test data;
- The test substances were not adequately identified, or were undefined mixtures; or,
- The publications contained insufficient information about the test method used.

NICEATM made a formal request in the *Federal Register* (Vol. 66, No. 57, pp. 16278 – 16279) for unpublished AR TA data and/or information from completed studies using or evaluating AR TA assays. A submission was received from Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan, in response to this request. The data from this submission are included in **Appendix D**, which also contains the *in vitro* AR TA data from the 26 peer-reviewed publications considered in this BRD.

Some companies involved in pharmaceutical discovery and development routinely use *in vitro* AR TA assays to screen substances for their potential androgenic activity. However, these data are not in the public domain and have not been provided to NICEATM.

While every effort was made to include all available, pertinent *in vitro* AR TA data in this BRD, the authors recognize that some data may have been inadvertently excluded.

### **9.2 Conclusions from Other Scientific Reviews of *In Vitro* AR TA Methods**

To date, no independent peer reviews of *in vitro* AR TA assays have been conducted. However, two recent workshops addressed the use of these assays as potential endocrine disruptor screening methods. Although the strengths and limitations of AR TA assays were discussed at both workshops, no effort was made to evaluate the reliability and performance of the assays. The conclusions from these workshops are summarized below.

### 9.2.1 1996 Endocrine Disruptor Screening Methods Workshop

*In vitro* AR TA assays were discussed at an Endocrine Disruptor Screening Methods Workshop held in July 1996 at Duke University in Durham, North Carolina. Gray et al. (1997) edited the proceedings of this workshop, which was cosponsored by the U.S. EPA, the Chemical Manufacturers Association (CMA), and the World Wildlife Fund (WWF).

An assessment was made of *in vitro* AR TA assays that use monkey kidney CV-1 cells. For these assays, CV-1 cells are transiently transfected with an expression vector containing cDNA for human AR and a reporter vector containing a reporter gene, typically luciferase, linked to an AR-inducible response element. The major advantages of these assays, as described by the authors, include:

- The use of human AR;
- CV-1 cells have some metabolic activity; and
- The assays can distinguish between agonists and antagonists.

The major disadvantages cited by the authors include:

- Transient co-transfections of expression and reporter vectors can be difficult to prepare and maintain;
- The assay requires both AR expression and reporter vectors;
- Reproducibility of the assay requires strict adherence to the protocol; and
- Metabolism of a test substance during the required 48-hour incubation period may confound results.

In addition, Gray et al. (1997) discussed the major advantages and disadvantages of yeast-based AR TA assays. These assays use recombinant, stably transformed yeast cells that contain AR from humans or other species of interest, and a reporter gene, typically  $\beta$ -galactosidase, linked to an AR-inducible response element.

The major advantages of yeast assays, as described by the authors, include:

- They are relatively easy to perform;
- A short incubation time, ranging from 4 hours to overnight, is used;

- Large number of samples can be processed relatively quickly; and
- Substances can be tested over a wide dose range.

The major disadvantages of yeast-based AR TA assays cited by the authors include:

- They do not appear to distinguish between agonists and antagonists (e.g., the known AR antagonist, hydroxyflutamide, is reported to induce TA in yeast-based AR assays);
- There may be significant metabolic differences between yeast and mammalian cells that could make it difficult to extrapolate data from these assays to humans;
- The cell wall and chemical transport systems of yeast cells are reported to selectively maintain low intracellular concentrations of some steroid hormones, a phenomenon that may apply to other types of substances;
- The porosity of the yeast cell wall versus that of mammalian cell membranes may be significantly different;
- The assay gives negative and/or weak positive results for *p,p'*-DDE, a substance that binds strongly to rat AR and hAR in COS and CV-1 cells.

### **9.2.2 1997 Workshop on Screening Methods for Detecting Potential (Anti-) Estrogenic/Androgenic Chemicals in Wildlife**

In March 1997 the U.S. EPA, the CMA, and the WWF cosponsored a workshop in Kansas City, Missouri, that addressed the use of “gene expression” assays as a type of *in vitro* screening methods for detecting potential (anti-)androgenic substances in wildlife. Ankley et al. (1998) edited the proceedings of this workshop.

The major advantages described by the authors for using gene expression assays as endocrine disruptor screens for wildlife include:

- Assays that use eukaryotic cell lines can distinguish between agonists and antagonists;
- The assays are amenable to automation using microtiter plates, which would allow for the rapid processing of large numbers of samples; and
- The methods are amenable to standardization.

The major disadvantages include:

- Require specialized equipment and training;
- Transient transfection of plasmids can be labor-intensive and may contribute to increased interassay variability;
- Poor correlation of results for some substances tested in yeast-based assays versus those using mammalian cells;
- These assays currently have limited applicability to nonmammalian species, which have been poorly studied with regard to development of suitable reporter gene assays for detection of (anti-)androgenic substances.

## **10.0 ANIMAL WELFARE CONSIDERATIONS**

### **10.1 Refinement, Reduction, and Replacement Considerations**

ICCVAM promotes the scientific validation and regulatory acceptance of new methods that refine, reduce, or replace animal use where scientifically feasible. Refinement, Reduction, and Replacement are known as the three Rs of animal protection. These principles of humane treatment of laboratory animals are described as:

- Refining experimental procedures such that animal suffering is minimized;
- Reducing animal use through improved science and experimental design; and
- Replacing animal models with nonanimal procedures (e.g., *in vitro* technologies), where possible.

Combes (2000) and Phillips (2000) recommended that adequate consideration be given to animal welfare concerns by careful development and validation of all proposed endocrine disruptor screening methods. With respect to the proposed use of *in vitro* AR TA assays as screening methods to detect substances that potentially exhibit androgenic or anti-androgenic activity, it is important to evaluate the current level of animal use in these assays, and to consider what opportunities exist for refining, reducing, or replacing procedures that use animals.

### **10.2 Use of Animals in *In Vitro* AR TA Assays**

All 18 of the *in vitro* AR TA assays addressed in this BRD utilize cultured whole cells containing androgen-inducible gene expression systems and, therefore, do not require use of animals. Of these assays, the following use five different human cell lines that either naturally express hAR or are transfected with hAR vectors: 1) HeLa hAR(T)+Luc(T); 2) HepG2 hAR(T)+Luc(T)+ -gal(T); 3) MDA-MB453 hAR(E)+Luc(T)\* (Transduced); MDA-MB453-kb2 hAR(E)+Luc(S); 4) PC-3 hAR(T)+Luc(T) and PALM hAR(S)+Luc(S); 5) LnCaP-FGC hAR(E)+CP. The first four assays require transfection with cDNA encoding the enzyme luciferase, which produces a luminescent signal that can be measured. The LnCaP-FGC+CP assay measures cell growth.

Four other groups of assays use mammalian cells from different species. One of these groups uses CV-1 monkey kidney cells that have been transfected with vectors encoding hAR and

luciferase or CAT (i.e., CV-1 hAR(T)+Luc(T)\*; CV-1 hAR(T)+CAT(T); CV-1 hAR(T)+Luc(T)). Another assay uses this same cell line that is transiently transfected with vectors encoding mouse AR and the enzyme CAT (i.e., CV-1 mAR(T)+CAT(T)). A different group of assays uses CHO cells that have been transfected with vectors encoding hAR and luciferase or CAT (i.e., CHO hAR(S)+Luc(S); CHO hAR(T)+Luc(T); CHO-K1 hAR(S)+Luc(S); CHO-K1 hAR(T)+Luc(T)+EGFP(T); CHO hAR(T)+CAT(T)+ gal(T)). Another assay uses EPC (carp skin tumor) cells that have been transfected with vectors encoding rainbow trout AR (i.e., EPC rtAR (T)+CAT(T)).

The last group of assays uses stably transformed yeast cells containing cDNA for hAR (i.e., Yeast (*S.cer*) hAR(S) + -gal(S)) and measures the production of the enzyme -galactosidase.

From an animal welfare perspective, all of these *in vitro* cell-based assays are equally advantageous. However, because none of these assays has been extensively used for the routine testing of substances, further development and validation are required.

## **11.0 PRACTICAL CONSIDERATIONS**

### **11.1 Test Method Transferability**

Test method transferability describes the ability of a new method to be accurately and reliably performed by multiple laboratories (ICCVAM, 1997). This definition includes laboratories with experience in the particular type of procedure, and otherwise competent laboratories with little or no experience in the particular procedure. It also addresses whether the necessary facilities, equipment, and trained staff to perform the method can be readily obtained, and whether the cost of the assay and the level of expertise or training needed are considered reasonable. The degree of transferability of a test method affects its interlaboratory reproducibility.

The ICCVAM Submission Guidelines (ICCVAM, 1999) request that an assessment of test method transferability be conducted with respect to the following factors that influence transferability:

- Availability of the facilities and the fixed major equipment needed to perform the test method.
- The training requirements for laboratory personnel to demonstrate proficiency with the test method.
- Costs involved in conducting the test.
- Time needed to conduct the test.

#### **11.1.1 Facilities and Fixed Major Equipment**

The facilities needed to conduct *in vitro* AR TA assays are widely available, and the necessary laboratory equipment is readily available from suppliers. To ensure personnel and community safety, facilities should adhere to pertinent State or Federal regulations for the handling of hazardous substances/wastes.

An issue that affects the transferability of many of the assays considered in this BRD is the use of patented technologies. The key patent claims are directed to an isolated or purified human AR protein that is produced by a cell transfected or transformed with a vector comprising certain DNA sequences. The detailed claims of the patent cannot be assessed unless the prosecution history of the patent is analyzed. While this technology is available for use by academic

institutions, government research laboratories, and not-for-profit organizations, it is not readily available to commercial laboratories, which are typically contracted to perform regulatory testing of substances.

The specific needs as related to the various *in vitro* AR TA procedures are essentially the same for all assays. These are described briefly below.

*Facilities:* Standard cellular or molecular biology laboratory with cell culture capabilities.

*Fixed Major Equipment:* Luminometer for assays requiring luciferase detection; incubator with temperature, CO<sub>2</sub>, and humidity controls; sterile biohazard hoods; and freezer.

## **11.2 Training Considerations**

*Assays Using Stably Transfected/Transduced Cell Lines:* Currently, mammalian and yeast lines containing both a stably transfected AR and a reporter are not available commercially. A high level of technical expertise would be required to establish such cell lines. However, once established in a laboratory, the cell lines could be readily used in a reporter gene assay that requires staff with basic laboratory skills and training in cell culture techniques.

The other major types of *in vitro* AR TA assays (i.e., those using transiently transfected mammalian cells and the cell proliferation assay) also require staff with basic laboratory skills and training in cell culture techniques. The assays requiring transient transfections would require special training in that technique.



### 11.3 Cost and Time Considerations

**Table 11-1** provides information on the expected time needed to perform a study, special equipment needed, and other considerations. Cost information was not available in the literature; however, it was provided upon request by one commercial laboratory that conducts these assays. It would be expected that the costs for the reporter gene assays using mammalian cells would be roughly equivalent.

**Table 11-1 Comparison of Costs, Time, and Special Equipment Needs of Different *In Vitro* AR TA Assays**

Assay	Cost/Test Substance	Duration	Special Equipment	Other Considerations
CHO Reporter Gene Assay – Transient	n.a.	3-4 days	Luminescence counter/luminometer for luciferase detection	Patented cDNA and co-transfection technology
CV-1 Reporter Gene Assay – Transient	n.a.	3-4 days	Luminescence counter/luminometer for luciferase detection	Patented cDNA and co-transfection technology
HeLa Reporter Gene Assay – Transient	n.a.	3-4 days	Luminescence counter/luminometer for luciferase detection	Patented cDNA and co-transfection technology
Hep-G2 Reporter Gene Assay – Transient	\$1950	3-4 days	Luminescence counter/luminometer for luciferase detection	Patented cDNA and co-transfection technology
MDA-MB-453-kb2 Reporter Gene Assay	n.a.	3-4 days	Luminescence counter/luminometer for luciferase detection	
PALM Reporter Gene Assay – Stable	n.a.	3-4 days	Luminescence counter/luminometer for luciferase detection	
PC-3 Reporter Gene Assay – Transient	n.a.	3-4 days	Luminescence counter/luminometer for luciferase detection	Patented cDNA and co-transfection technology
Yeast Reporter Gene Assay – Stable	\$1600	2-3 days		

n.a. = Cost estimates not available in the literature or from laboratories conducting the assay.

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## **12.0 MINIMUM PROCEDURAL STANDARDS FOR *IN VITRO* AR TA ASSAYS AND RECOMMENDED SUBSTANCES FOR USE IN VALIDATION STUDIES**

### **12.1 Introduction**

Few *in vitro* studies have been published on the ability of substances to act as AR agonists or antagonists. Furthermore, except for the report submitted by Otsuka Pharmaceutical Co. (2001), which evaluated the agonist activity of 65 substances, the number of substances tested for agonist or antagonist activity in each report ranged from one to 22, with most publications based on about ten substances. There are no published guidelines for conducting such studies, and no formal validation studies have been conducted to assess the performance or reliability of *in vitro* AR TA assays. To assist in the development and characterization of these assays, minimum procedural standards and a recommended list of test substances for use in validation studies are provided. The minimal procedural standards and recommended test substances are based on an evaluation of the specific *in vitro* AR TA assays considered in **Sections 6 and 7** of this BRD (**Appendix D**). For the reasons discussed in **Sections 6 and 11**, an assay with endogenous AR and a stably transfected reporter vector containing the *Luc* gene, is recommended as having the highest priority for future validation efforts.

### **12.2 Minimum Procedural Standards**

The minimum procedural standards listed below are recommended for standardized protocols developed for various types of AR TA assays. Adequate procedural details are essential to maximize interlaboratory reproducibility and minimize variation that may contribute to erroneous or nonreproducible results.

#### **12.2.1 Transcriptional Activation of the Reference Androgen**

Irrespective of the source of the cell line used, the transcriptional activation-inducing ability of the reference androgen (**Section 12.2.2**) must be demonstrated each time the test is conducted. Consistency in the level of the reporter gene product response induced by the reference androgen is used as a measure of the intralaboratory reproducibility of the assay, and as a criterion for assay acceptance. Since it has been demonstrated in *in vitro* AR TA antagonism assays that the ability to detect a weak antagonist depends on the concentration of the reference androgen, this concentration must be based on the dose response of that androgen in the particular cell line

being used for AR-induced transcriptional activation. It is suggested that the dose should give 70-80% of the maximal response in the cell line. This reference dose can be determined by measuring transcriptional activation in the cell line over a range of concentrations.

### 12.2.2 Reference Androgen

Similarly to the *in vitro* assays that measured AR binding, where four different reference androgens were used, the same four reference androgens have been used in *in vitro* AR TA assays (**Table 2-1**). For the majority of such studies, DHT has been used as the reference androgen. Since testosterone can be metabolized to DHT in some cell lines (e.g., CV-1, HeLa) used in *in vitro* AR TA studies, most investigators have avoided using it as the reference androgen. However, three investigators have used this substance as the reference androgen. R1881, a potent synthetic androgen, was used by four investigators (five publications) as the reference androgen. Mibolerone was used as the reference androgen by Takeo and Yamashita (2000) for studies involving AR from the rainbow trout. Since most investigators have used DHT as the reference androgen and the issue of DHT binding to a testosterone-estradiol binding globulin (TeBG) is not relevant to *in vitro* AR TA studies, DHT could be used as the reference androgen of choice. However, if a comparison of data between AR binding and AR TA assays is deemed important, it would be more appropriate if the same reference androgen were used for both types of assays. Thus, under these circumstances, R1881 would be recommended for both types of assays as the reference androgen.

### 12.2.3 Preparation of Test Substances

Test substances must be dissolved in culture medium or in a solvent that is miscible with the medium. For substances not sufficiently water soluble, absolute ethanol or DMSO are proposed as solvents. Preference is given to absolute ethanol since this solvent has been used in most of the studies conducted to date. Other solvents may be used as long as it can be demonstrated that they do not interact, or otherwise interfere, with the test system. A solvent control substance must be included in each assay.

#### 12.2.4 Concentration Range of Test Substances

To minimize effort and costs in screening/testing, and in recognition that adding excessive amounts of a test substance can perturb the test system through physicochemical mechanisms, most testing schemes include a limit dose (i.e., the highest dose that should be tested in the absence of solubility or toxicity constraints). An agreed upon limit dose for *in vitro* AR TA screening assays has not been established. Historically, the highest dose tested in such assays has ranged from 1 to 100  $\mu\text{M}$ , with most tests conducted using a maximum dose level of 100  $\mu\text{M}$ . The  $\text{EC}_{50}$  values reported for substances tested in various *in vitro* AR TA assays cover eight to nine orders of magnitude (from 20 pM to 8 mM) although the majority of  $\text{EC}_{50}$  values ranged from 20 pM to 100 nM. Thus, if the *in vitro* AR TA assay is required to detect substances with an  $\text{EC}_{50}$  that is at least 8 orders of magnitude higher than that of DHT, then the limit dose (unless precluded by chemical properties such as solubility) should be 100  $\mu\text{M}$ . However, if seven orders of magnitude are sufficient for detecting AR agonists, then the limit dose could be 10  $\mu\text{M}$ .

Therefore, for the *in vitro* screening for AR agonists, it is proposed that the limit dose be 100  $\mu\text{M}$  and that a concentration range from 10 pM to 100  $\mu\text{M}$ , in 10-fold increments, be used in each experiment. However, if it is suspected that the test substance binds weakly to the AR, the dose range should extend from 10 nM to 10 mM, in 10-fold increments.

For AR antagonism assays, the weakest AR antagonist, toxaphene, (see **Table 7-2**) had a reported  $\text{IC}_{50}$  value of 1.935 mM. Therefore, the range of substance concentrations tested in such studies should be from 10 nM to 10 mM.

For relatively insoluble substances, the highest dose should be at the limit of solubility; the concentration range should then decrease in 10-fold increments. Testing at concentrations that result in precipitation in the test medium should be avoided to minimize false positive results associated with the nonspecific interaction of the precipitate with the receptor (Gray et al., 1997).

### 12.2.5 Solvent and Positive Controls

Concurrent negative and solvent controls and a reference androgen must be included in each experiment. The negative control provides assurance that the solvent does not interact with the test system. The solvent should be tested at the highest concentration that is added with the test substance. The reference androgen in *in vitro* AR TA agonism assays is included to demonstrate the sensitivity of the assay in each experiment for detecting agonist activity and to allow for an assessment of variability in the conduct of the assay across time. A reference androgen for *in vitro* AR TA antagonism assays is required for the assay to function. In addition, to demonstrate the sensitivity of the *in vitro* AR TA antagonism assay, a substance with demonstrated AR antagonism activity (i.e., a positive control) is needed in each experiment. Hydroxyflutamide is suggested as the candidate AR antagonist as this substance has historically been shown to be negative as an agonist but positive as an antagonist.

### 12.2.6 Within-Test Replicates

Triplicate values should be obtained for each dose tested for each control and test substance.

### 12.2.7 Dose Spacing

Generally, to obtain a response curve to assess AR-induced transcriptional activation, the concentrations of the reference androgen and the test substances should be spaced by one order of magnitude (i.e., 1 nM, 10 nM, etc.) over the concentration range of interest (1 pM to 100  $\mu$ M). For antagonists, the concentration range should range from 10 nM to 1 mM. This results in the testing of nine concentrations of each substance for agonists and six concentrations of each substance for antagonism in each test. If the range of doses is reduced due to, for example, insolubility of the test substance at the limit dose, then equivalent spacing (e.g., half-log doses) of the nine or six doses over the smaller dose range should be used.

### 12.2.8 Data Analysis

Different investigators have used various approaches for analyzing data obtained from *in vitro* AR TA assays. For agonist assays, responses are compared to the concurrent vehicle control while for antagonist assays, treatments are compared to the response induced by the reference androgen alone. Data analysis approaches have varied from a visual inspection of the data only

to more formal statistical approaches using either one- or two-way analysis of variance (ANOVA) (with main effects being treatment or replicates and treatment, respectively) using a general linearized model. In some studies, the induced reporter gene response for each replicate has been converted to a fold induction above the concurrent control level, and means and variances of these data used as the basis for analysis.  $EC_{50}$  or  $IC_{50}$  values have been calculated using various curve fitting programs. One curve fitting approach was 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 mid-point is determined (see Deslypere et al., 1992; Gaido et al., 1997). Asymptotic standard errors of the parameter estimates are employed to perform two-sided “t” tests.

It would be useful for future validation studies to compare and evaluate the various methods used to analyze *in vitro* AR TA agonist and antagonist data in order to develop standard approaches.

#### **12.2.9 Assay Acceptance Criteria**

An *in vitro* AR TA assay testing for agonism activity should be accepted only if the response for the reference androgen occurs within the appropriate confidence limits based on historical data. An *in vitro* AR TA assay testing for antagonism activity should be accepted only if the response for the reference androgen and the positive antagonism control occur within the appropriate confidence limits based on historical data.

#### **12.2.10 Evaluation and Interpretation of Results**

A substance is classified as an AR agonist if the assay-specific response (e.g., luciferase activity) is significantly increased above the concurrent control level, as determined by an appropriate statistical test. A substance is classified as an AR antagonist if the substance induces a significant decrease in the ability of the reference androgen to induce TA, as determined by an appropriate statistical test.

#### **12.2.11 Test Report**

At a minimum, the test report must include the following information:

*Test substance:*

- Name, chemical structure, and CASRN, if known;
- Physical nature (solid or liquid), and purity, if known (every attempt should be made to obtain the purity); and
- Physicochemical properties relevant to the study (e.g., solubility, stability, volatility).

*Solvent:*

- Justification for choice of solvent if other than medium, absolute ethanol, or DMSO;
- Information to demonstrate that the solvent, if other than medium, absolute ethanol, or DMSO, does not affect the sensitivity of the assay.

*Androgen receptor:*

- Type and source of AR (if from a commercial source, the supplier must be identified);
- Isolation procedure or method for making constructs; and
- Nomenclature and components of the expression and reporter constructs.

*Reporter plasmid:*

- Type of reporter gene;
- Type and structure of response elements;
- Original plasmid used to make construct; and
- Description and methodology used to make plasmid that is transfected.

*Cell line:*

- Source of cell line and protocol for maintenance of the cell line;
- Growth parameters of the cell line before initiation of the assay; and
- Method used to transfect cells if transiently transfected cells are used.

*Test conditions:*

- Rationale for the concentration of the reference androgen used;
- Composition of media and buffers used;
- Concentration range of test substance with justification;



- Volume of solvent used to dissolve test substance and volume of test substance added;
- Incubation time and temperature;
- Type and composition of metabolic activation system, if added;
- Concentration range of positive and solvent controls;
- Method used to lyse cells after incubation;
- Method used to measure transcriptional activation;
- Methods used to determine fold induction, EC<sub>50</sub> value for agonism studies, or IC<sub>50</sub> value for antagonism studies; and
- Statistical methods used.

*Results:*

- Extent of precipitation of test substance;
- Reporter response for each replicate at each dose for all test substances, including confidence levels or other measure of intradose repeatability;
- Calculated EC<sub>50</sub> value for agonism studies or IC<sub>50</sub> value for antagonism studies, and confidence limits, if calculated, for the reference androgen (agonism studies), positive control (antagonism studies), and test substance; and
- Fold increase above control for each concentration.

*Discussion of the results:*

- Historical fold increases in activity and EC<sub>50</sub> values for reference androgen (agonism), including ranges, means, and standard deviations; and
- Reproducibility of IC<sub>50</sub> value of positive control antagonist compared to historical data.

*Conclusion:*

- Classification of test substance with regard to *in vitro* AR agonist or antagonist activity.

### **12.2.12 Replicate Studies**

Generally, replicate studies are not mandated for screening assays. However, in situations where questionable data are obtained (i.e., the fold increase is marginal, the EC<sub>50</sub> or IC<sub>50</sub> value is not

well defined, the call is equivocal, the test shows excess variability), repeat tests to clarify the results of the primary test would be prudent.

### 12.3 Standardization of *In Vitro* AR TA Assays for Validation

**Appendix B** provides *in vitro* AR TA assay protocols submitted by four investigators. The assay protocols, as titled by the investigators, are:

- Protocol for CV1 + hAR + Luciferase Assay, as provided by Dr. Elizabeth M. Wilson, Departments of Pediatrics and of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC, USA.
- Protocol for CHO Cells + hAR + Luciferase Assay as provided by Dr. Anne Marie Vinggaard, Institute of Food Safety and Toxicology, Danish Veterinary and Food Administration, Soborg, Denmark.
- Protocol for HepG2 Cells + Receptor + Reporter and/or -gal Plasmids for Use in Steroid Hormone Receptor Assays, as provided by Dr. Kevin Gaido, CIIT Centers for Health Research, Research Triangle Park, NC, USA.
- Protocol for Yeast-Based Androgen Receptor Assay, as provided by Dr. Kevin Gaido, CIIT Centers for Health Research, Research Triangle Park, NC, USA.
- Development of New Reporter Gene Assay Systems for Screening Endocrine Disrupters, as provided by Drs. Mitsuru Iida and Teruhisa Kato, Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan
- Development of Stably Transfected Cell Lines to Screen Endocrine Disrupters, as provided by Drs. Mitsuru Iida and Teruhisa Kato, Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan.

Inspection of these protocols provides a perspective on how various *in vitro* AR TA assays are conducted by different investigators. These protocols provide a basis for developing a more general protocol, one that takes into account the recommended minimum procedural standards provided in **Section 12.2**. Prior to developing that protocol, the protocols in **Appendix B** need to be reviewed for completeness and adequacy for their intended purpose.

One of the difficulties in recommending one of the mammalian cell assays as a screening test method for detecting substances with AR agonist or antagonist activity is the issue surrounding

the patents that exist for the AR gene sequence and the technology for transfecting this receptor into mammalian cells (**Section 11**). As a result, it has been difficult to further develop these *in vitro* assays as AR TA test methods. One approach to overcoming this restraint has been the use of cell lines that harbor an endogenous AR gene that then requires transfection or transduction of the reporter construct only. This has been the approach used by Gray and coworkers with the MDA-MB-453 cell line (Hartig et al., 2002; Wilson et al., 2002). Subsequently, these investigators developed a variant of this cell line (MDA-MB453-kb2) that was stably transfected with a luciferase reporter construct. A stably transfected cell line has also been developed by transfecting an AR expression vector and a reporter gene construct that also carry antibiotic resistant genes for selection (Terouanne et al., 2000). Neither of these cell lines has been validated for their intended use and both have associated limitations (**Table 6-2**). Nevertheless, at the present time, these cell lines or ones similar to them that might be developed in the future, have the greatest potential for use as an *in vitro* AR TA screening assay. One of the difficulties with the MDA-MB453-kb2 cell line is the use of the MMTV promoter. This response element contains sequences to which both the AR and GR can bind. Thus, the presence of the GR can alter the transcriptional activation responsiveness of the cell if the test substance binds to it as well as to the AR (e.g., medroxyprogesterone acetate). This is a problem with any cell line that has a GR that is transfected with reporter plasmid that contains the MMTV-Luc. However, the AR activity can be distinguished from that of GR with the use of selective competitors (Wilson et al, 2002).

#### **12.4 List of Recommended Substances for Validation of *In Vitro* AR TA Assays**

**Tables 12-1** and **12-2** provide lists of recommended substances to be used in the assessment of the reliability and comparative performance of *in vitro* AR TA agonist and antagonist assays, respectively. A number of factors were considered in developing the list for AR agonist studies, including the number of times the substance had been tested in any assay, the median EC<sub>50</sub> value (when available) of the substance in all the assays in which it was tested (see **Table 7-1**), the fold increase in response above the control substance, and whether it had been recommended for testing in the AR binding BRD. The latter was considered since it would be informative to assess whether a substance was positive for AR binding but did not elicit a positive transcriptional activation response or vice-versa. For antagonists, the median IC<sub>50</sub>, if available,

and the fold decrease in transcriptional activation compared to the reference androgen was used. Selection of the substances was based on the availability and concordance of multiple test results among the *in vitro* AR TA assays considered in **Appendix E**. Because quantitative data was available for only a few substances, consideration was given to qualitative responses (i.e., positive, weak positive, or negative). Methoxychlor (Gaido et al., 2000) and vinclozolin (Wilson et al., 2002) have been included in the substances to be tested for antagonism even though they have to be activated in the cell as they have been shown be active in HepG2 and MDA-MB-453-kb2 cells, respectively. It might be more appropriate to use the metabolites of these compounds for testing but they are not available from a commercial source.

In a validation study, it is important to include substances that cover the range of possible responses and, therefore, this list includes substances in each category. The variability in the numbers of strong, weak, and negative substances in each list reflects the available database.

**Table 12-1 List of Substances Recommended for Validation of *In Vitro* AR TA Assays for Agonism**

Substance	CASRN	Qualitative Response for AR Agonism <sup>a</sup>	EC <sub>50</sub> Value (μM) <sup>b</sup>	RBA <sup>c</sup>
Levonorgestrel	797-63-7	Positive (3)	0.000984	9.25
Methyltestosterone	58-18-4	Positive (2)	0.000812	
Androstenedione	63-05-8	Positive (3)	0.00153	1.03
Testosterone	58-22-0	Positive (8)	0.00245	29.2
Mifepristone	84371-65-3	Positive (4/5)*	0.0136	
Cortisol	50-23-7	Positive (3/5)*	0.043	HTD-1000 μM
Estrone	53-16-7	Positive (2)	0.0551	0.1
17 -Estradiol	50-28-2	Positive (11)	0.0861	1.9
Progesterone	57-83-0	Positive (6/8)*	2.6	3.05
Hydroxyflutamide	52806-53-8	Positive (5/6)	41.5	
Spirolactone	52-01-7	Positive (2)		33.8
Medroxyprogesterone acetate	71-58-9	Positive (4)		11.6
Cyproterone acetate	427-51-0	Positive (5)		3.0
Fluoxymestrone	76-43-7	Positive (1)		0.3
Linuron	330-55-2	Positive (1)		0.0055
Bicalutamide	90357-06-5	Weak (2)		
Fenitrothion	122-14-5	Weak (2)		
Nilutamide	63612-50-0	Weak (1)		
Atrazine	1912-24-9	Negative (1)		0.0018
Corticosterone	50-22-6	Negative (1)		0.000068
<i>o,p'</i> -DDT	789-02-6	Negative (2)		0.0045
<i>p,p'</i> -DDT	50-29-3	Negative (1)		0.0013
Procymidone	320809-16-8	Negative (1)		0.000068
Vinclozolin	50471-44-8	Negative (1)		0.018
Diethylstilbestrol	56-53-1	Negative (3)		0.010
Kepone	143-50-0	Negative (2)		0.00075
Methoxychlor	72-43-5	Negative (2)		0.00054
Flutamide	13311-84-7	Negative (3)		

<sup>a</sup>Numbers in parentheses refer to the number of different agonism assays in which the substance was tested. These counts exclude the cell proliferation assay and the assays using rainbow trout and mouse ARs.

<sup>b</sup>EC<sub>50</sub> values are medians of the EC<sub>50</sub> values presented in **Table 7-1**.

<sup>c</sup>RBA = Relative binding affinity reported only for substances recommended for use in validating *in vitro* AR binding assays (NIEHS, 2002). HTD = Highest tested dose.

\*Number of assays in which the substance was positive compared to the number of assays in which it was tested.

**Table 12-2 List of Substances Recommended for Validation of *In Vitro* AR TA Assays for Antagonism**

Substance	CASRN	Qualitative Response for AR Antagonism <sup>a</sup>	IC <sub>50</sub> Value (μM) <sup>b</sup>	RBA <sup>c</sup>
Mifepristone	84371-65-3	Positive (2)	0.05	
Hydroxyflutamide	52806-53-8	Positive (9)	0.1	
Cyproterone acetate	427-51-0	Positive (5)	0.1	3.0
Nilutamide	63612-50-0	Positive (2)	0.15	
Spirolactone	52-01-7	Positive (2/3)**	0.254	33.8
Vinclozolin	50471-44-8	Positive (3)	0.275	0.018
Diethylstilbestrol	56-53-1	Positive (2)	0.36	0.010
Bicalutamide	90357-06-5	Positive (4)	0.625	
<i>p,p'</i> -DDE*	72-55-9	Positive (5/6)**	3	0.016
Linuron	330-55-2	Positive (2)	5	0.0055
Procymidone	320809-16-8	Positive (2)	7.5	0.000068
Flutamide	13311-84-7	Positive (4)	115	
17 -Estradiol	50-28-2	Positive (4)	0.5	1.9
Medroxyprogesterone acetate	71-58-9	Positive (1)		11.6
Fenitrothion	122-14-5	Positive (2)		
<i>o,p'</i> -DDT	789-02-6	Positive (2)		0.0045
<i>p,p'</i> -DDT	50-29-3	Positive (2)		0.0013
Methoxychlor	72-43-5	Positive (2)		0.00054
Progesterone	57-83-0	Positive (3)	0.3	3.05
Kepone	143-50-0	Equivocal (1/2)**	6.9	0.00075
Testosterone	58-22-0	Negative (1)		29.2
Atrazine	1912-24-9	Negative (1)		0.0018
Fluoxymestrone	76-43-7	Negative (1)		0.3
Lindane*	58-89-9	Negative (2)		

<sup>a</sup>Numbers in parentheses refer to the number of different antagonism assays in which the substance was tested. These counts exclude the cell proliferation assay and the assays using rainbow trout and mouse ARs.

<sup>b</sup>IC<sub>50</sub> values are medians of the IC<sub>50</sub> values in **Table 7-2**.

<sup>c</sup>RBA = Relative binding affinity reported only for substances recommended for use in validating *in vitro* AR binding assays (NIEHS, 2002).

\*Substances NOT recommended to be tested in *in vitro* AR TA assays for agonism (**Table 12-1**).

\*\*Number of assays in which the substance was positive compared to the number of assays in which it was tested.

## 12.5 Summary and Conclusions

Currently, there are no published guidelines for conducting *in vitro* AR TA studies, and no formal validation studies have been conducted to assess the reliability or performance of the currently available assays. To support the further development and characterization of *in vitro* AR TA agonism and antagonism assays, minimum procedural standards for such assays and a recommended list of test substances for use in validation studies are provided. The minimum procedural standards and recommended test substances are based on an evaluation of the *in vitro* AR TA assays considered in **Sections 6 and 7** of this BRD. It is recommended that a mammalian cell assay with an endogenous AR and stably transfected reporter gene, as well as a stably transfected plasmid containing  $\beta$ -galactosidase to monitor toxicity be evaluated.

The minimum procedural standards include the following: methods for determining the ability of the reference androgen to induce transcriptional activation; methods for establishing a stable cell line; the concentration range of the test substance (including the limit dose) to test for agonists and antagonists; the use of negative, solvent, and positive controls; the number of replicates to use; dose spacing; data analysis; assay acceptance criteria; evaluation and interpretation of results; minimal information to include in the test report; and the potential need for replicate studies. These minimum procedural standards are provided to ensure that *in vitro* AR TA studies will be conducted in such a manner as to allow the results to be understandable and comparable among procedures.

Six submitted *in vitro* AR TA assay protocols developed by experts in the field are provided in **Appendix B**. Inspection of these protocols provides a perspective on how various *in vitro* AR TA assays are conducted by different investigators, and for developing a more general protocol, one that takes into account the recommended minimum procedural standards. Prior to developing that protocol, the appended protocols need to be evaluated for completeness and adequacy for their intended purpose.

A number of factors were considered in developing a list of substances to be used in validation efforts, including the EC<sub>50</sub> and IC<sub>50</sub> value of the substance in all of the assays in which it has

been tested. Because the number of substances with replicate quantitative agonist or antagonist data was insufficient to generate the desired number of substances for consideration, selection of most substances was based on results obtained in a single assay by a single investigator. The selected substances were sorted according to whether they were positive, weak positive, or negative in at least one *in vitro* AR TA assay.

It is anticipated that this BRD and the guidance it provides will help to stimulate validation efforts for *in vitro* AR TA assays.



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## 14.0 GLOSSARY<sup>1</sup>

**Accuracy<sup>2</sup>:** A measure of test performance. (a) The closeness of agreement between a test result and an accepted reference value; (b) The proportion of correct outcomes of a method. Often used interchangeably with **concordance**.

**Activation (of genes):** The interaction of specific molecules or molecular complexes with specific genes to initiate their expression (i.e., transcription of mRNA).

**Affinity (high; low):** The strength of binding of a molecule to a receptor protein.

**Agonism:** The binding of a substance to a receptor to initiate effects similar to those produced by the natural ligand for the receptor.

**Agonist:** A substance that mimics the action of an endogenous hormone.

**Androgen:** A class of steroid hormones, which includes testosterone and 5 -dihydrotestosterone, responsible for the development and maintenance of the male reproductive system.

**Antagonism:** The binding of a substance to a receptor to inhibit or counteract the effects produced by the natural ligand for the receptor.

**Antagonist:** A substance that blocks or diminishes the activity of an **agonist**.

**Complex mixture:** A mixture containing many, generally uncounted substances, a majority of which are undefined (e.g., plant homogenates, fuels).

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<sup>1</sup> The definitions in this Glossary are restricted to their uses with respect to endocrine mechanisms and actions.

<sup>2</sup> Definition used by the Interagency Coordinating Committee on the Validation of Alternative Methods.

**Concordance<sup>2</sup>:** A measure of test performance. The proportion of all chemicals that are correctly classified as positive or negative. Often used interchangeably with **accuracy**. The concordance is highly dependent on the **prevalence** of positives in the population being examined.

**COS:** A monkey kidney cell line

**C-Terminal region:** The end of a protein molecule that contains a free carboxylic acid moiety.

**CV-1:** A monkey kidney cell line

**Cytoplasm:** The material inside the cell, excluding the nucleus, that contains the intracellular fluid, organelles, soluble enzymes, membrane components and other factors.

**Cytosol:** see Cytoplasm.

**Detoxification:** Reduction of the toxicity (of a substance) by metabolism to a less toxic form, or by removal of the substance from the affected cell or organism.

**Domain:** A region of a protein defined by its activity.

**Endocrine disruption:** Activity by an exogenous chemical substance that alters the structure or function(s) of the endocrine system and causes adverse effects at the level of the organism, its progeny, populations, or subpopulations of organisms.

**Endocrine disruptor:** A substance determined to cause endocrine disruption.

**Endocrine system:** Made up of glands located throughout the body, the hormones that are synthesized and secreted by the glands into the bloodstream, and the receptors in the various tissues and organs that recognize and respond to the hormones.

**Endogenous:** Originating within the organism of interest.

**Endpoint:** The biological process, response, or effect assessed by a test method.

**EPC:** A carp cell line derived from a skin tumor.

**Estrogen:** A class of steroid hormones, which includes 17 $\beta$ -estradiol, responsible for regulation of specific female reproductive functions and for development and maintenance of the female reproductive system.

**Exogenous:** Originating outside the organism of interest.

**False negative<sup>2</sup>:** An active substance incorrectly identified as negative by a test.

**False negative rate<sup>2</sup>:** The proportion of all positive (active) substances falsely identified as negative. A measure of test performance.

**False positive<sup>2</sup>:** An inactive substance incorrectly identified as positive by a test.

**False positive rate<sup>2</sup>:** The proportion of all negative (inactive) substances falsely identified as positive. A measure of test performance.

**Frog metamorphosis assay:** A test method that measures the ability of a substance to affect the metamorphosis of frog larvae (tadpoles) to adults.

**Gonadal recrudescence assay:** A test method that measures the ability of a substance to produce effects in estrogen- and androgen-dependent accessory sex organs or gonad maturation in fish. A test method for potential estrogen- and androgen-related endocrine disruption.

**Half-life:** The time it takes for a chemical or radioactive substance to lose half its activity.

**Hazard:** An adverse health or ecological effect.

**HeLa:** A human cell line derived from a cervical cancer.

**HepG2:** A human cell line derived from a hepatoma.

**Hershberger assay:** Measures the ability of a substance to alter the weight of androgen-dependent accessory sex organs (e.g., ventral prostate or seminal vesicles) or tissues in castrated rats or mice. A test method for potential androgen and anti-androgen related endocrine disruption activity.

**Hormone:** A chemical substance produced in specific cells, or glands, that can either act locally or be released into the bloodstream to act on an organ or tissue in another part of the body.

**Hypospadias:** A clinical condition in newborns that manifests itself as a displaced opening of the urethra. Occurs in males only and is considered a fetal developmental anomaly.

**Interlaboratory reproducibility<sup>2</sup>:** A measure of whether different laboratories using the same protocol and test chemicals can produce qualitatively and quantitatively similar results. See **reliability**.

**Intralaboratory reproducibility<sup>2</sup>:** A measure of whether the same laboratory can successfully replicate results using a specific test protocol at different times. See **reliability**.

**In vitro:** In glass. Refers to assays that are carried out in an artificial system (e.g., in a test tube or petri dish) and typically use single-cell organisms, cultured cells, cell-free extracts, or purified cellular components.

**In vivo:** In the living organism. Refers to assays performed in multicellular organisms.

**Ligand:** A substance that is capable of binding to a specific receptor protein.

**Ligand-binding domain:** The area within a receptor molecule that is designed to attract and hold a ligand.

**LnCaP-FGC:** A cell line established from a supraclavicular lymph node removed from a patient with metastatic prostatic adenocarcinoma.

**MCF-7:** A cell line from a human breast adenocarcinoma.

**MDA (all variations):** A human cell line derived from a breast carcinoma.

**Metabolic activation:** Metabolism by an organism or a cell-free extract of a chemical to a biologically active form.

**Negative predictivity<sup>2</sup>:** The proportion of correct negative responses among substances testing negative.

**N-Terminal region:** The end of a protein molecule that contains a free amino acid moiety.

**PC-3:** A human cell line derived from a prostate adenocarcinoma.

**Peer review:** Objective review of data, a document, or proposal, and provision of recommendations, by an expert individual or group of individuals having no conflict of interest with the outcome of the review.

**pH:** A measure of the acidity or alkalinity of a solution. pH 7.0 is neutral; higher pHs are alkaline, lower pHs are acidic.

**Placental aromatase assay:** Measures the ability of a substance to induce or inhibit the activity of the aromatase enzyme which converts testosterone to estradiol. A test method for potential anti-estrogen related endocrine activity.

**Positive predictivity<sup>2</sup>:** The proportion of correct positive responses among substances testing positive.

**Protocol<sup>2</sup>:** The precise, step-by-step description of a test, including the listing of all necessary reagents, criteria and procedures for the evaluation of the test data.

**Pubertal female assay:** Measures the ability of a substance to induce or inhibit the onset of puberty in immature female rats and mice, measured as an early or late opening of the vagina. A test method for potential estrogenicity and anti-estrogenicity.

**Pubertal male assay:** Measures the ability of a substance to induce or inhibit prepubertal separation in immature male rats and mice. At recovery (53 days), various tissues are weighed and the thyroid examined histologically. A test method for potential androgen- and anti-androgen related endocrine disruption.

**R1881:** Methyltrienolone

**Receptor:** A protein or protein complex which binds to specific molecules for the purpose of transporting them elsewhere in the cell, or for producing a chemical signal.

**Receptor binding assay (competitive):** An assay to measure the ability of a substance to bind to a hormone receptor protein, which is typically performed by measuring the ability of the substance to displace the bound natural hormone.

**Receptor superfamily:** A family of related receptors with similar composition and reactivity (e.g., the estrogen, androgen, and glucocorticoid receptors).

**Relevance (of an assay)<sup>2</sup>:** The relationship of a test to the effect of interest and whether a test is meaningful and useful for a particular purpose. The extent to which an assay will correctly predict or measure the biological effect of interest. A measure of assay **performance**.

**Reliability (of an assay)<sup>2</sup>:** Reliability is a measure of the degree to which a test can be performed reproducibly within and among laboratories over time.

**Repression (of genes):** The interaction of specific molecules or molecular complexes with specific genes to prevent their expression (transcription of mRNA).

**Reproducibility:** The variability between single test results obtained in a single laboratory (intralaboratory reproducibility) or in different laboratories (interlaboratory reproducibility) using the same protocol.

**Screen/Screening Test<sup>2</sup>:** A relatively rapid, simple test conducted for the purposes of a general classification of substances according to general categories of hazard. The results of a screen are generally used for preliminary decision making and to set priorities for more definitive tests. A screening test may have a truncated response range (e.g., provides a qualitative response only).

**Sensitivity<sup>2</sup>:** The proportion of all positive substances that are correctly classified as positive in a test.

**Specificity<sup>2</sup>:** The proportion of all negative substances that are correctly classified as negative in a test.

**Steroidogenesis assay:** Measurement of the ability of chemicals to inhibit steroid hormone biosynthesis in testicular tissue or cells *in vitro*.

**Test battery:** A series of tests, usually performed at the same time or in close sequence. Each test in the battery usually measures a different component of a multifactorial toxic effect, or a mechanistically related effect.

**Tier 1 assay:** An assay that is a component of the EDSP screening battery of tests.

**Tier 1 battery:** Defined by the EDSP as a series of *in vitro* and *in vivo* tests to determine the ability of substances to interact with the endocrine system.

**Tier 2 assay:** An assay that is a component of the EDSP testing battery.

**Tier 2 battery:** Defined by the EDSP as a series of *in vivo* tests designed to confirm the endocrine disrupting ability of substances in laboratory animals and wildlife species.

**Transcriptional activation (assay):** An assay to measure the initiation of mRNA synthesis in a gene in response to a specific chemical signal, such as an androgen-androgen receptor complex.

**Transcriptional regulatory protein:** A protein that binds to a specific DNA sequence resulting in a change in the regulation of mRNA synthesis.

**Transfection:** The process by which foreign DNA is introduced into a cell to change the cell's genotype.

**Uterotrophic assay:** Measures the ability of a substance to cause uterine enlargement in an immature or ovariectomized rat or mouse. A test method for potential estrogenicity and anti-estrogenicity.

**Valid method<sup>2</sup>:** A method determined to be acceptable for a specific use.

**Validated method<sup>2</sup>:** A method for which the reliability and relevance for a specific purpose has been established.

**Validation<sup>2</sup>:** Validation is the process by which the reliability and relevance of an assay for a specific purpose are established.



**Vector:** A small segment of DNA (frequently a plasmid or viral DNA) that is used to carry a foreign gene or DNA sequence into a cell's nucleus.

**Weight of evidence (process):** The strengths and weaknesses of a collection of information are used as the basis for a conclusion that may not be evident from the individual data.

**Xenobiotic:** A substance that is not produced by the organism of interest.

**Zinc finger motif:** A configuration of a DNA-binding protein that resembles a finger and binds a zinc ion for its activity.

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# **Appendix A**

## **Methods for *In Vitro* AR TA Assays**

**A1 AR TA Assays Using CHO Cells**

**A2 AR TA Assays Using Human Cells**

**A3 AR TA Assays Using CV-1 Cells**

**A4 AR TA Assays Using Yeast Cells**

**A5 Miscellaneous AR TA Assays**

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## **Appendix A1**

### **AR TA Assays Using CHO Cells**

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## AR TA Assays Using CHO Cells

Reference	Bonefeld-Jorgensen et al. (2001)	Deckers et al. (2000)
<b>Characteristics of Cell Line</b>		
Name of cell line	CHO-K1	CHO-K1 clone 1G12-A5-CA
Cell source	Chinese hamster ovary	Chinese hamster ovary
<b>Transfection of Cells with Plasmids</b>		
Stable or transient transfection	Transient AR/Transient reporter	Stable AR/Stable reporter
AR expression vector	pSVAR0	hAR-MMTV-LUC
AR source	human	human
Reporter vector	pMMTV-LUC	hAR-MMTV-LUC
Endpoint measured	Luciferase activity	Luciferase activity
Plasmid transfected for cell toxicity	none	none
Endpoint measured for cell toxicity	n.a.	n.a.
<b>Preparation of Cells for Assay</b>		
<i>Transient</i>	Cells treated with test compound just prior to transfection	
Pregrowth of cells before transient transfection	24 hours pregrowth before treatment with test substance and transfection	n.p.
Time from transient transfection to treatment of cells	n.a.	n.p.
<i>Stable</i>		
Plating time prior to treatment with test substance	n.a.	n.a.
<b>Transcriptional activation assay</b>		
Test substance solvent	Ethanol	Ethanol
No. of replicates	4	n.p.
No. of times assay repeated	3	from 1 to 30
Test substance incubation time	24 hours	16 hours
<b>Agonism assay</b>		
Reference ligand	Methyltrienolone	5 $\alpha$ -Dihydrotestosterone
Final concentration of reference ligand	100 nM	n.p.
<b>Antagonism assay</b>		
Reference ligand	Methyltrienolone	n.a.
Final concentration of reference ligand	0.1 nM	n.a.

Abbreviations: n.a. = not applicable;  
n.p. = not provided

## AR TA Assays Using CHO Cells

Reference	Deslypere et al. (1992)	Otsuka Pharmaceutical Co. (2001)
<b>Characteristics of Cell Line</b>		
Name of cell line	CHO	CHO-K1 (AR-EcoScreen™)
Cell source	Chinese hamster ovary	Chinese hamster ovary
<b>Transfection of Cells with Plasmids</b>		
Stable or transient transfection	Transient AR/Transient reporter	Stable AR/Stable reporter
AR expression vector	pCMV3.1.hAR	pZeoSV2AR
AR source	human	n.p.
Reporter vector	MMTV-CAT	pIND ARE B10
Endpoint measured	CAT activity	Luciferase activity
Plasmid transfected for cell toxicity	SV40-pCH110 ( -gal)	none
Endpoint measured for cell toxicity	-galactosidase activity	n.a.
<b>Preparation of Cells for Assay</b>		
<i>Transient</i>		
Pregrowth of cells before transient transfection	Within 24 hours	n.a.
Time from transient transfection to treatment of cells	24 hours	n.a.
<i>Stable</i>		
Plating time prior to treatment with test substance	n.a.	24 hours
<b>Transcriptional activation assay</b>		
Test substance solvent	n.p.	DMEM/F12 medium
No. of replicates	2	n.p.
No. of times assay repeated	1 to 12	n.p.
Test substance incubation time	12, 24, 36, 48 hours	24 hours
<b>Agonism assay</b>		
Reference ligand	Testosterone	5 $\alpha$ -Dihydrotestosterone
Final concentration of reference ligand	n.p.	.001 $\mu$ M
<b>Antagonism assay</b>		
Reference ligand	n.a.	5 $\alpha$ -Dihydrotestosterone
Final concentration of reference ligand	n.a.	n.p.

Abbreviations: n.a. = not applicable;  
n.p. = not provided



## AR TA Assays Using CHO Cells

Reference	Otsuka Pharmaceutical Co. (2001)	Vinggaard et al. (1999)
<b>Characteristics of Cell Line</b>		
Name of cell line	CHO-K1 (EcoScreen™ High throughput transfection assay)	CHO-K1
Cell source	Chinese hamster ovary	Chinese hamster ovary
<b>Transfection of Cells with Plasmids</b>		
Stable or transient tranfection	Transient AR/Transient reporter	Transient AR/Transient reporter
AR expression vector	pZeoSV2AR	pSVAR0
AR source	n.p.	human
Reporter vector	pIND ARE B10	MMTV-LUC
Endpoint measured	Luciferase activity	Luciferase activity
Plasmid transfected for cell toxicity	pcDNA-EGFP	none
Endpoint measured for cell toxicity	Fluorescence	n.a.
<b>Preparation of Cells for Assay</b>		
<i>Transient</i>		Cells treated with test compound just prior to transfection
Pregrowth of cells before transient transfection	24 hours	24 hours pregrowth before treatment with test substance and transfection
Time from transient transfection to treatment of cells	3 hours	n.p.
<i>Stable</i>		
Plating time prior to treatment with test substance	n.a.	n.a.
<b>Transcriptional activation assay</b>		
Test substance solvent	DMSO + culture medium	DMEM/F12 medium
No. of replicates	4	4
No. of times assay repeated	2	3 to 5
Test substance incubation time	16 to 24 hours	24 hours
<b>Agonism assay</b>		
Reference ligand	n.a.	Methyltrienolone
Final concentration of reference ligand	n.a.	n.p.
<b>Antagonism assay</b>		
Reference ligand	5 $\alpha$ -Dihydrotestosterone	Methyltrienolone
Final concentration of reference ligand	5 nM	0.1 nM

Abbreviations: n.a. = not applicable;  
n.p. = not provided

## AR TA Assays Using CHO Cells

Reference	Vinggaard et al. (2000)
<b>Characteristics of Cell Line</b>	
Name of cell line	CHO-K1
Cell source	Chinese hamster ovary
<b>Transfection of Cells with Plasmids</b>	
Stable or transient transfection	Transient AR/Transient reporter
AR expression vector	pSVAR0
AR source	human
Reporter vector	MMTV-LUC
Endpoint measured	Luciferase activity
Plasmid transfected for cell toxicity	none
Endpoint measured for cell toxicity	n.a.
<b>Preparation of Cells for Assay</b>	
<i>Transient</i>	Cells treated with test compound just prior to transfection
Pregrowth of cells before transient transfection	24 hours pregrowth before treatment with test substance and transfection
Time from transient transfection to treatment of cells	n.p.
<i>Stable</i>	
Plating time prior to treatment with test substance	n.a.
<b>Transcriptional activation assay</b>	
Test substance solvent	DMEM/F12 medium
No. of replicates	4
No. of times assay repeated	3
Test substance incubation time	24 hours
<b>Agonism assay</b>	
Reference ligand	Methyltrienolone
Final concentration of reference ligand	n.p.
<b>Antagonism assay</b>	
Reference ligand	Methyltrienolone
Final concentration of reference ligand	0.1 nM

Abbreviations: n.a. = not applicable;  
n.p. = not provided

## **Appendix A2**

### **AR TA Assays Using Human Cells**

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## AR TA Assays Using Human Cells

Reference	Wang and Fondell (2001)	Wang and Fondell (2001)
<b>Characteristics of Cell Line</b>		
Name of cell line	HeLa (E19)	HeLa (E19)
Cell source	Human cervical tumor	Human cervical tumor
<b>Transfection of Cells with Plasmids</b>		
Stable or transient transfection	Stable AR/Transient reporter	Stable AR/Transient reporter
AR expression vector	pTetCMV-F0(S)-AR	pTetCMV-F0(S)-AR
AR source	human	human
Reporter vector	MMTV-Luc	ARE2-DS-Luc
Endpoint measured	Luciferase activity	Luciferase activity
Plasmid transfected for cell toxicity	none	none
Endpoint measured for cell toxicity	n.a.	n.a.
<b>Preparation of Cells for Assay</b>		
<i>Transient</i>		
Pregrowth of cells before transient transfection	One day prior to transfection with reporter vector	One day prior to transfection with reporter vector
Time from transient transfection to treatment of cells	3 hours	3 hours
<i>Stable</i>		
Plating time prior to treatment with test substance	n.a.	n.a.
<b>Transcriptional Activation Assay</b>		
Test substance solvent	n.p.	n.p.
No. of replicates	n.p.	n.p.
No. of times assay repeated	3	3
Test substance incubation time	48 hours	48 hours
<b>Agonism assay</b>		
Reference ligand	Testosterone	Testosterone
Final concentration of reference ligand	100 nM	100 nM
<b>Antagonism assay</b>		
Reference ligand	Testosterone	Testosterone
Final concentration of reference ligand	n.p.	n.p.

Abbreviations: n.a. = not applicable;

n.p. = not provided

## AR TA Assays Using Human Cells

Reference	Wang and Fondell (2001)	Gaido et al. (2000)
<b>Characteristics of Cell Line</b>		
Name of cell line	HeLa (E19)	HepG2
Cell source	Human cervical tumor	Human hepatoma
<b>Transfection of Cells with Plasmids</b>		
Stable or transient transfection	Stable AR/Transient reporter	Transient AR/Transient reporter
AR expression vector	pTetCMV-F0(S)-AR	n.p.
AR source	human	human
Reporter vector	PB(-285/+32)-Luc	MMTV-Luc
Endpoint measured	Luciferase activity	Luciferase activity
Plasmid transfected for cell toxicity	none	pCMV -gal
Endpoint measured for cell toxicity	n.a.	-galactosidase activity
<b>Preparation of Cells for Assay</b>		
<i>Transient</i>		
Pregrowth of cells before transient transfection	One day prior to transfection with reporter vector	overnight
Time from transient transfection to treatment of cells	3 hours	n.p.
<i>Stable</i>		
Plating time prior to treatment with test substance	n.a.	n.a.
<b>Transcriptional Activation Assay</b>		
Test substance solvent	n.p.	n.p.
No. of replicates	n.p.	3
No. of times assay repeated	3	At least 3
Test substance incubation time	48 hours	24 hours
<b>Agonism assay</b>		
Reference ligand	Testosterone	n.a.
Final concentration of reference ligand	100 nM	n.a.
<b>Antagonism assay</b>		
Reference ligand	Testosterone	5 $\alpha$ -Dihydrotestosterone
Final concentration of reference ligand	n.p.	n.p.

Abbreviations: n.a. = not applicable;

n.p. = not provided

## AR TA Assays Using Human Cells

Reference	Maness et al. (1998)	Tamura et al. (2001)
<b>Characteristics of Cell Line</b>		
Name of cell line	HepG2	HepG2
Cell source	Human hepatoma	Human hepatoma
<b>Transfection of Cells with Plasmids</b>		
Stable or transient transfection	Transient AR/Transient reporter	Transient AR/Transient reporter
AR expression vector	pRSAR	pRSAR
AR source	human	human
Reporter vector	MMTV-Luc	MMTV-Luc
Endpoint measured	Luciferase activity	Luciferase activity
Plasmid transfected for cell toxicity	pCMV -gal	pCMV -gal
Endpoint measured for cell toxicity	-galactosidase activity	-galactosidase activity
<b>Preparation of Cells for Assay</b>		
<i>Transient</i>		
Pregrowth of cells before transient transfection	overnight	n.p.
Time from transient transfection to treatment of cells	3 hours	n.p.
<i>Stable</i>		
Plating time prior to treatment with test substance	n.a.	n.a.
<b>Transcriptional Activation Assay</b>		
Test substance solvent	DMSO	DMSO
No. of replicates	3	3
No. of times assay repeated	3 to 9	3
Test substance incubation time	24 hours	24 hours
<b>Agonism assay</b>		
Reference ligand	5 -Dihydrotestosterone	n.a.
Final concentration of reference ligand	1 $\mu$ M	n.a.
<b>Antagonism assay</b>		
Reference ligand	5 -Dihydrotestosterone	5 -Dihydrotestosterone
Final concentration of reference ligand	n.p.	n.p.

Abbreviations: n.a. = not applicable;

n.p. = not provided

## AR TA Assays Using Human Cells

Reference	Wilson et al. (2002)	Hartig et al. (2002)
<b>Characteristics of Cell Line</b>		
Name of cell line	MDA-kb2	MDA-MB-453
Cell source	Derived from MDA-MB-453 cells (human breast carcinoma)	Human breast carcinoma
<b>Transfection of Cells with Plasmids</b>		
Stable or transient transfection	Stable reporter	Transduced reporter
AR expression vector	Endogenous hAR	Endogenous hAR
AR source	human	human
Reporter vector	pMMTV.Luc.neo	Ad/mLuc7
Endpoint measured	Luciferase activity	Luciferase activity
Plasmid transfected for cell toxicity	none	none
Endpoint measured for cell toxicity	n.a.	n.a.
<b>Preparation of Cells for Assay</b>		
<i>Transient</i>		
Pregrowth of cells before transient transfection	n.a.	24 hours
Time from transient transfection to treatment of cells	n.a.	4 hours
<i>Stable</i>		
Plating time prior to treatment with test substance	4 to 6 hours	n.a.
<b>Transcriptional Activation Assay</b>		
Test substance solvent	Ethanol	Ethanol
No. of replicates	At least 4	3 to 4
No. of times assay repeated	3	n.p.
Test substance incubation time	overnight	48 hours
<b>Agonism assay</b>		
Reference ligand	n.a.	5 $\alpha$ -Dihydrotestosterone
Final concentration of reference ligand	n.a.	0.1 nM
<b>Antagonism assay</b>		
Reference ligand	5 $\alpha$ -Dihydrotestosterone	5 $\alpha$ -Dihydrotestosterone
Final concentration of reference ligand	0.1 or 1.0 nM	0.1 nM

Abbreviations: n.a. = not applicable;

n.p. = not provided



## AR TA Assays Using Human Cells

Reference	Lambright et al. (2000)	Sultan et al. (2001)
<b>Characteristics of Cell Line</b>		
Name of cell line	MDA-MB-453-kb2	PALM
Cell source	Human breast carcinoma	Derived from PC-3 cells (human prostate adenocarcinoma)
<b>Transfection of Cells with Plasmids</b>		
Stable or transient transfection	Stable reporter	Stable AR/Stable reporter
AR expression vector	Endogenous hAR	pSG5-puro-hAR
AR source	human	human
Reporter vector	MMTV.neo.luciferase	pMMTV-neo-Luc
Endpoint measured	Luciferase activity	Luciferase activity
Plasmid transfected for cell toxicity	pCMV -gal	none
Endpoint measured for cell toxicity	-galactosidase activity	n.a.
<b>Preparation of Cells for Assay</b>		
<i>Transient</i>		
Pregrowth of cells before transient transfection	n.a.	n.a.
Time from transient transfection to treatment of cells	n.a.	n.a.
<i>Stable</i>		
Plating time prior to treatment with test substance	5 to 6 hours	n.p.
<b>Transcriptional Activation Assay</b>		
Test substance solvent	Ethanol	n.p.
No. of replicates	n.p.	n.p.
No. of times assay repeated	n.p.	n.p.
Test substance incubation time	overnight	n.p.
<b>Agonism assay</b>		
Reference ligand	n.a.	n.a.
Final concentration of reference ligand	n.a.	n.a.
<b>Antagonism assay</b>		
Reference ligand	5 $\alpha$ -Dihydrotestosterone	Methyltrienolone
Final concentration of reference ligand	0.1 nM	0.1 nM

Abbreviations: n.a. = not applicable;

n.p. = not provided

## AR TA Assays Using Human Cells

Reference	Terouanne et al. (2000)	Terouanne et al. (2000)
<b>Characteristics of Cell Line</b>		
Name of cell line	PALM	PC-3
Cell source	Derived from PC-3 cells (human prostate adenocarcinoma)	Human prostate adenocarcinoma
<b>Transfection of Cells with Plasmids</b>		
Stable or transient transfection	Stable AR/Stable reporter	Transient AR/Transient reporter
AR expression vector	pSG <sub>5</sub> -puro-hAR	pSG <sub>5</sub> -puro-hAR
AR source	human	human
Reporter vector	pMMTV-neo-Luc	pMAMneo-Luc
Endpoint measured	Luciferase activity	Luciferase activity
Plasmid transfected for cell toxicity	none	pCMV <sub>5</sub> -galactosidase
Endpoint measured for cell toxicity	n.a.	-galactosidase activity
<b>Preparation of Cells for Assay</b>		
<i>Transient</i>		
Pregrowth of cells before transient transfection	n.a.	n.p.
Time from transient transfection to treatment of cells	n.a.	n.p.
<i>Stable</i>		
Plating time prior to treatment with test substance	12 hours	n.a.
<b>Transcriptional Activation Assay</b>		
Test substance solvent	F12 medium	n.p.
No. of replicates	2	2
No. of times assay repeated	At least 3	At least 3
Test substance incubation time	30 hours	n.p.
<b>Agonism assay</b>		
Reference ligand	Methyltrienolone	n.a.
Final concentration of reference ligand	0.1 nM	n.a.
<b>Antagonism assay</b>		
Reference ligand	Methyltrienolone	Methyltrienolone
Final concentration of reference ligand	0.1 nM	0.1 nM

Abbreviations: n.a. = not applicable;

n.p. = not provided

## AR TA Assays Using Human Cells

Reference	Schrader and Cooke (2000)
<b>Characteristics of Cell Line</b>	
Name of cell line	PC-3 LUC <sup>AR+</sup>
Cell source	Derived from PC-3 cells (human prostate adenocarcinoma)
<b>Transfection of Cells with Plasmids</b>	
Stable or transient transfection	Stable AR/Stable reporter
AR expression vector	pCMV5-hAR
AR source	human
Reporter vector	MMTV.pMAMneo-Luc
Endpoint measured	Luciferase activity
Plasmid transfected for cell toxicity	none
Endpoint measured for cell toxicity	n.a.
<b>Preparation of Cells for Assay</b>	
<i>Transient</i>	
Pregrowth of cells before transient transfection	n.a.
Time from transient transfection to treatment of cells	n.a.
<i>Stable</i>	
Plating time prior to treatment with test substance	24 hours
<b>Transcriptional Activation Assay</b>	
Test substance solvent	DMSO
No. of replicates	n.p.
No. of times assay repeated	n.p.
Test substance incubation time	18 hours
<b>Agonism assay</b>	
Reference ligand	5 $\alpha$ -Dihydrotestosterone
Final concentration of reference ligand	n.p.
<b>Antagonism assay</b>	
Reference ligand	5 $\alpha$ -Dihydrotestosterone
Final concentration of reference ligand	n.p.

Abbreviations: n.a. = not applicable;

n.p. = not provided

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## **Appendix A3**

### **AR TA Assays Using CV-1 Cells**

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## AR TA Assays Using CV-1 Cells

Reference	Hartig et al. (2002)	Kelce et al. (1995)
<b>Characteristics of Cell Line</b>		
Name of cell line	CV-1	CV-1
Cell source	Monkey kidney	Monkey kidney
<b>Transfection of Cells with Plasmids</b>		
Stable or transient transfection	Transient AR/Transient reporter	Transient AR/Transient reporter
AR expression vector	Ad5 hAR	pCMVhAR
AR source	human	human
Reporter vector	MMTV-Luc	MMTV.luciferase
Endpoint measured	Luciferase activity	Luciferase activity
Plasmid transfected for cell toxicity	none	none
Endpoint measured for cell toxicity	n.a.	n.a.
<b>Preparation of Cells for Assay</b>		
Pregrowth of cells before transient transfection	24 hours	n.p.
Time from transient transfection to treatment of cells	4 hours	24 and 48 hours
<b>Transcriptional activation assay</b>		
Test substance solvent	Ethanol	n.p.
No. of replicates	3 to 4	n.p.
No. of times assay repeated	n.p.	At least 4
Test substance incubation time	48 hours	53 hours
<b>Agonism assay</b>		
Reference ligand	5 -Dihydrotestosterone	n.a.
Final concentration of reference ligand	0.1 nM	n.a.
<b>Antagonism assay</b>		
Reference ligand	5 -Dihydrotestosterone	5 -Dihydrotestosterone
Final concentration of reference ligand	0.1 nM	0.1 nM

Abbreviations: n.a. = not applicable;  
n.p. = not provided

## AR TA Assays Using CV-1 Cells

Reference	Kemppainen and Wilson (1996)	Kemppainen et al. (1992)
<b>Characteristics of Cell Line</b>		
Name of cell line	CV-1	CV-1
Cell source	Monkey kidney	Monkey kidney
<b>Transfection of Cells with Plasmids</b>		
Stable or transient transfection	Transient AR/Transient reporter	Transient AR/Transient reporter
AR expression vector	pCMVhAR	pCMVhAR
AR source	human	human
Reporter vector	MMTV-Luc	pMTV29VTM
Endpoint measured	Luciferase activity	CAT activity
Plasmid transfected for cell toxicity	none	none
Endpoint measured for cell toxicity	n.a.	n.a.
<b>Preparation of Cells for Assay</b>		
Pregrowth of cells before transient transfection	n.p.	24 hours
Time from transient transfection to treatment of cells	n.p.	n.p.
<b>Transcriptional activation assay</b>		
Test substance solvent	n.p.	n.p.
No. of replicates	n.p.	n.p.
No. of times assay repeated	At least 3	At least 6
Test substance incubation time	30 hours	n.p.
<b>Agonism assay</b>		
Reference ligand	5 $\alpha$ -Dihydrotestosterone	Methyltrienolone
Final concentration of reference ligand	n.p.	n.p.
<b>Antagonism assay</b>		
Reference ligand	5 $\alpha$ -Dihydrotestosterone	Methyltrienolone
Final concentration of reference ligand	0.1 nM	n.p.

Abbreviations: n.a. = not applicable;  
n.p. = not provided



## AR TA Assays Using CV-1 Cells

Reference	Kemppainen et al. (1999)	Lambright et al. (2000)
<b>Characteristics of Cell Line</b>		
Name of cell line	CV-1	CV-1
Cell source	Monkey kidney	Monkey kidney
<b>Transfection of Cells with Plasmids</b>		
Stable or transient transfection	Transient AR/Transient reporter	Transient AR/Transient reporter
AR expression vector	pCMVhAR	pCMVhAR
AR source	human	human
Reporter vector	MMTV-Luc	MMTV-Luc
Endpoint measured	Luciferase activity	Luciferase activity
Plasmid transfected for cell toxicity	none	none
Endpoint measured for cell toxicity	n.a.	n.a.
<b>Preparation of Cells for Assay</b>		
Pregrowth of cells before transient transfection	24 hours	n.p.
Time from transient transfection to treatment of cells	4 hours	24 and 48 hours
<b>Transcriptional activation assay</b>		
Test substance solvent	n.p.	n.p.
No. of replicates	n.p.	n.p.
No. of times assay repeated	At least 3	n.p.
Test substance incubation time	4 hours	5-6 hours
<b>Agonism assay</b>		
Reference ligand	5 -Dihydrotestosterone	n.a.
Final concentration of reference ligand	n.p.	n.a.
<b>Antagonism assay</b>		
Reference ligand	5 -Dihydrotestosterone	5 -Dihydrotestosterone
Final concentration of reference ligand	0.1 nM	0.1 nM

Abbreviations: n.a. = not applicable;  
n.p. = not provided

## AR TA Assays Using CV-1 Cells

Reference	Van Dort et al. (2000)
<b>Characteristics of Cell Line</b>	
Name of cell line	CV-1
Cell source	Monkey kidney
<b>Transfection of Cells with Plasmids</b>	
Stable or transient tranfection	Transient AR/Transient reporter
AR expression vector	mouse AR (not defined)
AR source	mouse
Reporter vector	pSV2-CAT
Endpoint measured	CAT activity
Plasmid transfected for cell toxicity	none
Endpoint measured for cell toxicity	n.a.
<b>Preparation of Cells for Assay</b>	
Pregrowth of cells before transient transfection	n.p.
Time from transient transfection to treatment of cells	n.p.
<b>Transcriptional activation assay</b>	
Test substance solvent	n.p.
No. of replicates	n.p.
No. of times assay repeated	3
Test substance incubation time	n.p.
<b><i>Agonism assay</i></b>	
Reference ligand	5 $\alpha$ -Dihydrotestosterone
Final concentration of reference ligand	n.p.
<b><i>Antagonism assay</i></b>	
Reference ligand	n.a.
Final concentration of reference ligand	n.a.

Abbreviations: n.a. = not applicable;  
n.p. = not provided

## **Appendix A4**

### **AR TA Assays Using Yeast Cells**

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## AR TA Assays Using Yeast Cells

Reference	Gaido et al. (1997)	Moffat et al. (2001)
<b>Characteristics of Yeast</b>		
Species	<i>S. cerevisiae</i>	<i>S. cerevisiae</i>
Strain	YPH500	n.p.
<b>Transfection of Cells with Plasmids</b>		
Stable or transient transfection	Stable	Stable
AR expression vector	CUP1-Met hAR	hAR
AR source	human	human
Reporter vector	ARE- gal	lacZ
Endpoint measured	-galactosidase activity	-galactosidase activity
Plasmid transfected for cell toxicity	none	none
Endpoint measured for cell toxicity	n.a.	n.a.
Plating time prior to treatment with test substance	Cultured overnight, then grown to early-mid log phase	n.p.
<b>Transcriptional activation assay</b>		
Test substance solvent	n.p.	Ethanol
No. of replicates	n.p.	n.p.
No. of times assay repeated	3 to 5	n.p.
Test substance incubation time	overnight	2 days
<b>Agonism assay</b>		
Reference ligand	5 $\alpha$ -Dihydrotestosterone	5 $\alpha$ -Dihydrotestosterone
Final concentration of reference ligand	n.p.	n.p.
<b>Antagonism assay</b>		
Reference ligand	n.a.	5 $\alpha$ -Dihydrotestosterone
Final concentration of reference ligand	n.a.	.005 $\mu$ M

Abbreviations: n.a. = not applicable;

n.p. = not provided

## AR TA Assays Using Yeast Cells

Reference	O'Connor et al. (1998)	O'Connor et al. (1999)
<b>Characteristics of Yeast</b>		
Species	<i>S. cerevisiae</i>	<i>S. cerevisiae</i>
Strain	YPH500	YPH500
<b>Transfection of Cells with Plasmids</b>		
Stable or transient transfection	Stable	Stable
AR expression vector	CUP1-Met hAR	CUP1-Met hAR
AR source	human	human
Reporter vector	n.p.	n.p.
Endpoint measured	-galactosidase activity	-galactosidase activity
Plasmid transfected for cell toxicity	none	none
Endpoint measured for cell toxicity	n.a.	n.a.
Plating time prior to treatment with test substance	overnight	overnight
<b>Transcriptional activation assay</b>		
Test substance solvent	Methanol	Methanol
No. of replicates	3	3
No. of times assay repeated	n.p.	n.p.
Test substance incubation time	3 hours	3 hours
<b>Agonism assay</b>		
Reference ligand	5 -Dihydrotestosterone	5 -Dihydrotestosterone
Final concentration of reference ligand	n.p.	n.p.
<b>Antagonism assay</b>		
Reference ligand	5 -Dihydrotestosterone	5 -Dihydrotestosterone
Final concentration of reference ligand	n.p.	n.p.

Abbreviations: n.a. = not applicable;

n.p. = not provided

## AR TA Assays Using Yeast Cells

Reference	O'Connor et al. (2000)
<b>Characteristics of Yeast</b>	
Species	<i>S. cerevisiae</i>
Strain	YPH500
<b>Transfection of Cells with Plasmids</b>	
Stable or transient tranfection	Stable
AR expression vector	CUP1-Met hAR
AR source	human
Reporter vector	n.p.
Endpoint measured	-galactosidase activity
Plasmid transfected for cell toxicity	none
Endpoint measured for cell toxicity	n.a.
Plating time prior to treatment with test substance	overnight
<b>Transcriptional activation assay</b>	
Test substance solvent	Methanol
No. of replicates	3
No. of times assay repeated	n.p.
Test substance incubation time	3 hours
<b><i>Agonism assay</i></b>	
Reference ligand	5 $\alpha$ -Dihydrotestosterone
Final concentration of reference ligand	n.p.
<b><i>Antagonism assay</i></b>	
Reference ligand	5 $\alpha$ -Dihydrotestosterone
Final concentration of reference ligand	n.p.

Abbreviations: n.a. = not applicable;

n.p. = not provided

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## **Appendix A5**

### **Miscellaneous AR TA Assays**

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## Miscellaneous AR TA Assays

Reference	Sonnenschein et al. (1989)	Takeo and Yamashita (2000)
<b>Characteristics of Cell Line</b>		
Name of cell line	LnCaP-FGC	EPC
Cell source	Human metastatic lymph node/prostatic adenocarcinoma	Carp skin tumor (epithelioma papulosum cyprini)
<b>Transfection of Cells with Plasmids</b>		
Stable or transient transfection	n.a.	reporter
AR expression vector	endogenous	pCMV-rtAR
AR source	human mutant AR	rainbow trout
Reporter vector	n.a.	pARE3TK-CAT
Endpoint measured	Cell proliferation	CAT activity
Plasmid transfected for cell toxicity	n.a.	none
Endpoint measured for cell toxicity	n.a.	n.a.
<b>Preparation of Cells for Assay</b>		
<i>Transient</i>		
Pregrowth of cells before transient transfection	n.a.	n.p.
Time from transient transfection to treatment of cells	n.a.	24 hours
<b>Transcriptional Activation Assay</b>		
Test substance solvent	n.p.	n.p.
No. of replicates	n.p.	n.p.
No. of times assay repeated	n.p.	3
Test substance incubation time	8 days	48 hours
<b>Agonism assay</b>		
Reference ligand	5 $\alpha$ -Dihydrotestosterone	n.a.
ligand	6 nM	n.a.
<b>Antagonism assay</b>		
Reference ligand	n.a.	n.a.
ligand	n.a.	n.a.

Abbreviations: n.a. = not applicable;  
n.p. = not provided

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# Appendix B

## *In Vitro* AR TA Assay Protocols

- B1 Protocol for CV1 + hAR + Luciferase Assay**  
(Provided by Dr. Elizabeth M. Wilson, Departments of Pediatrics and of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC, USA)
- B2 Protocol for CHO Cells + hAR + Luciferase Assay**  
(Provided by Dr. Anne Marie Vinggaard, Institute of Food Safety and Toxicology, Danish Veterinary and Food Administration, Soborg, Denmark)
- B3 Protocol for HepG2 Cells + Receptor + Reporter and/or  $\beta$ -gal Plasmids for Use in Steroid Hormone Receptor Assays**  
(Provided by Dr. Kevin Gaido, CIIT Centers for Health Research, Research Triangle Park, NC, USA)
- B4 Protocol for Yeast-Based Androgen Receptor Assay**  
(Provided by Dr. Kevin Gaido, CIIT Centers for Health Research, Research Triangle Park, NC, USA)
- B5 Development of new reporter gene assay systems for screening Endocrine Disrupters**  
(Provided by Drs. Mitsuru Iida and Teruhisa Kato, Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan)
- B6 Development of stably transfected cell lines to screen Endocrine Disrupters**  
(Provided by Drs. Mitsuru Iida and Teruhisa Kato, Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan)
- B7 Technical Perspective on the U.S. EPA Endocrine Disruptor Screening Program: *In Vitro* EDSTAC Guideline Protocols**  
(Provided by Dr. Grantley Charles, Toxicology and Environmental Research and Consulting, The Dow Chemical Company, Midland, MI, USA, and Dr. William Kelce, Pharmacia Corporation, Kalamazoo, MI, USA)

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## **Appendix B1**

### **Protocol for CV1 + hAR + Luciferase Assay**

**(Provided by Dr. Elizabeth M. Wilson, Departments of  
Pediatrics and of Biochemistry and Biophysics,  
University of North Carolina, Chapel Hill, NC, USA)**

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**6 cm dish CV1 LUCIFERASE ASSAY (hAR)**

Revised 1-25-02

Monday

1. recount cells: best to count 50-100 cells per 5x5 in hemocytometer, count 2 5x5 grids and average, count  $\times 10^4 =$  cells/ml
2. plate  $0.42 \times 10^6$  CV1 cells/6 cm dish by preparing large mix of cells and media so 4 ml media/plate containing 5% bovine calf serum, DMEM-H/20 mM Hepes (2 M Hepes stock, pH 7.2, filter), penicillin and streptomycin, 2 mM L-glutamine, spread cells evenly. Cells usually added from master mix; swirl often while adding cells to the plates.

Tuesday

prepare  $\text{CaPO}_4$  precipitates for groups of up to 6 plates using freshly made solutions: for large assays of same DNA, pool the precipitates before adding to the plates.

(a) make 2 M  $\text{CaCl}_2$ : 2.94 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  bring to 10 ml with sterile autoclaved  $\text{dH}_2\text{O}$ , filter sterilize

(b) make 2X HBS: 500 ml    8.2 g NaCl  
    12.5 ml 2 M Hepes  
    0.2 g  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$

bring to 500 ml with sterile autoclaved  $\text{dH}_2\text{O}$  from TC room, pH with 5 N NaOH (takes 150-200  $\mu\text{l}$ ), pH to 7.11-7.14, sterile filter, make 27.5 ml aliquots (25 ml needed for 100 6 cm plates), store frozen at  $-20^\circ\text{C}$

[for 50 ml 2XHBS: 14 ml 1 M NaCl, 0.25 ml 2 M Hepes Na salt, 750  $\mu\text{l}$  0.1 M  $\text{Na}_2\text{HPO}_4$ , bring to 45 ml with sterile  $\text{ddH}_2\text{O}$  (use sterile autoclaved water), add about 45  $\mu\text{l}$  5 M NaOH, pH to 7.11-7.14, filter sterilize, store pH electrode in pH 7 buffer, NOT  $\text{H}_2\text{O}$ ]

(c) prepare DNA one or more days before assay

make dilutions of DNA stocks so additions are  $\sim 3$ -10  $\mu\text{l}$

add expression and reporter DNA to bottom of 14 ml polystyrene round-bottom (17x100 mm) Falcon tubes, store frozen  $-20^\circ\text{C}$

50 ng pCMVhAR (or 10 ng pCMVhAR1-660 (ABC))

5  $\mu\text{g}$  MMTV-luciferase

[For PSA-luciferase use 5  $\mu\text{g}$  reporter/dish, 100 ng pCMVhAR/dish]

(d) per 6 cm dish, add to tubes containing DNA:

210  $\mu\text{l}$  sterile  $\text{H}_2\text{O}$

30  $\mu\text{l}$  2 M  $\text{CaCl}_2$  (final 0.125 M  $\text{CaCl}_2$ )

240  $\mu\text{l}$  2X HBS, vortex briefly, let sit 30 min at RT

vortex briefly, add 475  $\mu\text{l}$  of mix per well, return plates to incubator, incubate 4 h

example for 6 dishes:

DNA	$\text{H}_2\text{O}$	2 M $\text{CaCl}_2$	2XHBS
0.3 $\mu\text{g}$ pCMVhAR	1.26 ml (2 x 630 $\mu\text{l}$ )	180 $\mu\text{l}$	1.44 ml (2 x 720 $\mu\text{l}$ )
30 $\mu\text{g}$ MMTV-Luc			

- (e) aspirate plates, add 1.5 ml glycerol shock medium, incubate 3 min RT, aspirate, wash 4 ml PBS, aspirate, add 4 ml serum free, phenol red free DMEM-H, Hepes, P/S, glutamine  $\pm$  hormone, return to incubator for overnight

Glycerol Shock Medium: use 5% DMEM-H red

# dishes x 1.5 ml/dish = total volume (make extra)

total volume x 15% = amount of glycerol

total volume - amount of glycerol = amount of media to add with glycerol

Wednesday aspirate media, add 4 ml fresh phenol red-free, serum-free DMEM-H, P/S, Hepes, glutamine ± hormone, add DHT to stock media as needed and add to plates, incubate overnight 37°C

Thursday remove media, wash with 4 ml PBS, aspirate to dry; add 0.5 ml/plate lysis buffer; rock plates 20-30 min, not much longer at RT. Transfer 100 µl from each well to 96 well Nunc flat bottom standard nontreated white plate. Read on LumiStar automated luminometer that injects 100 µl luciferin stock and 100 µl reading buffer

Lysis buffer: 2 mM EDTA, 1% Triton X-100, 25 mM Trizma (Tris base) phosphate, pH 7.8

D-Luciferin: prepare 1 mM D-luciferin (K<sup>+</sup> salt, MW 318.41) in dH<sub>2</sub>O store in 10 ml aliquots at -20°C covered with foil (D-luciferin is light sensitive) use 100 µl/sample, save extra at -20°C, Na<sup>+</sup> salt sometimes turns yellow but is probably still good, pH of H<sub>2</sub>O might be off, better to use K<sup>+</sup> salt) (from Analytical Luminescence)

Reading buffer: The optimal pH for the reaction is pH 7.8 cold; if glycylglycine and ATP are carefully pH cold, then the final will be pH 7.8

Stock	Amount to <u>20 ml final</u>	Amount to <u>100 ml final</u>	<u>Final conc</u>
0.5 M glycylglycine, pH 7.8 cold	1 ml	5 ml	25 mM
1 M MgCl <sub>2</sub>	300 µl	1.5 ml	15 mM
100 mM ATP in dH <sub>2</sub> O (bring to pH 7.8 cold with 1 M NaOH, CRITICAL, store -80°C 1 ml aliquots) Sigma - tissue culture grade	1 ml	5 ml	5 mM
50 mg/ml BSA dH <sub>2</sub> O	200 µl	1 ml	0.5 mg/ml
dH <sub>2</sub> O	17.5 ml	87.5 ml	
need 40 ml for 100 plates			

## **Appendix B2**

### **Protocol for CHO Cells + hAR + Luciferase Assay**

**(Provided by Dr. Anne Marie Vinggaard, Institute of Food Safety and Toxicology, Danish Veterinary and Food Administration, Soborg, Denmark)**

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**Protocol for CHO Cells + hAR + Luciferase Reporter Gene Assay**

**Day 1** A suspension of CHO cells (ATCC batch no. ) is made in DMEM/F12 media supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma, St.Louis,MO) and with 10% DCC serum (BioWhitaker). A 75cm<sup>2</sup> cell culture flask with almost confluent CHO cells is washed with 5 ml PBS and is trypsinated with 3 ml 0.05 % trypsin containing 0.02 M EDTA at 37 °C. Cells are diluted and cell number is determined (approx.  $120 * 10^4$  cells/ml). Calculate how much cell suspension, that is necessary and dilute the cell suspension with media so that every well is added 7000 cells (70,000 cells/ml). Cells are seeded in white microtiter plates from Packard with 100 µl / well.

**Passage of cells**

**Day 2** 9a.m.: Cells are approx. 90 % confluent. Cells are transfected with cDNA and FuGene 6 (Roche) according to the table. Cells are incubated for 5 hours (14 a.m.) Turn the plates on paper towel and add media +/- hormones and chemicals (p.3). Cells are incubated for 20 hours (11a.m)

**Day 3** Remove media by turning the plates on paper towel.

MgCl<sub>2</sub> is added to lysis buffer. All wells are added 20µl lysis buffer and incubated for 15 min on a shaker. Prepare luciferin solution containing (2 ml luciferin/ATP is added 2 ml lysis buffer with Mg Cl<sub>2</sub>). Protect from light. Measure luciferase activity on the BioOrbit Galaxy Luminometer directly in the plates by injection of 40 µl luciferin solution per well. The chemiluminiscense generated from each well is measured over a 1 sec interval after an incubation time of 2 sec.

Transfection scheme

The expression vector pSVAR0, AR13 and the MMTV-LUC reporter plasmid were both provided by Dr. Albert Brinkmann, Erasmus University, Rotterdam.

Optimum AR reporter gene assay conditions:

For 200 wells:

DMEM/F12	: 940 $\mu$ l	} 5 $\mu$ l / well
Fugene	: 60 $\mu$ l	
DNA (totally)	: 15 $\mu$ g	

75 ng DNA per well

FuGene ( $\mu$ l) / DMEM+FuGene ( $\mu$ l) = 0.06

DNA  $\mu$ g / Fugene  $\mu$ l = 0.25

psvAR0 , MMTV-Luc = 1 : 100

AR13 , MMTV-Luc = 2 : 100 (for cytotoxicity determination)

	$\mu$ g DNA	Plate 1 - 6	Plate x (cytotoxicity)
DMEM/F12 without serum		3290 $\mu$ l	600 $\mu$ l
FuGene		210 $\mu$ l	38.3 $\mu$ l
psvAR0 0.1 $\mu$ g/ $\mu$ l (batch no. )	0.53 $\mu$ g	5.3 $\mu$ l	AR13(0.082 $\mu$ g/ $\mu$ l) 3.11 $\mu$ l
MMTV-Luc 2.252 $\mu$ g/ $\mu$ l (batch no. )	51.97 $\mu$ g	23.1 $\mu$ l	6.087 $\mu$ l

DMEM/F12 without serum is added to a 15 ml plastic vial. FuGene is added without touching the walls of the vial. Gently mix and incubate for 5 min at room temperature. cDNA is added to another 15 ml vial. The diluted FuGene solution is added drop-wise to the cDNA. Gently mixing. The solution incubates for 15 min at room temperature. 8\* 250  $\mu$ l is added to a column in a microtiter plate. 5  $\mu$ l is added to the each well containing the cells using a 100 $\mu$ l 8-channel pipette. Be sure that the cDNA is distributed well (DNA should lie as grain of sand in the media instantly or the day after).

## Overview of plates

Don't use row A and H

**50  $\mu$ l compound + 50  $\mu$ l media** is added according to the scheme

	<b>Final conc.:</b>	
<b>Plate 1</b>	<b>Compound x</b>	
Row B:	<b>0.01 nM R1881</b>	<b>0.02 nM R1881</b> x 12 wells + media
Row C:	0.025, 0.05, 0.10	<b>uM</b> x 4 wells + <b>0.02 nM R1881</b>
Row D:	0.20, 0.39, 0.78	-----” -----
Row E:	1.56, 3.13; 6.25	-----” -----
Row F:	12.5, 25, 50	-----” -----
Row G:	<b>0.01 nM R1881</b>	<b>0.02 nM R1881</b> x 12 wells + media
<b>Plate 2</b>		
Row B:	0; 0.001; 0.0023 nM	R1881 x 4 wells + media
Row C:	0.01; 0.023; 0.1 nM	R1881 x 4 wells + media
Row D:	0.23; 1.0; 2.3 nM	R1881 x 4 wells + media
Row E:	0; 1; 5 nM	OHF x 4 wells + <b>0.02 nM R1881</b>
Row F:	10; 50; 100 nM	OHF x 4 wells + <b>0.02 nM R1881</b>
Row G:	500; 1000; 5000nM	OHF x 4 wells + <b>0.02 nM R1881</b>

Solvents

Hydroxyflutamide and R1881: Stock solutions in freezer no.

**Positive antagonism control: Hydroxyflutamide (2 x conc.)**

10.000 nM:	5 $\mu$ l 5 mM + 2.5 ml media
2000 nM:	5 $\mu$ l 1 mM + 2.5 ml media
1000 nM:	5 $\mu$ l 500 $\mu$ M + 2.5 ml media
200 nM:	5 $\mu$ l 100 $\mu$ M + 2.5 ml media
100 nM:	5 $\mu$ l 50 $\mu$ M + 2.5 ml media
20 nM:	5 $\mu$ l 10 $\mu$ M + 2.5 ml media
10 nM:	5 $\mu$ l 5 $\mu$ M + 2.5 ml media
2 nM:	5 $\mu$ l 1 $\mu$ M + 2.5 ml media

**Positive agonism control: R1881 (2 x conc.)**

20 nM:	5 $\mu$ l 10 $\mu$ M + 2.5 ml media – (not to be added)
4.6 nM:	500 $\mu$ l 20 nM + 1.665 ml media
2 nM:	5 $\mu$ l 1 $\mu$ M + 2.5 ml media
0.46 nM	500 $\mu$ l 2 nM + 1.665ml media
0.2 nM:	5 $\mu$ l 0.1 $\mu$ M + 2.5 ml media
0.02 nM:	<b>40 <math>\mu</math>l 0.01 <math>\mu</math>M + 20 ml media</b>
0.046 nM	500 $\mu$ l 0.2 nM + 1.665 ml media
0.002 nM:	5 $\mu$ l 0.001 $\mu$ M + 2.5 ml media
0.0046 nM:	500 $\mu$ l 0.02 nM + 1.665 ml media
0 nM:	5 $\mu$ l EtOH + 2.5 ml media.

**Compound x (2 x conc.)**

100 $\mu$ M:	25 $\mu$ l 20 mM + 5 ml media
50.0 $\mu$ M:	1 ml 100 $\mu$ M + 1 ml media
25.0 $\mu$ M:	1 ml 50 $\mu$ M + 1 ml media
12.5 $\mu$ M:	1 ml 25 $\mu$ M + 1 ml media
6.25 $\mu$ M:	1 ml 12.5 $\mu$ M + 1 ml media
3.13 $\mu$ M:	1 ml 6.25 $\mu$ M + 1 ml media
1.56 $\mu$ M:	1 ml 3.13 $\mu$ M + 1 ml media
0.78 $\mu$ M:	1 ml 1.56 $\mu$ M + 1 ml media
0.39 $\mu$ M:	1 ml 0.78 $\mu$ M + 1 ml media
0.20 $\mu$ M:	1 ml 0.39 $\mu$ M + 1 ml media
0.10 $\mu$ M:	1 ml 0.20 $\mu$ M + 1 ml media
0.05 $\mu$ M:	1 ml 0.10 $\mu$ M + 1 ml media



Media, buffers, compounds etc.

Lysis buffer: 25 mM Trisphosphate pH 7.8 (adjusted with phosphoric acid)  
 15 % glycerol  
 1 % Triton X-100  
 1 mM DTT  
 Stored in sterile 50 ml vials at -20°C  
 Before use: add 8 mM MgCl<sub>2</sub> (8 µl 1M MgCl<sub>2</sub>/ml buffer)

Requirement: x \* 10 ml lysis buffer + x \* 4.5 ml luciferin/ATP  
 (Freezer no. )

Requirement of media: DMEM/F12 + 10 % DCC x ml + x ml for counting xx ml  
 DMEM/F12 + 10 % FBS for a 25cm<sup>2</sup> flask xx ml  
 DMEM/F12 + 1% PSF xx ml

Compounds: Comp.1 Mw. xx g/mol  
 Supplier: Lot no.: Purity:  
 Stocksolution of 20 mM (x mg to x ml EtOH)  
 Date: Person:  
 Remarks:

Ethanol: Merck pro analysis UN 1170, K 27773283-020

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## **Appendix B3**

### **Protocol for HepG2 Cells + Receptor + Reporter and/or $\beta$ -gal plasmids for Use in Steroid Hormone Receptor Assays**

**(Provided by Dr. Kevin Gaido, CIIT Centers for Health Research, Research Triangle Park, NC, USA)**

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## TransIT Transfection Method of HepG2 Cells for Use in Steroid Hormone Receptor Assays

### 1. MATERIALS AND SOURCES:

- a. TransIT-LT1 Transfection Reagent, supplier: Mirus Corporation, CAT. #: MIR 2300.
- b. 1X Phosphate Buffered Saline Solution.
- c. Plasmid DNA's of choice: i.e., receptor, reporter, and/or -gal plasmids.
- d. Phenol red-free Minimum Essential Medium (MEM).
- e. Complete phenol red-free Minimum Essential Medium (MEM), with stripped (or charcoal/dextran treated) fetal bovine serum.
- f. 0.02% EDTA.
- g. Trypsin, 2.5%.
- h. Dimethyl sulfoxide.
- i. 1M Sodium pyruvate.
- j. L-glutamine (100X).

### 2. EQUIPMENT AND SUPPLIES:

- a. Incubator with 5% CO<sub>2</sub>/air, 37°C
- b. Vortexer
- c. 10 µl, 100 µl, 200 µl, and 1000 µl Eppendorf pipettor or equivalent
- d. pipet tips
- e. 1, 2, 5, 10, 25, and 50 ml pipets
- f. 500 ml screw cap glass bottles, sterile
- g. 24 well tissue culture plates
- h. 15 and 50 ml centrifuge tubes, sterile, polypropylene
- i. 17x100, polypropylene snap-cap tubes, sterile, round bottom
- j. 1.5 ml siliconized polypropylene screw-cap vials

### 3. PREPARATION:

#### a. 0.12% Trypsin/0.02% EDTA.

In 500 ml sterile screw cap glass bottle, sterilely transfer 190 ml 0.02% EDTA. Add 10 ml of 2.5% trypsin. Store at 4°C.

#### b. Complete phenol red-free MEM.

To 500 ml of phenol red-free MEM, add 0.5 ml 1M sodium pyruvate solution, 10.0 ml glutamine, and 50 ml resin-stripped (or charcoal dextran treated) fetal bovine serum. Store 4°C.

### c. Chemicals.

Dissolve chosen chemical to make a 0.1M stock solution using appropriate vehicle. Make serial dilutions in 1.5 ml polypropylene screw-cap vials to yield a standard curve of concentrations varying from  $10^{-5}$  M to  $10^{-11}$  M (may be changed as necessary).

## 4. PROCEDURE:

### Plating Cells.

- a. Aspirate medium from 150 mm plate of 75-80% confluent HepG2 cells and rinse with 10 ml of 0.02% EDTA.
- b. Place 10 ml of 0.12% trypsin/0.02% EDTA on plate.
- c. Place in incubator until cells begin to detach (~5 min).
- d. After cells have detached, pipette vigorously to remove the cells and transfer to 50 ml polypropylene centrifuge tube containing complete phenol red-free MEM.
- e. Rinse plate with complete phenol red-free MEM and add to tube.
- f. Centrifuge at 1000 RPM for 5 min at 4°C.
- g. Carefully aspirate supernatant and resuspend the pellet in phenol red-free complete MEM.
- h. Take cell count. Plate cells in 24-well tissue culture dishes at  $10^5$  cells/0.5 ml complete phenol red-free MEM. Swirl the plate gently to spread cells evenly in wells.
- i. Place cells in 37°C incubator with 5% CO<sub>2</sub>/air for 18 hours.

### Transfecting Cells.

In a 17x100 ml round bottom, polypropylene, snap cap tube, add the following reagents: (For transfection of a 24-well tissue culture plate)

1. 0.65 ml of phenol red-free MEM without any additives.
2. Appropriate amount of TransIT LT1 reagent. For every µg of DNA plasmid, add 2 µl of TransIT LT1 reagent. (11 µl of TransIT LT-1 reagent is needed for the suggested amounts of plasmid listed in 3. Below.) Mix very gently and let sit at RT for at least 5 min.
3. Carefully add appropriate amounts of receptor, promoter, and -gal plasmids. This may vary depending on the application. A suggestion for amounts is as follows:

Androgen Assay

Receptor Plasmid: 7 ng/well  
pCMV Plasmid ( -gal): 30 ng/well  
Promoter Plasmid: 200 ng/well

4. Mix very gently and let sit at RT for at least 5 min.
5. To each well of the 24 well plate containing HepG2 cells, carefully add 25  $\mu$ l of the TransIT/DNA complex.
6. Place plate in incubator and allow to incubate for 3 hr at 37°C.

Treating cells.

1. Dilute chosen chemicals 1:1000 in complete phenol red-free MEM, to create final concentrations ranging from  $10^{-5}$  to  $10^{-11}$ M (this may vary as necessary).
2. After the 3 hr incubation, aspirate the media and add 0.5 ml/well of the chemical diluted in media.
3. Return plate to incubator and incubate for 24 hr. Collect cell lysate for -gal and luciferase assays.

## Lysis Procedure

### 1. MATERIALS:

- D. Phosphate Buffered Saline (1X PBS).
- E. Tris base.
- F. Trans-1, 2-diaminocyclohexane-N, N, N', N'-tetraacetic acid (CDTA).
- G. Glycerol.
- H. Phosphoric Acid.
- I. Triton X-100.
- J. 1M Dithiothreitol (DTT).
- K. Transfected cells plated in 24-well plate.

### 2. EQUIPMENT AND SUPPLIES:

- k. 1-200  $\mu$ l Pipettor
- l. Multi-channel pipettor, 1-100  $\mu$ l
- m. 1-200  $\mu$ l pipette tips
- n. Pipette aid
- o. Vacuum system with hazardous waste flask attached
- p. pH meter
- q. 5 3/4" Pasteur pipette
- r. 500 ml squeeze water bottle
- s. 96 well ELISA plate
- t. 96 well Plate, white
- u. 250 ml Glass beakers
- v. 100 and 200 ml Graduated cylinders
- w. Stirrer and stir bars

### 3. PREPARATION:

- a. **5X Lysis Solution.**
  - d. Weigh out 3.03g Tris Base and 0.695g CDTA and place in 250 ml beaker.
  - e. Dissolve completely in 60 ml of dH<sub>2</sub>O.
  - f. Measure 100 ml glycerol in 100 ml graduated cylinder, pour into fresh 250 ml beaker.
  - g. Rinse 100 ml cylinder with Tris base/CDTA. Add to glycerol in 250 ml beaker. Mix well.
  - h. pH to 7.8 with phosphoric acid (H<sub>3</sub>PO<sub>4</sub>) if necessary.
  - i. Add dH<sub>2</sub>O to 200 ml.



- j. Add 5 ml of 100% Triton X-100 (solution will look cloudy/milky). Store room temperature.
  - b. **1X Lysis Solution.**  
In 50 ml centrifuge tube, dilute 5X Lysis Solution to 1X by diluting 1 ml 5X lysis solution into 4 ml dH<sub>2</sub>O. Add 30  $\mu$ l 1M DTT per 10 ml 1X lysis solution. Make fresh each time. Make up enough 1X lysis solution to dispense 65 $\mu$ l per well.
4. **PROCEDURE:**
  - a. Aspirate media from wells and rinse with 0.5ml of PBS per well.
  - b. Aspirate PBS from wells and with multi-channel pipettor; dispense 65  $\mu$ l of 1X lysis solution per well.
  - c. Let sit at room temperature for 20 min, rocking occasionally.
  - d. Transfer 30  $\mu$ l of cell lysate to 96 well ELISA plate. This will be used for the  $\beta$ -galactosidase assay.
  - e. Transfer 20  $\mu$ l of cell lysate to a 96 well white plate. This will be used for the luciferase assay.

## **$\beta$ -Galactosidase Assay Using Chlorophenol Red- $\beta$ -D-galactopyranoside**

### **1. MATERIALS AND SOURCES:**

- a. Chlorophenol red- $\beta$ -D-galactopyranoside (CPRG).
- b. Disodium phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ ).
- c. Monosodium phosphate ( $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ).
- d. Potassium chloride (KCl).
- e. Magnesium sulfate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ).
- f. -Mercaptoethanol (2-ME).

### **2. EQUIPMENT AND SUPPLIES:**

- A. Spectrophotometric microplate reader, with a 575 nm filter and kinetics capability
- B. Multi-channel pipettor
- C. Graduated cylinder, 1000 ml
- D. Balance
- E. Stir plate
- F. Magnetic stir bar
- G. 1-100  $\mu\text{l}$  pipettor
- H. 1-100  $\mu\text{l}$  pipet tips
- I. Pipettor reservoirs
- J. 0.2  $\mu$  Filter unit
- K. 96 well ELISA plate
- L. 1 L beaker
- M. 50 ml centrifuge tube, polypropylene, sterile

### **3. CPRG BUFFER PREPARATION:**

- j. Weigh out in 1 L beaker:

16.1 g	$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$
5.5 g	$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$
0.75 g	KCl
0.25 g	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
- k. Dissolve in 800 ml of distilled water with stirring.
- l. Adjust pH to 7.8.
- m. Transfer to 1000 ml graduated cylinder. Bring up to 1000 ml with distilled water.
- n. Filter sterilize. Store at room temperature.

**4. ASSAY PROCEDURE:**

- a. Pipet 30  $\mu$ l of cell lysate into a 96 well plate (usually done in triplicate).
- b. PER WELL OF 96 WELL PLATE , add 170  $\mu$ l of CPRG reagent made up as follows: 80  $\mu$ g CPRG dissolved in 20  $\mu$ l distilled water, 150  $\mu$ l of CPRG buffer, and 0.84  $\mu$ l 2-ME (1/200 dilution).
- c. Using multi-channel pipettor, dispense 170  $\mu$ l of CPRG reagent into each well containing lysate. For plate blank, use 30  $\mu$ l of lysis solution and add 170  $\mu$ l of CPRG reagent.

Set spectrophotometer microplate reader to kinetic endpoint and read the plate at 575 nm at 1 min intervals for 30 min to obtain Vmax. Samples will change from yellow to dark red as reaction occurs.

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## **Appendix B4**

### **Protocol for Yeast-Based Androgen Receptor Assay**

**(Provided by Dr. Kevin Gaido, CIIT Centers for Health  
Research, Research Triangle Park, NC, U.S.)**

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## Yeast-Based Androgen Receptor Assay

### 1. MATERIALS:

- a. Yeast Nitrogen Base without Amino Acids
- b. D-(+)-Glucose (Dextrose)
- c. Copper Sulfate Pentahydrate
- d. 2-Mercaptoethanol
- e. Oxalyticase, EnzoGenetics, Corvallis, OR, USA, catalog# 0-105, 5 mg
- f. Glycerol
- g. Di-sodium phosphate ( $\text{Na}_2\text{HPO}_4$ )
- h. Monosodium phosphate ( $\text{NaH}_2\text{PO}_4$ )
- i. Potassium Chloride (KCl)
- j. Magnesium Sulfate ( $\text{MgSO}_4$ )
- k. 0-Nitrophenyl  $\beta$ -D-Galacto-Pyranoside (ONGP)
- l. Lauryl Sulfate (Sodium dodecyl sulfate)
- m. Sodium Chloride (NaCl)
- n. L-Lysine-HCl
- o. Adenine Sulfate
- p. L-Tryptophan
- q. Uracil

### 2. EQUIPMENT:

- a. Microplate reader with kinetics capability using 590 and 420 nm filters
  - b. Multi-channel pipetter
  - c. Graduated cylinders, 100, 500, and 1000 ml
  - d. Balance
  - e. Stir plate
  - f. Magnetic stir bars
  - g. 1-100  $\mu\text{l}$  pipetter
  - h. 1-10  $\mu\text{l}$  pipetter
  - i. 50 ml centrifuge tube racks
  - j. Spectrophotometer with a 600 nm filter
- k. pH meter
- l. Beakers, 1000 ml
- m. 30°C incubator with ability to shake 300 rpm
- n. Pipette aid
- o. Autoclave
- p. Culture flask, 125 ml

**3. SUPPLIES:**

- a. 1-100  $\mu$ l pipette tips
- b. 1-10  $\mu$ l pipette tips
- c. Multi-channel pipette reservoirs
- d. 96 well plate
- e. 50 ml centrifuge tubes, polypropylene, sterile
- f. 100, 500, and 1000 ml glass bottles, with screw cap, sterile
- g. 100, 500, and 1000 ml-0.2  $\mu$  filter units for sterilization
- h. 1.5 ml semi-micro cuvettes
- i. 1, 2, 5, 10, 25 ml pipettes
- j. Weigh boats
- k. 1.5 ml microfuge tube

**PREPARATION:****1. 10X Yeast Nitrogen Base without Amino Acids (YNB)**

- a. Weigh out 67g Yeast Nitrogen Base without Amino Acids.
- b. Place in 1000 ml graduated cylinder.
- c. Bring up to 1000 ml with distilled water.
- d. Mix with magnetic stir bar on stir plate.
- e. Filter sterilize with 1000 ml-0.2  $\mu$  filter unit. Transfer to 1000 ml sterile glass bottle.

**2. 20% Dextrose Stock**

- a. In 1000 ml beaker, dispense 800 ml distilled water, add magnetic stir bar, and place on magnetic stirrer.
- b. Weigh out 200g Dextrose
- c. Add Dextrose slowly to vigorously stirring distilled water.  
Filter sterilize with 1000 ml-0.2  $\mu$  filter unit. Transfer to 1000 ml sterile glass bottle.

**3. 10 mM Copper Sulfate**

- a. Weigh out 0.25g Copper Sulfate pentahydrate. Place in 100 ml graduated cylinder.
- b. Bring up to 100 ml with distilled water.
- c. Filter sterilize with 100 ml-0.2  $\mu$  filter unit. Transfer to 100 ml sterile glass bottle.



**4. 10% SDS**

- a. Weigh out 10g Lauryl Sulfate. Place in 100 ml graduated cylinder.
- N. Bring up to 100 ml with distilled water. Mix well.
- O. Transfer to 100 ml sterile glass bottle.

**5. 1M Sodium Chloride**

- a. Weigh out 58.44g NaCl. Place in 1000 ml graduated cylinder.
- b. Bring to 1000 ml with distilled water. Mix well.
- c. Filter sterilize with 1000 ml-0.2  $\mu$  filter unit. Transfer to 1000 ml sterile glass bottle.

**6. 50% Glycerol with 100mM NaCl**

- a. Put 50 ml glycerol into 100 ml graduated cylinder.
- b. Add 10 ml of 1M NaCl solution.
- c. Bring up to 100 ml with distilled water. Mix well.
- d. Transfer into 100 ml sterile glass bottle.

**7. Oxalyticase**

To 5 mg bottle of oxalyticase, add 1.11 ml of 50% Glycerol solution, making a 200U/ $\mu$ l solution. Mix well. Store at 4 °C.

**8. Z Buffer**

- a. Weigh out:
 

16.1 g	Na <sub>2</sub> HPO <sub>4</sub>
5.5 g	NaH <sub>2</sub> PO <sub>4</sub>
0.75 g	KCl
0.25 g	MgSO <sub>2</sub>
- b. Place in 1000 ml graduated cylinder.
- c. Bring up to 800 ml with distilled water.
- d. Adjust pH to 7.0 while stirring with stir bar on stir plate.
- e. Bring up to 1000 ml with distilled water.
- f. Filter sterilize with 1000 ml-0.2  $\mu$  filter unit. Transfer to 1000 ml sterile glass bottle.

**9. Amino Acids**

- a. LYS-1.8g L-lysine-HCl in 500 ml of distilled water. Autoclave.
- b. TRP-2.4 g L-tryptophan in 500 ml of distilled water. Filter sterilize with 500 ml-0.2  $\mu$  filter unit.
- c. URA-1.2 g uracil in 500 ml of distilled water. Autoclave.
- d. ADE-0.6 g adenine sulfate in 500 ml of distilled water. Autoclave.

**10. Growth Media for AR Transformed Yeast**

- a. Measure out 50 ml 10X YNB, 50 ml 20% Dextrose, 5 ml Lysine, 5 ml Tryptophan, 5 ml Uracil, and 17 ml Adenine in 500 ml graduated cylinder. Mix well.
- b. Bring up to 500 ml with distilled water.
- c. Filter sterilize with 500 ml-0.2  $\mu$  filter unit. Transfer to 500 ml sterile glass bottle.

## ASSAY:

1. Start an overnight culture of androgen receptor transformed yeast in growth media by making a 1:10 dilution of a log-phase culture of yeast.
2. Dilute the overnight culture of yeast in the morning by half in growth media. Start the assay in the afternoon
3. Dilute cells to an OD<sub>600</sub> of 0.06 in growth media.
4. Add 100  $\mu$ l 10 mM Copper Sulfate solution/20 ml growth media.
5. Dispense 5 ml diluted yeast solution into a 50 ml **polypropylene** centrifuge tube (1 tube per dose of chemical being tested and 1 tube per dose in dihydrotestosterone standard curve).
6. Add 5  $\mu$ l chemical or standard/50 ml tube. This is a 1:1000 dilution of the chemical to the diluted yeast cells.
7. Incubate over night (~18 hours) at 30 °C in shaking incubator at 300 rpm.
8. Following overnight incubation:
  - a. Make a 1:10 dilution of each tube in growth media and determine OD<sub>600</sub>.
  - b. Dilute samples to OD<sub>600</sub> of 0.25 in 1.5 ml microfuge tube.
  - c. Dispense 100  $\mu$ l of diluted yeast/well of a 96 well plate. Do each dose of chemical or standard in triplicate.
  - d. Determine OD<sub>590</sub> on microplate reader.
9. Set up plate reader to read blank and unknowns at 420 nm, for 20 minutes, with readings every minute.
10. Add 100  $\mu$ l of Assay Buffer to each well.

For 11 ml of Assay Buffer:

2mg/ml ONGP	22 mg
0.1% SDS	110 $\mu$ l 10% SDS
50 mM 2-Mercaptoethanol	29.7 $\mu$ l 2-ME
200 U/ml oxalyticase	11 $\mu$ l 200 U/ $\mu$ l oxalyticase
Z-Buffer	10.9 ml

- Make sure ONGP is in solution before adding SDS. Dilute ONGP in Z buffer in 50 ml polypropylene tube and vortex to mix.
  - Stable for 1 hour. Use immediately after preparation.
11. Start reading immediately on microplate reader set at 420 nm every minute for 20 minutes. Samples will turn yellow as reaction occurs.
  12. Determine V<sub>max</sub> (change in OD<sub>420</sub>/minute) for the linear portion of the reaction.
  13. Normalize the activity by calculating V<sub>max</sub>/OD<sub>590</sub>.

## **Appendix B5**

### **Development of new reporter gene assay systems for screening Endocrine Disrupters**

**(Provided by Drs. Mitsuru Iida and Teruhisa Kato, Otsuka  
Pharmaceutical Co. Ltd., Tokushima, Japan)**

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# Development of new reporter gene assay systems for screening Endocrine Disrupters

EcoScreen assay™ (high throughput transfection assay)

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## INTRODUCTION

There is a great need for effective in vivo screening methods for detecting (anti)estrogenic and (anti)androgenic chemicals. We have developed rapid and sensitive reporter gene assays for detection of the chemicals that have agonist and antagonist activity against the estrogen, androgen, and thyroid hormone receptors. We believe that these methods have the potential to become powerful tools for identifying endocrine disrupters.

## MATERIALS and METHODS

### Chemicals

17-beta-estradiol, 5-alpha-dehydroxy testosterone, T3, T4, dimethylsulfoxide (DMSO) and rat S-9/Cofactor A Set were from Wako (Osaka, Japan). MTT was from Dojin (Osaka Japan) ALAMABLUE™ from Serotec (Oxford UK). The test solutions were prepared from stock solutions in DMSO and then 10 times serial dilutions were made with DMSO and finally diluted 100 times in the culture media with no supplement (the final DMSO concentration in the media was 1.0%). The test samples were adjusted to the concentrations ranging from  $10^{-11}$  M to  $10^{-5}$  M.

### Samples for Estrogen and Androgen reporter assay (agonist activity detection)

The estrogen receptor agonist assay and androgen receptor agonist assay were carried out on 61 chemical compounds (See Appendix1 CHEMICAL LIST) designated by the Japanese Ministry of Economy and Industry for studies on the feasibility of screening for endocrine disrupters. All measurements were done in quadruplicate. We repeated this assay 2 times, and the results were in very good agreement. The configuration of the samples on a 96 well plate is shown in Figure 1-a and 1-b. The wells in row H contained positive and negative controls. Results are shown in figures in the appendix. Samples were identified as HTS “No”.

### **Samples for Estrogen and Androgen receptor antagonist activity detection assay**

The samples listed in Table 1a, and 1b were examined for activity as antagonists for ER and AR as described below.

#### **Plasmids**

##### **For estrogen receptor reporter gene assay**

*pGL3ERE-7*: an estrogen responsive reporter plasmid harboring the TATA box from herpes simplex virus thymidine kinase (tk) promoter (1) and four copies of estrogen response element (2), linked to the luciferase gene.

*pcDNA ER-alpha*: mammalian expression vector for estrogen receptor-alpha with Zeocin resistant gene.

##### **For androgen receptor reporter gene assay**

*pIND ARE B10*: contains the hygromycin resistant gene and 4 copies of the androgen response element: (AGTACG nnn TGTTCT) from the C3 gene (3), linked to the luciferase gene.

*pZeoSV2AR*: An expression plasmid with the androgen receptor driven by the SV40 promoter, and the Zeocin resistance gene.

##### **For thyroid hormone receptor reporter gene assay**

*PINDTRE*: contains 4 copies of the thyroid response element TRE pal: GGTCATGACC(5) linked to the luciferase gene.

*pZeoSV2TR-beta*: expression plasmid containing the thyroid hormone receptor-beta driven by SV40 promoter and the Zeocin resistance gene

##### **For cell viability/non specific inhibition assay**

*pGL3 control*: luciferase expression vector driven by CMV promoter

*pcDNA-EGFP*: mammalian expression vector containing the green fluorescence protein cDNA.

### **Estrogen Receptor Agonist Activity detection assay.**

**1st day:** Chinese Hamster Ovary cells (CHO- K1) were maintained in DMEM/F12 supplemented with 100 U/ml penicillin, 100ug/ml streptomycin, and 10% fetal bovine serum. The cells were trypsinized and suspended at  $1 \times 10^5$ /ml. They were seeded with 84 ul of culture medium in 96 well microtiter plates (Nunclon™ #137101, NalgeNunc Denmark) in DMEM/F12 containing 5% charcoal-treated fetal bovine serum (Hyclone, Logan, UT) and incubated for 24 hr at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>.

#### **2nd day:**

**Preparation of Plasmid cocktail:** For one 96 well plate assay, 6 ug of pGL3ERE-7 and 60 ng of pcDNA ER-alpha (100:1) was added to a 1.5 ml eppendorf tube. The total volume of DNA solution was kept below 50 ul.

**Preparation of transfection mix per one 96 well plate:** 18 ug of nonliposomal transfection reagent Fugene™ (Roche Diagnostic Corp. IN USA) were added to 660 ul of DMEM/F12 (with no supplement) in a small sterile tube. Then the plasmid cocktail (see above) was added to the tube and incubated for 25 min at room temperature.

**Transfection:** 6 ul of the transfection mix were added into each well of the seeded 96 well plate by multi channel pipet, and then incubated 3 hr. After incubation, 10 ul of

each chemical diluted with the culture media (see Chemicals) were added, and the cells incubated for 16-24 hr.

**3rd day:** Followed incubation, 100 ul of the luciferase substrate with cell lysis reagent Steady-Glo™ (Promega) was added to all assay wells. After shaking at room temperature for 5 min. the luminescence was measured in an ARVO multi-label counter (Perkin-Elmer).

(see Appendix 2 for the scheme of the high throughput transfection assay)

#### Note 1

We found that the most stable and reproducible data were obtained when Nunclon™ plates (NalgeNunc, Denmark) were used. Plates from other manufacturers often gave high backgrounds, perhaps because ingredients in the plastic were stimulatory.

#### Note 2

Another source of variability is in the accuracy of dispensing the transfection mixture into the wells, if the distribution is done manually. The use of devices for automated delivery can reduce this source of error. However, if this is not feasible we have found that another seeding and transfection protocol is useful.

#### ***Alternative method for transfection***

**1st day:** CHO-K1 cells were trypsinized and prepared at a density of  $1 \times 10^5$ /ml. 11ml of cell suspension were placed in a sterile 50 ml conical tube (for one plate). The transfection mix was added (see original protocol) to the 50 ml conical tube, mixed gently, and incubated for 15 minutes. Then each well was seeded with 90 ul of cell suspension and incubated for 16-24 hr.

**2nd day:** 10 ul of test sample (see original protocol) were added to the wells and incubated for 16-24 hr.

**3rd day:** Same as original protocol.

(see Appendix 3 for the scheme of the alternative transfection method)

This method is easy to perform. Although the signal intensity may decrease because the transfection efficiency decreases, there is still sufficient intensity for measurement.

#### **Estrogen Receptor Antagonist Activity detection assay**

The protocol for antagonist activity detection assay is the same as agonist detection assay except that cell viability is evaluated by measuring the fluorescence of EGFP prior to the luminescence measurement. Only the differences between the protocols are described here.

1. Plasmid cocktail: 6 ug of pGL3ERE-7 and 60 ng of pcDNA ER-alpha + 480 ng of pcDNA-EGFP.
2. The test solutions were prepared using "Spiked Media" that contains  $5 \times 10^{-11}$  M of 17-beta-estradiol.
3. After the final incubation period of 24 hr, green fluorescence was measured (excitation: 485 nm, emission: 535 nm) prior to the luminescence measurement by the ARVO multi-label counter (Berthold).

### **Androgen Receptor and Thyroid Hormone Receptor Agonist / Antagonist Activity detection assay**

They are the same as described above except for the use of different plasmids for expression of each receptor and the luciferase-reporter containing the corresponding hormone response element.

#### ***For Androgen Agonist detection assay***

Plasmid cocktail: 6 ug of pIND ARE B10 and 240 ng of pZeoSV2AR.  
5-alpha dehydroxy-testosterone (DHT) was used as a positive control.

#### ***For Androgen Antagonist detection assay***

Plasmid cocktail: 6 ug of pIND ARE B10 and 240 ng of pZeoSV2AR + 480 ng of pcDNA-EGFP. The test solutions were prepared using "Spiked Media" that contains  $5 \times 10^{-9}$  M of 5-alpha-DHT

#### ***For Thyroid Hormone Receptor Agonist detection assay***

Plasmid cocktail: 6 ug of pINDTRE and 120 ng of pZeoSV2TR-beta.  
T3 was used as a positive control.

#### ***For Thyroid Hormone Receptor Antagonist detection assay***

Plasmid cocktail: 6 ug of pIND TRE and 120 ng of pZeoSV2TR-beta and 480 ng of pcDNA-EGFP  
The test solutions were prepared using "Spiked Media" which contains  $5 \times 10^{-8}$  M of T3.

#### ***Cell proliferation assay for evaluation of cell viability in antagonist activity assay***

CHO-K1 cells were transfected with 6 ug of pGL3 control plasmid and 480 ng of pcDNA-EGFP by the same method as the above-mentioned protocol with Fugene<sup>TM</sup>, and cultured with different concentrations of DMSO, from 0% to 10%, for 24 hr. DMSO inhibits cell growth, and thus serves as a model for a non specific expression and growth inhibitor. Then the luciferase activity and EGFP fluorescence were measured. The MTT assay and ALAMARBLUE<sup>TM</sup> cell proliferation assay were also done in order to determine the reliability of the GFP assay as an indicator of nonspecific inhibition/cytotoxicity in the actual antagonist detection assays.

### **Data Analysis**

#### ***Definition of PC50 (50% of Positive Reaction)***

Chemicals that can be used to determine an EC50 (half maximal activity of a particular compound) are limited to a small number that have a similar activity/toxicity profile as E2. This is because the activity curves of many compounds do not reach a plateau before the maximum tolerated dose is reached. In order to compare the activity of chemicals whose activity does not reach a plateau at the maximum tolerated dose we have defined the PC50 as the concentration of compound that corresponds to  $\frac{1}{2}$  the value of the transcriptional activity of the positive control ( $10^{-9}$  M of 17-beta-estradiol). Thus the PC50 can be used to rank compounds when true half maximal values cannot be determined because of toxicity (Refer to fig. 2). This PC50 concept is based on the guideline of the Japanese Ministry of Economy and Industry. The use of the PC50 removes the requirement for a standard curve at every measurement. (Only a solvent control and positive control at plateau level are needed). We have found that there is



very little variance in the PC50 value from experiment to experiment, even with discernable variation in luciferase activity due to differences in culture condition and transfection efficiency.

To determine the PC 50, one concentration of the standard at the maximal activity level was included in each assay as a positive control (for ER assay:  $10^{-9}$  M of 17-beta estradiol; for AR assay:  $10^{-8}$  M of 5alpha-DHT). In each assay the reaction curve of the sample (ranging from  $10^{-12}$  M to  $10^{-6}$  M), and the  $\frac{1}{2}$  maximal point was determined by analysis of the data by a Cubic Spline Curve Fitting Method using software designed by us.

## RESULTS AND DISCUSSION

### *Estrogen receptor agonist activity*

Table 2 shows the rank order of compounds that have ER agonist activity on the basis of the PC50 determination. DDT (HST0099) and DEE (HST0100) have detectable ER agonist activity, but do not reach the PC50 level (see Appendix 4: results of ER agonist assay). Assessment of compounds with weak activity requires the use of comparison standards adjusted to lower activity such as PC40 (40% of maximal positive reaction) or PC30 (30% of maximal positive reaction).

### *Androgen receptor agonist activity*

Table 3 shows the rank of the compounds that have AR agonist activity on the basis of the PC50. Most of the listed compounds were natural ligands or synthetic steroid hormones. The results are shown by the graph in appendix 3. Progesterone (HST0008) and Aldosterone (HST0009) showed only slight reaction, and RU486 (HST0087) and Cortisol (HST0099) were about 40% of the reaction of positive control at the highest concentration ( $10^{-6}$  M).

### *Thyroid hormone receptor agonist activity*

Four sub types of the thyroid hormone receptor (TR) are known: alpha1, alpha2, alpha3, and beta. We have performed assays with reporter plasmids for TR-alpha1 and beta. Figure 3 shows the result of agonist assay of TR-beta receptor. There was about a 20 fold induction relative to the solvent control (0.1% DMSO) with 100nM T3, with a detection limit of 500 pM and PC50 of 2 nM. We have not carried out large-scale screening for TR receptor. However, after testing about 100 compounds, we found that only T3 and T4 had clear agonist activity.

### **Determination of non-specific inhibition/cell toxicity in the antagonist activity assay**

In the antagonist activity assay a constant amount of the standard ligand was added to the test sample containing the unknown. The antagonist activity was observed as a decline in the luciferase activity. It is essential to distinguish a decline in luciferase activity due to true receptor antagonism from the non-specific inhibition of expression or cell toxicity that some compounds display.

Fig.4 shows how the reporter activity was affected by the nonspecific inhibitory activity of a test sample, using DMSO as an example. CHO-K1 cells were transfected with pcDNA-EGFP (green fluorescence protein expression plasmid) and pGL3 control (luciferase expression plasmid), and were cultured in various concentrations of DMSO. Expression of the markers in both plasmids is constitutive. In 4% DMSO the activity of EGFP and Luciferase fell to 5% or less of control. However, the ALAMABLUE™ assay and MTT assay reported 56% and 88%, respectively, of the 0% of DMSO control. This experiment showed that expression of genes on the plasmids was more sensitive to DMSO than the other assays. The MTT assay, which measures the reduction activity of the intracellular dehydrogenases, is widely used as an index of the cell proliferation or cell number. The ALAMABLUE™ assay is a simple method suitable for measuring large number of samples, and is said to be well correlated with the MTT assay. The ALAMABLUE™ method measures change of the reduction/oxidation state of the culture environment as a result of cell proliferation. However, our results indicate that these assays are not reliable indicators of nonspecific inhibition of plasmid gene expression. The pattern of decline in expression of EGFP was in good agreement with that of luciferase. Consequently we monitor nonspecific inhibitory/cell toxicity effects of the samples by measuring the expression of EGFP in the receptor activity assays. This is straightforward, and can be performed on living cells in a 96 well plate format. Usually this measurement is taken just before measuring the activity of luciferase. The advantage of this strategy is that both EGFP and luciferase assays can be performed on the same cells.

#### ***Estrogen receptor antagonist activity***

Fig.5a shows the result of the estrogen receptor antagonist assay for tamoxifen (CAS No.10540-29-1: anti-cancer drug). The GFP fluorescence is shown by the green line, and the luciferase activity by the yellow line. At the concentration of  $10^{-7}$  M, the luciferase activity was 18% of the control, while the GFP activity was about 100%. This shows that tamoxifen is an antagonist of the estrogen receptor. The antagonist activity of 4-hydroxy tamoxifen is about 100 times stronger than that of the tamoxifen (Fig.5b). With triphenyltin chloride (fig.5c), at  $10^{-6}$  M, GFP showed 93% of activity, while the luciferase activity was about 75% of control. In another set of experiments we compared the antagonistic activity of a styrene dimer with, or without, metabolic activation by incubation with a rat liver S9 preparation. We found that without S9 treatment 1-Methyl-1-phenylindan (styrene dimer,  $10^{-6}$  M) showed no receptor antagonistic activity, while with S9 treatment the activity of GFP (red triangle) was 91% of control while the luciferase activity (blue triangle) fell to 60%. Although this requires additional study, metabolites of this compound may have weak antagonist activity against the estrogen receptor, while the parent compound does not.

#### ***androgen receptor agonist activity***

Fig 6a-h shows the results of the androgen receptor antagonist detection assay. Cyproterone acetate (fig.6a) showed the strongest antagonist activity to the androgen receptor of all the compounds we have tested. Consequently we use cyprotenone acetate as a positive control in every measurement. Two pesticides, hydramethylnone (fig.6b) and tralomethrin (fig.6d), were judged to have no true antagonist activity, because the

decline in the luciferase activity was matched by the decline in GFP activity. Two pesticides (CNP: fig.6c, fenitroton: fig.6e) were clear antagonists. At  $10^{-6}$  M GFP expression was unaffected while luciferase activity was reduced to 23% (CNP) and 13% (fenitroton). Weaker antagonistic activity was shown by prothiofos (fig.6f) and vinclozolin (fig.6g).

Spironolactone (fig.6h) gave a biphasic activity curve. At low concentrations ( $10^{-8}$ – $10^{-6}$  M) in the presence of testosterone it was an antagonist, while at higher concentrations the antagonistic activity was reversed. In the absence of testosterone it was an agonist at high concentrations ( $10^{-5}$ – $10^{-6}$  M). These results suggest that for some compounds the definition of antagonist and agonist will have to be qualified by an indication of concentration and the presence of other ligands.

## CONCLUSION

Other reporter cell lines that constitutively express steroid receptors, in some cases several receptors, have been developed. For example, T47D expresses ER-alpha and ER-beta, Androgen, Progesterone, and Retinoic acid receptors. There is a Hela cell derivative that expresses the glucocorticoid receptor, while MCF-7 naturally expresses ER-alpha and ER-beta receptors. These lines will report the activity of compounds that stimulate any of the receptors and cannot distinguish which receptor(s) have been stimulated. The strategy we have employed measures the signal from only the receptor introduced by transfection since the CHO cells do not express any endogenous steroid receptor.

Our method can be considered a “ high throughput transfection assay ”. Generally these methods are thought to suffer from variability and lack of reproducibility, due in part to toxicity of the transfection reagent, and uncontrollable variation in cell culture conditions. However we have found that recently developed transfection reagents solve many of these problems. FuGene™ is in one of these reagents. A reporter gene assay using FuGene™ has been reported previously by Vingaard (4). This reagent does not show any toxicity to the cells and if methods for accurate delivery of reagents are established there is little intra-assay variation in measurement. We have found that the average intra-assay coefficient of variation was only 5.9% (CV5.9%) in assays of over a hundred compounds. Our method is simple and affords a significant reduction of lab work and produces reliable data. Actually our method does not have any medium exchange and plate washing step after seeding a cell on 96 well plate until measuring luminescence. If the measurement is carried out on the concentration of 4 doses in duplicate the cost per sample (except for personnel expenses) will be \$10 or less. In conclusion, our method is suitable for pre-screening a large number of environmental chemicals and should identify compounds that need further testing.

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## **Appendix B6**

### **Development of stably transfected cell lines to screen Endocrine Disrupters**

**(Provided by Drs. Mitsuru Iida and Teruhisa Kato, Otsuka  
Pharmaceutical Co. Ltd., Tokushima, Japan)**

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## Development of stably transfected cell lines to screen Endocrine Disrupters

**ER-EcoScreen assay™ and AR-EcoScreen assay™ (Stable CHO clones containing luciferase based reporter gene and expressing hormone receptors)**

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### INTRODUCTION

We have developed genetically engineered stable transfected cell lines, expressing hormone receptor and luciferase based reporter genes, for screening compounds and compound mixtures for endocrine disrupter activity. We have named the lines “**ER-EcoScreen™**” (expressing estrogen receptor) and “**AR-EcoScreen™**” (expressing androgen receptor). To establish these cell lines we introduced the plasmids used in our transient transfection Eco-Screen Assay™ systems. We have demonstrated that the cells have the same reactivity to the samples tested in the Eco-Screen Assay™ system. We also have confirmed that these cell lines do not lose reporter activity during continuous cell passage.

### METHODS

#### ***Stable transfection of Hormone Receptor and Reporter Gene in CHO-K1 cell***

About 16 hr prior to transfection, CHO-K1 cell were seeded at 50% confluence in a 6-well plate in 2 ml culture medium per well. Transfections were carried out with Fugene™ according to the Instruction Manual. For the estrogen receptor (ER) reporter assay, 12 ug of pINDERE-15 (containing the luciferase gene, under the control of the minimal heat shock promoter with 4 copies of the estrogen response element, as well as the hygromycin resistant gene) and 120 ng of pcDNA ER-alpha (estrogen receptor expression plasmid). For the androgen receptor (AR) reporter assay, 12 ug of pIND ARE B10 (4 copies of the androgen response element linked to luciferase, and the hygromycin resistance gene) and 480 ng of pZeoSV2AR (androgen receptor expression plasmid) were transfected per well. After 24 hr the cells were trypsinized and the cells from each well plated in two 100-mm petri dishes. The culture medium was replaced every three days with medium containing 200 ug/ml of Zeocin and 200 ug/ml of Hygromycin until colonies were large enough to isolate (about 10 days). Luciferase-positive clones were isolated using a photon detecting CCD camera (Night OWL, Perkin-Elmer). Briefly, clones were exposed to 0.2 nM luciferin and 1 nM E2 for the ER assay, 10 nM testosterone for the AR assay, for 24 hr, and then introduced into the CCD camera. Luminescence intensity was monitored for 10 min per dish and the

luminescence image from cell was superimposed on to the light field image of the cell clones in the dish. Positive clones were isolated using cloning rings and further cultured in 24 well plates. After growth each clone was trypsinized and seeded into 2 wells in two 96 well plate (Nunclon™ NalgeNunc Denmark) and further cultured. After 24 hr culture, cells in one plate were exposed to 0.1% DMSO as a control, while the cells in the other plate were incubated with 1nM of E2 for ER assay and 1nM of 5-alpha-dehydrotestosterone for AR assay, respectively. Followed 24 hr culture, 100 ul of the luciferase substrate with cell lysis reagent Steady-Glo™ (Promega) were added to all assay wells. After shaking at room temperature for 5 min the chemiluminescence was measured in the ARVO multi-label counter (Perkin-Elmer). The most responsive clone was selected.

#### ***Procedure for ER/AR-Eco screen assay™***

**1st day:** The most responsive CHO-K1 stable clone was maintained in DMEM/F12 supplemented with 100 U/ml penicillin, 100 ug/ml streptomycin, and 10% fetal bovine serum. The cell were trypsinized and prepared at a density of  $1 \times 10^5$ /ml, and then seeded with 90 ul of culture medium in 96 well microtiter plates (Nunclon™ #137101, NalgeNunc Denmark) in DMEM/F12 containing 5% charcoal-treated fetal bovine serum (Hyclone, Logan, UT) and incubated for 24 hr at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>/ air.

**2nd day:** After 24 hr culture 10 ul of sample solution from serial dilutions of each chemical with the culture media (see Chemicals on protocol 1) were added to the plates and cultured for 16-24 hr.

**3rd day:** Followed 24 hr culture, 100 ul of the luciferase substrate with cell lysis reagent Steady-Glo™ (Promega) were added to all assay wells. After shaking at room temperature for 5 min the chemiluminescence was measured by ARVO multi-label counter (Perkin-Elmer).

#### **Chemicals for Estrogen and Androgen reporter assay (agonist activity detection)**

The estrogen receptor agonist assay and androgen receptor agonist assay were carried out with 12 chemicals for ER-EcoScreen™ and AR-EcoScreen™, respectively (Table 1 and 2).

#### **Data Analysis**

We used the criteria of PC50 for data analysis. Refer to the “Definition of PC50” on a report 1 “Development of new reporter gene assay systems for screening Endocrine Disrupters.” The data were analyzed with software by applying Cubic Spline Curve Fitting Method. EC50 is also shown for reference.

## **RESULTS**

### **Clone stability for ER-Eco Screen™**

The cloned line (ER-Eco Screen™) that was stably transfected with pINDERE-15 and pcDNA ER-alpha showed stable expression over at least 15 passages during more than two months of culture (fig.1-a).



In over 10 assays, this clone reported about 3.5 fold induction with 100 pM E2 compared to solvent treatment (0.1%DMSO), with a detection limit of 0.5 pM. The PC50 was 9.2 pM.

#### **Clone stability for AR-Eco Screen™**

Our cloned AR-Eco Screen™ was stably transfected with pIND ARE B10 and pZeoSV2AR. It was responsive to DHT for 30 passages over three months culture (fig.1-b), as observed in over 10 assays. This clone showed about a 5 fold induction with 1nM DHT compared to solvent (0.1%DMSO), with a detection limit of 15 pM. The PC50 was 153 pM.

#### ***Estrogen receptor agonist activity***

Table 1 shows the rank of the compounds that had ER agonist activity on the basis of the PC50. Although there were slight differences, the ranking on the basis of PC50 was almost the same as that of the high throughput transfection assay. (Refer to Table 1 on the report of “high throughput transfection assay”) The reaction curves for all measurements are shown in appendix 1. Although DDT (HST0099) and DEE (HST0100) had detectable ER agonist activity, they did not reach the PC50 (see appendix). As noted before, with weakly active compounds, standards such as PC40 (40% of positive reaction) or PC30 (30% of positive reaction) are more useful for ranking purposes.

#### ***Androgen receptor agonist activity***

Table 2 shows the rank of the compounds, which have AR agonist activity, on the basis of the PC50. The reaction curves for all measurements are shown in appendix 2. Most of listed compounds that showed high agonistic activity were natural ligands or synthetic steroid hormones. Progesterone (HST0008) and Aldosterone (HST0009) showed only slight activity. RU486 (HST0087) and Cortisol (HST0099) at the highest concentration ( $10^{-6}$  M) examined were about 40% of the reaction of the positive control. The results were almost same as that of high throughput transfection assay. (Refer to Table 2 on the report of “high throughput transfection assay”).

## **DISCUSSION**

In our presentation of the high throughput assay we discussed the problem of ranking weakly active compounds, those whose reaction curves failed to reach one half of the plateau level of the positive control, and for which a PC50 could not be calculated. In some cases, although a PC50 could not be determined, the reaction curves did plateau (Estrone (THS00022), RU486 (HTS00087) and Cortisol (HTS00088), in the androgen agonist assay), and so an EC50 could be calculated (see table 2 and HTS00022, HTS00087, HTS00088 on Appendix 7). Comparing chemicals with weak and strong activity on the basis of EC50 determinations can be controversial and cause some with genuine activity to be disregarded. The PC50 ranking is a practical approach and weaker compounds can be qualified in terms of PC40 (40% of positive reaction) or PC30 (30% of positive reaction). The results presented above show that

both cell lines can distinguish compounds with strong activity and with weak activity, and the results can be used to rank the compounds.

As pointed out in our report on high throughput screening, others have developed cell lines with stable transfected reporter genes. These include MCF-7 (4), HeLa (5), T47D (6), and PC-3 (7) cells. These lines all express multiple steroid receptors. For example, T47D cells express ER-alpha and ER-beta, androgen, progesterone and retinoic acid receptors. Therefore, in the assays, cross-reaction may be observed, and it is impossible to distinguish whether ER-alpha or ER-beta has bound ligand. PC-3 cells actively metabolize steroids, and so natural ligands like testosterone and 5alpha-DHT cannot be used as standards. In contrast the CHO-K1 cells do not metabolize steroid hormones and do not express endogenous steroid receptors. Thus it is possible to measure the signal from only the transfected receptor.

## CONCLUSION

This method is suitable for high throughput screening applications, and generates reliable data.

## POSTSCRIPT

We continue to improve our system. Recently we have derived clones that give stronger signals on receptor activation, and thus are more sensitive. We are now preparing cell lines that express both EGFP and the reporter system simultaneously, and our preliminary results are promising. These will be developed for receptor antagonist activity assays, similar to those described in the transfection assay system.

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## **Appendix B7**

### **Technical Perspective on the U.S. EPA Endocrine Disruptor Screening Program: *In Vitro* EDSTAC Guideline Protocols**

**(Provided by Dr. Grantley Charles, Toxicology and Environmental  
Research and Consulting, The Dow Chemical Company, Midland,  
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## Technical Perspective on the U.S. EPA Endocrine Disruptor Screening Program:

### *In Vitro* EDSTAC Guideline Protocols<sup>1</sup>

#### I. Introduction

The Food Quality Protection Act of 1996, amending the Federal Food, Drug and Cosmetic Act, directed the Environmental Protection Agency (EPA) to develop a screening program to evaluate whether or not certain chemical agents could potentially have hormone-like effects in humans. The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) convened by the EPA recommended a tiered testing approach for the evaluation of endocrine, androgen and thyroid related effects of commercial chemicals and environmental contaminants (EDSTAC, 1998).

Under this testing paradigm, Tier I screening would identify chemicals with a potential to affect the estrogen, androgen and thyroid systems. The recommendations of the EDSTAC for a Tier I screening battery encompassed the utilization of *in vitro* test system methodologies that recognize known mechanisms by which chemicals can interact directly with the estrogen, androgen and thyroid hormone systems. These *in vitro* assays included evaluations of direct binding to the hormone receptors as well the ability of test compounds to activate marker response genes (reporters), linked to hormone responsive genetic elements. The Tier I assays are intended for use in rapid initial screening and prioritization of chemicals for further definitive *in vivo* Tier II testing to determine any potential adverse effects of an endocrine-active substance.

Tier I *in vitro* assays are used as screening tools to provide mechanistic data. These data should not be used as the sole element in a risk assessment regulatory context for test compounds. The *in vitro* screening assays are intended to be used in a hierarchical system which includes, as appropriate, *in vivo* Tier I screening assays and *in vivo* Tier II tests. In this hierarchical system a negative Tier II outcome would supercede a positive Tier I finding (EPA, 2000).

There are limitations inherent in the recommended *in vitro* assays that restrict their effectiveness as large scale, precise, valid, screening tools (Holmes *et al.*, 1998; Zacharewski, 1998). These include but are not limited to:

- Inability to distinguish agonists from antagonists (receptor binding)
- Issues of limited metabolic capacity and bioaccumulation
- Limited/variable chemical uptake

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<sup>1</sup> This technical perspective was prepared by experienced scientists engaged in *in vitro* and *in vivo* toxicological research and testing of industrial chemicals/ pesticides/pharmaceuticals. The primary authors of this commentary are listed under acknowledgements.

Dependence on specific receptor or response element interactions not mimicked *in vivo*

Lack of 'gold standard' protocols/methodologies for evaluation of assay results across laboratories

Issues of proprietary and/or restricted use under US patent law regarding the use of human cDNA sequences coding for human nuclear hormone receptors (and/or simultaneous co-transfection of receptor and reporter constructs; cis-trans technology) for use in reporter gene transactivation assays

These limitations need to be addressed in order to maximize the potential use of these assays/methodologies in a properly functional, tiered, screening paradigm required for the assessment of adverse chemical effects on the endocrine system. This paper seeks to aid in moving forward the process of producing sensitive, specific, accurate and properly validated Tier I *in vitro* methods that could be used as screening assays for hormonal activity.

## II. Major Elements To Be Considered for Standardization and Validation of *In Vitro* Assays

The following factors need to be taken into consideration in developing, validating and implementing *in vitro* assays for hormonal activity:

There are at present several different methodologies for the performance of estrogen and androgen receptor binding (Nikov *et al.*, 2000; Blair *et al.*, 2000; Nagel *et al.*, 1997) and reporter gene transactivation assays (Pons *et al.*, 1990; Zacharewski *et al.*, 1994; Kelce *et al.*, 1995; Gaido *et al.*, 1997; Maness *et al.*, 1998; Vinggaard *et al.*, 1999). To date, the inter-laboratory variability, sensitivity, reproducibility and precision of these techniques have not been sufficiently evaluated. Furthermore, alterations in specific assay parameters can also lead to significant variability (Beresford *et al.*, 2000; Charles *et al.*, 2000). A single methodology therefore needs to be properly standardized and validated as the 'gold standard' by which other alternative protocols can be reliably compared.

This gold standard *in vitro* protocol/methodology should be validated under an Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) type process in which several laboratories utilize identical protocols to assess the robustness of the assay in terms of reproducibility and accuracy. An agreed upon set of reference chemicals should be used to assist in the validation especially with regard to specificity and sensitivity.

*In vitro* assays performed as part of the Tier I screening methodology should be performed in compliance with Good Laboratory Practice (GLP) provisions of the USEPA, OECD and/or MAFF so as to ensure the quality of the data derived from the studies. This includes the proper characterization of the test material for potential purity and/or contamination prior to assay utilization.

A definite set of pass-fail criteria should be elaborated for each *in vitro* test system/methodology so as to minimize the potential confusion that may result from individual laboratory determinations. These would include criteria such as acceptable coefficients of variation (CVs), techniques for assessing cytotoxicity and definition of acceptable levels of cytotoxicity, required numbers of replicate data points per experiment, as well as cutoffs for designating a positive/negative response relative to defined controls.

In light of the desire to minimize the number of animals that will be used in the implementation of any new toxicological testing procedures, the utilization of methodologies which make limited use of animals (e.g. recombinant receptor proteins for binding assays) should be promoted.

The following discussion provides technical perspectives and recommendations on the design, methodology, and evaluation criteria of nuclear hormone receptor binding assays and nuclear hormone transcriptional activation assays. In addition, the limitations of the testicular steroidogenesis assay are described. These perspectives and recommendations have been developed to promote technical discussions among the scientists engaged in the development, standardization and validation of *in vitro* methods for use as Tier I screening assays for hormonal activity.

### **III. Nuclear Hormone Transcriptional Activation Assays**

#### **III. A. Purpose & Background**

The purpose of this procedure is to screen chemicals for the capacity to activate or inhibit ligand-induced transcription mediated by the mammalian estrogen and androgen nuclear receptors. The general premise is that nuclear hormone receptors bind ligand, which leads to alteration of their conformation, and subsequent binding to specific response element sequences on DNA and the initiation of transcription of the downstream gene. For convenience, the downstream gene codes for a protein (e.g., luciferase) that can be easily and accurately measured (i.e., a reporter gene) and therefore signals the potency of various ligands/chemicals to bind the receptor and either initiate or inhibit receptor-induced transcription of the reporter. Reporter gene assays then assess both agonist (test chemical alone) and antagonist (test chemical in the presence of stimulating ligand) activity.

In order to avoid potential US patent restrictions regarding the use of human cDNA sequence coding for human nuclear hormone receptors (and/or simultaneous co-transfection of receptor and reporter constructs; cis-trans technology), cell lines known to express endogenous human nuclear receptors are recommended. Cells expressing the human nuclear receptor of interest need only have the reporter gene introduced into them in order to be used for transcriptional activation assays.

Reporter genes can be transiently introduced into cells and used over the course of several days or stably integrated into the cells genomic DNA and used indefinitely, provided their responses to known ligands are stable and verified on a periodic basis. The response variability of transient expression systems is, however, an issue for routine use. Few stable cells lines for nuclear (estrogen and androgen) hormone receptor reporter gene assays are currently available, therefore the protocol recommended here uses accepted methods for transient reporter gene transfections.

### **III. B. General Assay Design**

In brief, cells should be seeded into tissue culture plates, transiently transfected with the reporter gene, fed media containing treatment compounds with and without stimulating ligand. Following a defined treatment period, cell lysates are harvested and assessed for reporter (e.g. luciferase, -galactosidase) activity. Concurrent with the reporter assay, an identically transfected and treated, satellite assays should be run and evaluated for cytotoxicity. For the screening of test chemicals, a dose-response assay is recommended in order to discriminate between highly potent ligands that may be cytotoxic at high concentrations from weak non-cytotoxic ligands that exhibit agonist activity at higher concentrations.

### **III. C. Recommended Design Features**

The dose range should encompass the low pM range to the chemical solubility limit as the upper concentration to be evaluated. Alternatively, the upper limit should also be defined as that below which no cytotoxicity is observed.

Cells should be cultured aseptically in appropriate media using standard cell culture techniques. The optimal number of cells seeded into each dish or well should be determined empirically by each laboratory and is directly dependent on the transfection efficiency of the reporter gene. It is critical that seeding density is uniform, as alterations in cell number per well will introduce unnecessary variability in the assay.

The use of charcoal stripped serum is important to remove endogenous steroids from the serum which can activate transcription of the reporter gene and confound the experiment. A steroid free environment is especially important for estrogen receptor mediated transactivation experiments as many general laboratory procedures and supplies have been shown to artificially induce estrogen receptor mediated responses. In this context, laboratories should strive for an estrogen free environment.

Higher transfection efficiencies using lipofectin, FUGene or electroporation mean that fewer cells are necessary to induce an easily measured response and the assay can be completed using an efficient 96-well format. These transfection methods are recommended over more traditional calcium-phosphate precipitation and DEAE-Dextran that generally give much reduced transfection efficiencies.

Since the assays are generally performed in large multi-well formats, each plate should have its own positive and negative controls and should be considered a single experiment for data



analysis purposes. A concentration of the inhibitor control should be selected that reduces transcriptional activation by at least 90% in the presence of stimulatory ligand. Duplicate evaluations of each test chemical concentration should be assessed per experiment. Experiments should be replicated at least three times on different days.

17  $\beta$ -estradiol and 5  $\alpha$ -dihydrotestosterone are recommended stimulatory ligands for the estrogen and androgen receptor assays, respectively. The concentration of stimulatory ligand used in test article antagonism studies should induce transcriptional activity to levels approximately 80-90% of maximum; use of submaximal levels insures that the receptor is not saturated with agonist ligand and incapable of responding to inhibitory compounds. ICI-182,780 and hydroxyflutamide (Wakeling *et al.*, 1991; Clark *et al.*, 1981; Kelce *et al.*, 1995) are the respective recommended antagonism controls and should be used at concentrations that inhibit transcriptional activation by 90% or more. Other stimulatory and antagonist controls are acceptable provided that they are appropriately validated against the standard controls.

Control and test chemicals should be solubilized in ethanol or DMSO and added to the media in each well to a final concentration determined empirically as part of the initial standardization and validation effects for that cell line. Particular attention should be given to the solubility of test chemicals especially at the high doses. Any precipitate, discoloration, or persistent light refractive changes on the media surface should be noted and included in the final report indicating potential solubility problems. Other vehicles may be used provided appropriate determination of its effects on the cell line and reporter activity are properly standardized and validated.

### III. D. Data Presentation and Pass-Fail Criteria

The percent coefficient of variation (%CV) of replicate samples at each concentration of test or control chemical cannot exceed 20% in any assay. Data which exceeds the 20%CV at any concentration of test or control chemical within an assay will fail these criteria and all data for that concentration of test or control chemical for that particular assay must be excluded from the data analysis. All data failing these criteria should be so indicated in the data tables. The antagonist control must reduce transactivation by at least 90% within a 20%CV or the assay will be considered unacceptable.

Data from transactivation experiments should be replicated at least three times each on different days. Data should be tabulated and graphed as reporter activity (relative light units) on the ordinate versus log dose of test chemical on the abscissa. For convenience, reporter data can be presented as %-control (%-maximal activity induced by stimulatory ligand) provided actual control values are clearly indicated.

The EC<sub>50</sub> (agonist experiments) is calculated as the concentration of test chemical that activates transcription by 50% relative to the maximal activity induced by stimulatory ligand.

The IC<sub>50</sub> (antagonist experiments) is calculated as the concentration of test chemical that inhibits transcription by 50% relative to the maximal activity induced by stimulatory ligand. EC<sub>50</sub> and IC<sub>50</sub> values for each test chemical and the positive and antagonist controls, respectively, should be tabulated for each assay and the means together with a measure of the variability (e.g., standard deviation) from all assays clearly indicated.

An efficacy of 25% of the positive control (or the negative control in the case of antagonist activity) should be considered a positive response for that test chemical in that assay.

### III. E. Limitations

The following limitations of transcriptional activation studies should be recognized:

Differences in sensitivity exist among clones of a given cell line (Villalobos *et al.*, 1995) in terms of their endocrine responses. Hence adequate characterization of cell lines are necessary and the testing methodology should address factors such as drift in responsiveness, sensitivity and specificity to minimize variability in response across laboratories.

Test end points are dependent upon interactions with a given receptor structure or engineered response element. Therefore, results from any single gene transactivation system for a given chemical may vary significantly from that of another.

Reproducibility of results will always be a potential concern, consequently, test systems should be widely available to enable confirmatory findings by other laboratories. It is therefore essential to perform an ICCVAM-type validation on a specific estrogen and androgen transactivation systems to act as gold standards to which other assay systems could be compared.

It should be noted that transiently transfected cell lines exhibit some degree of variability across experiments in terms of their responses making stable cell lines a potentially more appealing alternative for validation purposes. In the event that new, stable cell lines are developed and are generally available, it is recommended that they be used with the caveat that they are properly validated in accordance with the ICCVAM principles already outlined. Their sensitivity, accuracy, precision and specificity should also be reviewed on a periodic basis to protect against genetic drift and cellular mutations that may compromise the integrity of the assay system.

### IV. Acknowledgements

The primary authors of this technical perspective are Grantley Charles (Dow), William Kelce (Pharmacia) and Leonard Davis (DuPont).

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**Appendix C**  
**Chemical and Product Class Information for the**  
**Substances Tested in the *In Vitro* AR TA Assays**

**Information Sorted by Substance Name**

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### Information Sorted by Substance Name

Substance Name	Synonyms	CASRN	Chemical Class	Product Class
<b>Aldosterone</b>		52-39-1	Steroid, nonphenolic	Pharmaceutical
<b><i>t</i>-Allethrin</b>	(+)-Allethrethonyl (+)- <i>cis,trans</i> -chrysanthemate	584-79-2	Pyrethrin	Pesticide
<b>Allopregnenedione</b>	3,20-Allopregnenedione	566-65-4	Steroid, nonphenolic	Chemical intermediate
<b>Anastrozole</b>	Zeneca ZD 1033	120511-73-1	Nitrile	Pharmaceutical
<b>5<math>\alpha</math>-Androstane-3<math>\alpha</math>,17<math>\beta</math>-diol</b>	3,17-Dihydroxy-5 $\alpha$ -androstane	1852-53-5	Steroid, nonphenolic	Pharmaceutical
<b>5<math>\alpha</math>-Androstane-3<math>\beta</math>,17<math>\beta</math>-diol</b>		126061-67-4	Steroid, nonphenolic	Pharmaceutical
<b>Androstenedione</b>	4-Androstene-3,17-dione; delta-4-Androstenedione	63-05-8	Steroid, nonphenolic	Natural product
<b>Anthracene</b>		120-12-7	Polycyclic aromatic hydrocarbon	None
<b>Ascorbic acid</b>	Vitamin C; L-Ascorbic acid	50-81-7	Carboxylic acid; Lactone	Pharmaceutical
<b>Atrazine</b>	1,3,5-Triazine-2,4-diamine, 6-chloro-N-ethyl-N'-(1-methylethyl)-	1912-24-9	Aromatic amine; Triazine	Pesticide
<b>Benz[a]anthracene</b>		56-55-3	Polycyclic aromatic hydrocarbon	None
<b>Benzo[a]pyrene</b>		50-32-8	Polycyclic aromatic hydrocarbon	None
<b>Bicalutamide</b>	Casodex; ICI 176,334	90357-06-5	Anilide; Nitrile	Pharmaceutical
<b>2,2-Bis-(<i>p</i>-hydroxyphenyl)-1,1,1-trichloroethane</b>	HPTE	2971-36-0	Organochlorine	Pesticide
<b>Bisphenol A</b>		80-05-7	Bisphenol	Chemical intermediate
<b>Bisphenol B</b>		77-40-7	Bisphenol	Adhesives; Chemical intermediate; Coatings
<b>Bisphenol E</b>	1,1-Bis(4-hydroxyphenyl)ethane	6052-84-2	Diphenolalkane	Chemical intermediate
<b>4,4'-Bisphenol F</b>	Bis(4-hydroxyphenyl)methane	620-92-8	Diphenolalkane	Chemical intermediate; Pharmaceutical
<b>Butylated hydroxyanisole</b>	BHA; (1,1-Dimethylethyl)-4-methoxyphenol	25013-16-5	Ether; Phenol	Preservative (foods, cosmetics, and pharmaceuticals)
<b>Butylated hydroxytoluene</b>	BHT	128-37-0	Phenol	Preservative (foods and cosmetics)
<b>Butyl benzyl phthalate</b>	Benzyl butyl phthalate	85-68-7	Phthalate	Plasticizer
<b>Chlornitrofen</b>	CNP; 2,4,6-Trichlorophenyl 4-nitrophenyl ether	1836-77-7	Biphenyl; Ether	Pesticide
<b>11<math>\beta</math>-Chloromethyl estradiol</b>	Org 4333	71794-60-0	Steroid, phenolic	None
<b>Chrysene</b>		218-01-9	Polycyclic aromatic hydrocarbon	None
<b>Clomiphene</b>		911-45-5	Stilbene derivative	Pharmaceutical

### Information Sorted by Substance Name

Substance Name	Synonyms	CASRN	Chemical Class	Product Class
<b>Corticosterone</b>	17-Deoxycortisol; 11 $\beta$ ,21-Dihydroxyprogesterone	50-22-6	Steroid, nonphenolic	Pharmaceutical
<b>Cortisol</b>	Hydrocortisone	50-23-7	Steroid, phenolic	Pharmaceutical
<b>Coumestrol</b>		479-13-0	Coumarin	Natural product
<b><i>p</i>-Cumyl phenol</b>	2-Phenyl-2-(4-hydroxyphenyl)propane	599-64-4	Phenol	Chemical intermediate
<b>Cyproterone acetate</b>	1,2 $\beta$ -Methylene-6-chloro-(sup 4,6)-pregnadiene-17 $\beta$ -ol-3,20-dione 17 $\beta$ -acetate	427-51-0	Steroid, nonphenolic	Pharmaceutical
<b><i>o,p'</i>-DDD</b>	1-(2-Chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane	53-19-0	Organochlorine	Pesticide metabolite; Pharmaceutical
<b><i>p,p'</i>-DDD</b>	1,1-Dichloro-2,2-bis( <i>p</i> -chlorophenyl) ethane; TDE	72-54-8	Organochlorine	Pesticide metabolite
<b><i>o,p'</i>-DDE</b>		3424-82-6	Organochlorine	Pesticide metabolite
<b><i>p,p'</i>-DDE</b>	4,4'-DDE	72-55-9	Organochlorine	Pesticide metabolite
<b><i>o,p'</i>-DDT</b>	2-( <i>o</i> -Chlorophenyl)-2-( <i>p</i> -chlorophenyl)-1,1,1-trichloroethane	789-02-6	Organochlorine	Pesticide
<b><i>p,p'</i>-DDT</b>	1,1,1-Trichloro-2,2-bis( <i>p</i> -chlorophenyl)ethane	50-29-3	Organochlorine	Pesticide
<b>15-Dehydroetogestrel</b>			Steroid, nonphenolic	None
<b>15-Dehydronorethisterone</b>			Steroid, nonphenolic	None
<b>Dexamethasone</b>	(11 $\beta$ ,16 $\alpha$ )-9-Fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione	50-02-2	Steroid, nonphenolic	Pharmaceutical
<b>Dibenzo[<i>a,h</i>]anthracene</b>		53-70-3	Polycyclic aromatic hydrocarbon	None
<b>Dibutyl phthalate</b>		84-74-2	Phthalate	Plasticizer
<b>3',5'-Dichloro-2-hydroxy-2-methylbut-3-enanilide</b>	Vinclozolin metabolite M2	16776-82-1	Organochlorine	Pesticide metabolite
<b>(4-[2,2-Dichloro-1-(4-hydroxyphenyl)vinyl]phenol</b>			Organochlorine; Phenol	None
<b>2-[[3,5-(Dichlorophenyl)-carbamoyloxy]-2-methyl-3-butenic acid</b>	Vinclozolin metabolite M1	119209-27-7	Organochlorine	Pesticide metabolite
<b>Dicofol</b>	Keltane	115-32-2	Organochlorine	Pesticide
<b>Di-(2-ethylhexyl)phthalate</b>		117-81-7	Phthalate	Chemical intermediate; Plasticizer
<b>Diethylstilbestrol</b>	DES	56-53-1	Stilbene	Pharmaceutical
<b>5<math>\alpha</math>-Dihydrotestosterone</b>	Dihydrotestosterone; Androstanolone; Stanolone	521-18-6	Steroid, nonphenolic	Pharmaceutical
<b>5<math>\beta</math>-Dihydrotestosterone</b>	17 $\beta$ -Hydroxy-5 $\alpha$ -androstan-3-one	571-22-2	Steroid, nonphenolic	Pharmaceutical
<b>4,4'-Dihydroxybenzophenone</b>	Bis(4-hydroxyphenyl)ketone	611-99-4	Benzophenone	Chemical intermediate; Pharmaceutical

### Information Sorted by Substance Name

Substance Name	Synonyms	CASRN	Chemical Class	Product Class
Dihydroxy-DDE			Organochlorine	Pesticide derivative
4,4-Dimethoxybenzhydrol	Bis(4-methoxyphenyl)methanol; <i>p,p'</i> -Dimethoxybenzhydrol alcohol	728-87-0	Alcohol; Ether	None
Dimethoxy-DDE			Organochlorine	Pesticide derivative
Dimethylbenz[ <i>a</i> ]anthracene		57-97-6	Polycyclic aromatic hydrocarbon	None
Diphenylphthalate		84-62-8	Phthalate	Chemical intermediate
DTIB	4-[4,4-Dimethyl-3-(4-hydroxybutyl)-5-oxo-2-thioxo-1-imidazolidinyl]-2-iodobenzonitrile		Imidazole	None
Epitestosterone	(17 $\beta$ )-17-Hydroxyandrost-4-en-3-one	481-30-1	Steroid, nonphenolic	Natural product
Equilin		474-86-2	Steroid, phenolic	Pharmaceutical
Equol		531-95-3	Isoflavone	Pharmaceutical
17 $\alpha$ -Estradiol		57-91-0	Steroid, phenolic	None
17 $\beta$ -Estradiol	Estradiol	50-28-2	Steroid, phenolic	Pharmaceutical
Estriol	Estratriene-3,16,17-triol	50-27-1	Steroid, phenolic	Pharmaceutical
Estrone	Estra-1,3,5(10)-trien-17-one, 3-hydroxy-	53-16-7	Steroid, phenolic	Pharmaceutical
17 $\alpha$ -Ethinyl estradiol	Ethinyl estradiol	57-63-6	Steroid, phenolic	Pharmaceutical
Etonogestrel	3-Keto-desogestrel	54048-10-1	Steroid, phenolic	Pharmaceutical
Fenbuconazole	RH-7592; 4-(4-Chlorophenyl)-2-phenyl-2-(1H-1,2,4-triazol-1-ylmethyl)butyronitrile	114369-43-6	Azole; Nitrile	Pesticide
Fenitrothion		122-14-5	Organothiophosphate	Pesticide
Fenvalerate	Fenoxin	51630-58-1	Pyrethrin	Pesticide
Finasteride	Proscar	98319-26-7	Steroid, nonphenolic	Pharmaceutical
Fluoranthene		206-44-0	Polycyclic aromatic hydrocarbon	None
Fluoxymesterone	Androfluorene; Halotestin	76-43-7	Steroid, nonphenolic	Pharmaceutical
Flutamide	4'-Nitro-3'-trifluoromethylisobutyranilide	13311-84-7	Anilide	Pharmaceutical
Gestodene	Gestoden	60282-87-3	Steroid, nonphenolic	Pharmaceutical
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid	7365-45-9	Alkyl sulfonate	Buffer
2,2',3,4,4',5,5'-Heptachlorobiphenyl	PCB #180	35065-29-3	Polychlorinated biphenyl	Dielectric fluid
2,2',3,4,4',5'-Hexachlorobiphenyl	PCB #138	35065-28-2	Polychlorinated biphenyl	Dielectric fluid
2,2',4,4',5,5'-Hexachlorobiphenyl	PCB #153	35065-27-2	Polychlorinated biphenyl	Dielectric fluid

### Information Sorted by Substance Name

Substance Name	Synonyms	CASRN	Chemical Class	Product Class
$\alpha$ -Hexachlorocyclohexane	-HCH	319-84-6	Organochlorine	Pesticide; Pharmaceutical
$\beta$ -Hexachlorocyclohexane (1a,2b,3a,4b,5a,6b)	-Lindane; -HCH	319-85-7	Organochlorine	Pesticide
$\delta$ -Hexachlorocyclohexane	-HCH	319-86-8	Organochlorine	Pesticide
Hydramethylnone		67485-29-4	Pyrimidine	Pesticide
Hydroxyflutamide	2-Hydroxy-2-methyl-N-(4-nitro-3-(trifluoromethyl)phenyl)-propanamide	52806-53-8	Anilide	Pharmaceutical metabolite
4-Hydroxytamoxifen		68047-06-3	Triphenylethylene	Pharmaceutical
ICI 182,780		129453-61-8	Steroid, phenolic	Pharmaceutical
Inocoterone		83646-86-0	Indene; Polycyclic aromatic hydrocarbon	Pharmaceutical
Kepone	Chlordecone	143-50-0	Organochlorine	Pesticide
Ketoconazole	Nizoral	65277-42-1	Imidazole	Pharmaceutical
11-Keto-15-dehydronorethisterone			Steroid, nonphenolic	None
11-Ketonorethisterone		5210-27-5	Steroid, nonphenolic	None
11-Ketotestosterone	Androst-4-ene-3,11-dione, 17-hydroxy-, (17 $\alpha$ )-	564-35-2	Steroid, nonphenolic	None; Natural androgen in fish
Levonorgestrel		797-63-7	Steroid, nonphenolic	Pharmaceutical
Lindane	-Hexachlorocyclohexane	58-89-9	Organochlorine	Pesticide; Pharmaceutical
Linuron	1-Methoxy-1-methyl-3-(3,4-dichlorophenyl)urea	330-55-2	Urea	Pesticide
Medroxyprogesterone acetate	Medroxyprogesterone 17-acetate; MPA	71-58-9	Steroid, nonphenolic	Pharmaceutical
Methoxychlor	Benzene, 1,1'-(2,2,2-trichloroethylidene)bis(4-methoxy-	72-43-5	Organochlorine	Pesticide
Methyltestosterone		58-18-4	Steroid, nonphenolic	Pharmaceutical
Methyltrienolone	R1881; 17 $\alpha$ -Methyl-17 $\beta$ -hydroxy-estra-4,9,11-trien-3-one	965-93-5	Steroid, nonphenolic	Pharmaceutical
Mibolerone	7 $\alpha$ ,17 $\beta$ -Dimethyl-19-nortestosterone	3704-09-4	Steroid, nonphenolic	Pharmaceutical
Mifepristone	RU-486	84371-65-3	Steroid, nonphenolic	Pharmaceutical
Mirex		2385-85-5	Organochlorine	Pesticide; Pharmaceutical
Monohydroxy-DDE			Organochlorine	Pesticide derivative
Monohydroxymethoxychlor		28463-03-8	Organochlorine	Pesticide derivative
Moxestrol	11 $\beta$ -Methoxyethinylestradiol; R2858	34816-55-2	Steroid, phenolic	Pharmaceutical
Nafoxidine		1847-63-8	Stilbene; Triphenylethylene	Pharmaceutical

### Information Sorted by Substance Name

Substance Name	Synonyms	CASRN	Chemical Class	Product Class
<b>Neburon</b>	1-Butyl-3-(3,4-dichlorophenyl)-1-methylurea	555-37-3	Urea	Pesticide
<b>Nilutamide</b>	Anandron; RU 23908; Nilandron	63612-50-0	Imidazole	Pharmaceutical
<b>cis -Nonachlor</b>		5103-73-1	Organochlorine	Pesticide
<b>trans -Nonachlor</b>		39765-80-5	Organochlorine	Pesticide
<b>p -Nonylphenol</b>	4-Nonylphenol	104-40-5	Alkylphenol	Chemical intermediate
<b>1-O-(Nonylphenyl)-a,b-D-glucopyranosiduric acid</b>			Alkylphenol; Glucuronic acid	None
<b>Norethisterone</b>	Norethindrone	68-22-4	Steroid, nonphenolic	Pharmaceutical
<b>Norgestrel</b>	13-Ethyl-17 -hydroxy-18,19-dinor-pregn-4-en-20-yn-3-one	6533-00-2	Steroid, nonphenolic	Pharmaceutical
<b>19-Nortestosterone</b>	19-NT; Nandrolone	434-22-0	Steroid, nonphenolic	Pharmaceutical
<b>p-tert -Octylphenol</b>	4-(1,1,3,3-Tetramethylbutyl)phenol	140-66-9	Alkylphenol	Chemical intermediate
<b>1-O-(Octylphenyl)-a,b-D-glucopyranosiduronic acid</b>			Alkylphenol; Glucuronic acid	None
<b>Oxandrolone</b>		53-39-4	Steroid, nonphenolic	Pharmaceutical
<b>Oxychlorthane</b>		27304-13-8	Organochlorine	Pesticide metabolite
<b>Permethrin</b>		52645-53-1	Pyrethrin	Pesticide
<b>Phenanthrene</b>		85-01-8	Polycyclic aromatic hydrocarbon	None
<b>Phenobarbital, sodium salt</b>		57-30-7	Pyrimidine	Pharmaceutical
<b>Phenothrin</b>		26002-80-2	Phenyl ether	Pesticide
<b>Photomirex</b>		39801-14-4	Organochlorine	Pesticide degradation product
<b>Pregnenolone</b>	3 -Hydroxypregn-5-en-20-one	145-13-1	Steroid, nonphenolic	Pharmaceutical
<b>Procymidone</b>	N-(3',5'-Dichlorophenyl)-1,2-dimethylcyclopropane-1,2-dicarboximide	32809-16-8	Imide	Pesticide
<b>Progesterone</b>	Pregn-4-ene-3,20-dione	57-83-0	Steroid, nonphenolic	Pharmaceutical
<b>Promegestone</b>	R5020; 17,21-Dimethyl-19-nor-4,9-pregnadiene-3,20-dione	34184-77-5	Steroid, nonphenolic	Pharmaceutical
<b>Propylthiourea</b>		927-67-3	Urea	Pesticide
<b>Prothiofos</b>	O-Ethyl-O-(2,4-dichlorophenyl)-S-n-propyl-dithiophosphate	34643-46-4	Organothiophosphate	Pesticide
<b>Pyrene</b>		129-00-0	Polycyclic aromatic hydrocarbon	Dye
<b>R2956</b>	17 -Hydroxy-2,2,-17-trimethylestra-4,9,11-trien-3-one	42438-88-0	Steroid, phenolic	Pharmaceutical
<b>Rimsulfuron</b>		122931-48-0	Sulfonylurea	Pesticide

### Information Sorted by Substance Name

Substance Name	Synonyms	CASRN	Chemical Class	Product Class
RU 56187	4-(3,4,4-Trimethyl-5-oxo-2-thioxo-1-imidazolidinyl)-2-(trifluoromethyl)benzotrile	143782-25-6	Imidazole; Nitrile	Pharmaceutical
RU 59063	4-[4,4-Dimethyl-3-(4-hydroxybutyl)-5-oxo-2-thioxo-1-imidazolidinyl]-2-trifluoromethylbenzotrile	155180-53-3	Imidazole; Nitrile	Pharmaceutical
$\beta$ -Sitosterol		83-46-5	Steroid, nonphenolic	Natural product; Pharmaceutical
Spironolactone	17-Hydroxy-7-mercapto-3-oxo-17-pregn-4-ene-21-carboxylic acid, gamma-lactone acetate	52-01-7	Steroid, nonphenolic	Pharmaceutical
Tamoxifen		10540-29-1	Stilbene; Triphenylethylene	Pharmaceutical
Testosterone	Androst-4-en-3-one, 17-hydroxy-, (17 $\alpha$ )-	58-22-0	Steroid, nonphenolic	Pharmaceutical
2,3,7,8-Tetrachlorodibenzo- <i>p</i> -dioxin	TCDD; dioxin	1746-01-6	Dioxin	None
Toxaphene		8001-35-2	Organochlorine	Pesticide
Tralomethrin		66841-25-6	Pyrethrin	Pesticide
Trihydroxymethoxychlor			Organochlorine	Pesticide derivative
Trimethoxymethoxychlor			Organochlorine	Pesticide derivative
Vinclozolin	3-(3,5-Dichlorophenyl)-5-ethenyl-5-methyl-2,4-oxazolidinedione	50471-44-8	Organochlorine	Pesticide
Zearalenone		17924-92-4	Resorcylic acid lactone; Phenol	Chemical intermediate; Natural product
Zearanol		26538-44-3	Resorcylic acid lactone; Phenol	Natural product

# **Appendix D**

## **Substances Tested in the *In Vitro* AR TA Assays**

**D1 Information Sorted by Substance Name and Assay**

**D2 References**

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## **Appendix D1**

### **Substances Tested in the *In Vitro* AR TA Assays**

### **Information Sorted by Substance Name and Assay**

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## Information Sorted by Substance Name and Assay

Assay Type*	Reference Androgen**	Substance Name	CASRN†	AGONISM <sup>b</sup> Qualitative††	AGONISM Maximum Fold x (conc. $\mu$ M)††	AGONISM EC50 ( $\mu$ M)††	ANTAG. <sup>a,b</sup> Qualitative††	ANTAG. <sup>a,b</sup> IC50 ( $\mu$ M)††	Prolif. Response <sup>a</sup> ††	RPP <sup>a,b,†</sup> ††	Reference
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Aldosterone	52-39-1	neg.							Otsuka Pharmaceutical Co. (2001)
EPC rtAR ( ) +CAT(T)	DHT	Aldosterone	52-39-1	neg.							Takeo and Yamashita (2000)
PALM hAR(S)+Luc(S)	R1881	Aldosterone	52-39-1	neg.	2x (1 $\mu$ M)						Terouanne et al. (2000)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	<i>t</i> -Allethrin	584-79-2	neg.							Gaido et al. (1997)
CHO hAR(T)+CAT(T)+ gal(T)	DHT	Allopregnanedione	566-65-4	neg.	1x (2nM)						Deslypere et al. (1992)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	Anastrozole	120511-73-1	neg.			neg.				O'Connor et al. (1998)
LNCaP-FGC hAR(E)+CP	DHT	5 -Androstane-3 ,17 -diol	1852-53-5						weak	0.1	Sonnenschein et al. (1989)
LNCaP-FGC hAR(E)+CP	DHT	5 -Androstane-3 ,17 -diol	126061-67-4						pos.	10	Sonnenschein et al. (1989)
CHO hAR(T)+CAT(T)+ gal(T)	DHT	Androstenedione	63-05-8	pos.	65x (1nM)						Deslypere et al. (1992)
CHO-K1 hAR(S)+Luc(S)	DHT	Androstenedione	63-05-8	pos.		0.00242					Otsuka Pharmaceutical Co. (2001)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Androstenedione	63-05-8	pos.		0.000645					Otsuka Pharmaceutical Co. (2001)
CHO hAR(T)+Luc(T)	R1881	Anthracene	120-12-7				neg.				Vinggaard et al. (2000)
Yeast( <i>S. cer</i> ) hAR(S)+ gal(S)	DHT	Ascorbic acid	50-81-7	neg.			neg.				Moffat et al. (2001)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Atrazine	1912-24-9	neg.							Otsuka Pharmaceutical Co. (2001)
PALM hAR(S)+Luc(S)	R1881	Atrazine	1912-24-9				neg.				Sultan et al. (2001)
CHO hAR(T)+Luc(T)	R1881	Benz[ <i>a</i> ]anthracene	56-55-3				pos.	3.2			Vinggaard et al. (2000)
CHO hAR(T)+Luc(T)	R1881	Benzo[ <i>a</i> ]pyrene	50-32-8				pos.	3.9			Vinggaard et al. (2000)
CV-1 hAR(T)+Luc(T)	DHT	Bicalutamide	90357-06-5	weak			pos.				Kempainen and Wilson (1996)
PALM hAR(S)+Luc(S)	R1881	Bicalutamide	90357-06-5				pos.	0.75			Terouanne et al. (2000)
PALM hAR(S)+Luc(S)	R1881	Bicalutamide	90357-06-5	weak	5x (1 $\mu$ M)		pos.	18			Terouanne et al. (2000)
PC-3 hAR(T)+Luc(T)	R1881	Bicalutamide	90357-06-5				pos.	0.5			Terouanne et al. (2000)
CHO hAR(T)+Luc(T)	R1881	Bicalutamide	90357-06-5				pos.	0.5			Vinggaard et al. (1999)

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CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	2,2-Bis( <i>p</i> -hydroxyphenyl)-1,1,1-trichloroethane	2971-36-0	neg.							Otsuka Pharmaceutical Co. (2001)
CV-1 hAR(T)+Luc(T)*	DHT	2,2-Bis( <i>p</i> -hydroxyphenyl)-1,1,1-trichloroethane	2971-36-0	pos.			pos.				Hartig et al. (2002)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	2,2-Bis( <i>p</i> -hydroxyphenyl)-1,1,1-trichloroethane	2971-36-0				pos.				Gaido et al. (2000)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	2,2-Bis( <i>p</i> -hydroxyphenyl)-1,1,1-trichloroethane	2971-36-0				pos.				Maness et al. (1998)
MDA-MB-453 hAR(E)+Luc(T)*	DHT	2,2-Bis( <i>p</i> -hydroxyphenyl)-1,1,1-trichloroethane	2971-36-0	pos.			pos.	0.1			Hartig et al. (2002)
MDA-MB-453-kb2 hAR(E)+Luc(S)	DHT	2,2-Bis( <i>p</i> -hydroxyphenyl)-1,1,1-trichloroethane	2971-36-0				pos.	10			Wilson et al. (2002)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Bisphenol A	80-05-7	neg.							Otsuka Pharmaceutical Co. (2001)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	Bisphenol A	80-05-7				neg.				Gaido et al. (2000)
PALM hAR(S)+Luc(S)	R1881	Bisphenol A	80-05-7				pos.	1			Sultan et al. (2001)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Bisphenol B	77-40-7	neg.							Otsuka Pharmaceutical Co. (2001)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	Bisphenol E	6052-84-2				pos.				Gaido et al. (2000)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	4,4'-Bisphenol F	620-92-8				pos.				Gaido et al. (2000)
PALM hAR(S)+Luc(S)	DHT	Butylated hydroxyanisole	25013-16-5	neg.			pos.	7.6			Schrader and Cooke (2000)
PALM hAR(S)+Luc(S)	DHT	Butylated hydroxytoluene	128-37-0	neg.			pos.	5.7			Schrader and Cooke (2000)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Butyl benzyl phthalate	85-68-7	neg.							Otsuka Pharmaceutical Co. (2001)
PALM hAR(S)+Luc(S)	R1881	Butyl benzyl phthalate	85-68-7				neg.				Sultan et al. (2001)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	Butyl benzyl phthalate	85-68-7	neg.							Gaido et al. (1997)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Chlornitrofen	1836-77-7	pos.			weak				Otsuka Pharmaceutical Co. (2001)
LNCAp-FGC hAR(E)+CP	DHT	11 -Chloromethyl estradiol	71794-60-0						pos.	1	Sonnenschein et al. (1989)
CHO hAR(T)+Luc(T)	R1881	Chrysene	218-01-9				pos.	10.3			Vinggaard et al. (2000)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Clomiphene	911-45-5	neg.							Otsuka Pharmaceutical Co. (2001)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Corticosterone	50-22-6	neg.							Otsuka Pharmaceutical Co. (2001)

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CHO hAR(T)+CAT(T)+ gal(T)	DHT	Cortisol	50-23-7	pos.	35x (1 $\mu$ M)						Deslypere et al. (1992)
CHO-K1 hAR(S)+Luc(S)	DHT	Cortisol	50-23-7	pos.		0.0427					Otsuka Pharmaceutical Co. (2001)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Cortisol	50-23-7	neg.							Otsuka Pharmaceutical Co. (2001)
PALM hAR(S)+Luc(S)	DHT	Cortisol	50-23-7	pos.							Schrader and Cooke (2000)
PALM hAR(S)+Luc(S)	R1881	Cortisol	50-23-7	pos.	20x (1 $\mu$ M)						Terouanne et al. (2000)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	Cortisol	50-23-7	neg.							Gaido et al. (1997)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Coumestrol	479-13-0	neg.							Otsuka Pharmaceutical Co. (2001)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	Coumestrol	479-13-0	neg.							Gaido et al. (1997)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	Coumestrol	479-13-0	neg.							O'Connor et al. (2000)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	<i>p</i> -Cumylphenol	599-64-4	neg.							Otsuka Pharmaceutical Co. (2001)
CHO hAR(T)+Luc(T)	R1881	Cyproterone acetate	427-51-0				pos.	0.5			Vinggaard et al. (1999)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Cyproterone acetate	427-51-0	pos.							Otsuka Pharmaceutical Co. (2001)
CV-1 hAR(T)+CAT(T)	R1881	Cyproterone acetate	427-51-0	pos.	15x (10nM)		pos.				Kemppainen et al. (1992)
CV-1 hAR(T)+Luc(T)	DHT	Cyproterone acetate	427-51-0	pos.	10x (0.1 $\mu$ M)		pos.	0.1			Kemppainen et al. (1999)
HeLa hAR(S)+DS-Luc(T)	DHT	Cyproterone acetate	427-51-0	weak	2x (100nM)						Wang and Fondell (2001)
HeLa hAR(S)+M-Luc(T)	DHT	Cyproterone acetate	427-51-0	weak	0.5x (100nM)						Wang and Fondell (2001)
PALM hAR(S)+Luc(S)	R1881	Cyproterone acetate	427-51-0	pos.			pos.	0.01			Terouanne et al. (2000)
PALM hAR(S)+Luc(S)	R1881	Cyproterone acetate	427-51-0	pos.	30x (1 $\mu$ M)		pos.	45			Terouanne et al. (2000)
PC-3 hAR(T)+Luc(T)	R1881	Cyproterone acetate	427-51-0	pos.			pos.	0.01			Terouanne et al. (2000)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	<i>o,p'</i> -DDD	53-19-0				pos.				Maness et al. (1998)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	<i>p,p'</i> -DDD	72-54-8	neg.							Otsuka Pharmaceutical Co. (2001)
CV-1 hAR(T)+Luc(T)	DHT	<i>p,p'</i> -DDD	72-54-8				pos.				Kelce et al. (1995)

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HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	<i>p,p'</i> -DDD	72-54-8				pos.				Maness et al. (1998)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	<i>p,p'</i> -DDD	72-54-8	neg.							Gaido et al. (1997)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	<i>o,p'</i> -DDE	3424-82-6	neg.							Otsuka Pharmaceutical Co. (2001)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	<i>o,p'</i> -DDE	3424-82-6				pos.				Maness et al. (1998)
PALM hAR(S)+Luc(S)	R1881	<i>o,p'</i> -DDE	3424-82-6				pos.	1.5			Sultan et al. (2001)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	<i>o,p'</i> -DDE	3424-82-6	neg.							Gaido et al. (1997)
CHO hAR(T)+Luc(T)	R1881	<i>p,p'</i> -DDE	72-55-9				pos.	1			Vinggaard et al. (1999)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	<i>p,p'</i> -DDE	72-55-9	neg.							Otsuka Pharmaceutical Co. (2001)
CV-1 hAR(T)+Luc(T)	DHT	<i>p,p'</i> -DDE	72-55-9				pos.				Kelce et al. (1995)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	<i>p,p'</i> -DDE	72-55-9				pos.				Gaido et al. (2000)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	<i>p,p'</i> -DDE	72-55-9	weak			pos.				Maness et al. (1998)
MDA-MB-453-kb2 hAR(E)+Luc(S)	DHT	<i>p,p'</i> -DDE	72-55-9				pos.	5			Wilson et al. (2002)
PALM hAR(S)+Luc(S)	DHT	<i>p,p'</i> -DDE	72-55-9	pos.			pos.	15.2			Schrader and Cooke (2000)
PALM hAR(S)+Luc(S)	R1881	<i>p,p'</i> -DDE	72-55-9				pos.	0.75			Sultan et al. (2001)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	<i>p,p'</i> -DDE	72-55-9	weak		8820					Gaido et al. (1997)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	<i>p,p'</i> -DDE	72-55-9	neg.							Gaido et al. (1997)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	<i>p,p'</i> -DDE	72-55-9	pos.		350	neg.				O'Connor et al. (1999)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	<i>o,p'</i> -DDT	789-02-6	neg.							Otsuka Pharmaceutical Co. (2001)
CV-1 hAR(T)+Luc(T)	DHT	<i>o,p'</i> -DDT	789-02-6				pos.				Kelce et al. (1995)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	<i>o,p'</i> -DDT	789-02-6				pos.				Maness et al. (1998)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	<i>o,p'</i> -DDT	789-02-6	neg.							Gaido et al. (1997)
CV-1 hAR(T)+Luc(T)	DHT	<i>p,p'</i> -DDT	50-29-3				pos.				Kelce et al. (1995)

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HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	<i>p,p'</i> -DDT	50-29-3				pos.				Maness et al. (1998)
Yeast( <i>S. cere</i> YPH500) hAR(S)+ gal(S)	DHT	<i>p,p'</i> -DDT	50-29-3	neg.							Gaido et al. (1997)
CHO hAR (S)+Luc (S)	DHT	15-Dehydroetionogestrel		neg.							Deckers et al. (2000)
CHO hAR (S)+Luc (S)	DHT	15-Dehydronorethisterone		neg.							Deckers et al. (2000)
CHO hAR(T)+Luc(T)	R1881	Dexamethasone	50-02-2	neg. <sup>b</sup>							Vinggaard et al. (1999)
CV-1 hAR(T)+Luc(T)*	DHT	Dexamethasone	50-02-2		3x (0.1µM)						Hartig et al. (2002)
EPC rtAR ( ) +CAT(T)	DHT	Dexamethasone	50-02-2	neg.							Takeo and Yamashita (2000)
MDA-MB-453 hAR(E)+Luc(T)*	DHT	Dexamethasone	50-02-2	pos. <sup>b</sup>	248x (1µM)						Hartig et al. (2002)
PALM hAR(S)+Luc(S)	DHT	Dexamethasone	50-02-2	pos.							Schrader and Cooke (2000)
PALM hAR(S)+Luc(S)	R1881	Dexamethasone	50-02-2	pos.	30x (1µM)						Terouanne et al. (2000)
CHO hAR(T)+Luc(T)	R1881	Dibenzo[ <i>a,h</i> ]anthracene	53-70-3	pos.							Vinggaard et al. (2000)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Dibutyl phthalate	84-74-2	neg.							Otsuka Pharmaceutical Co. (2001)
CV-1 hAR(T)+Luc(T)*	DHT	3',5'-Dichloro-2-hydroxy-2-methylbut-3-enanilide	16776-82-1	pos.	17x (10µM)		pos.				Hartig et al. (2002)
MDA-MB-453 hAR(E)+Luc(T)*	DHT	3',5'-Dichloro-2-hydroxy-2-methylbut-3-enanilide	16776-82-1	pos.			pos.	0.1			Hartig et al. (2002)
MDA-MB-453-kb2 hAR(E)+Luc(S)	DHT	3',5'-Dichloro-2-hydroxy-2-methylbut-3-enanilide	16776-82-1				pos.	0.2			Wilson et al. (2002)
PALM hAR(S)+Luc(S)	R1881	3',5'-Dichloro-2-hydroxy-2-methylbut-3-enanilide	16776-82-1				pos.	0.02			Sultan et al. (2001)
CV-1 hAR(T)+Luc(T)*	DHT	(4-[2,2-Dichloro-1-(4-hydroxyphenyl)vinyl]phenol)		pos.	8.4x (10µM)						Hartig et al. (2002)
MDA-MB-453 hAR(E)+Luc(T)*	DHT	(4-[2,2-Dichloro-1-(4-hydroxyphenyl)vinyl]phenol)		pos.				0.1			Hartig et al. (2002)
MDA-MB-453-kb2 hAR(E)+Luc(S)	DHT	(4-[2,2-Dichloro-1-(4-hydroxyphenyl)vinyl]phenol)					pos.	5			Wilson et al. (2002)
CV-1 hAR(T)+Luc(T)*	DHT	2-[[3,5-Dichlorophenyl]carbamoyl]oxy]-2-methyl-3-butenic acid	119209-27-7	pos.	9.1x (10µM)		pos.				Hartig et al. (2002)
MDA-MB-453 hAR(E)+Luc(T)*	DHT	2-[[3,5-Dichlorophenyl]carbamoyl]oxy]-2-methyl-3-butenic acid	119209-27-7	pos.			pos.	0.1			Hartig et al. (2002)

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MDA-MB-453-kb2 hAR(E)+Luc(S)	DHT	2-[[3,5-Dichlorophenyl]carbamoyl]oxy]-2-methyl-3-butenic acid	119209-27-7				pos.	0.2			Wilson et al. (2002)
PALM hAR(S)+Luc(S)	R1881	2-[[3,5-Dichlorophenyl]carbamoyl]oxy]-2-methyl-3-butenic acid	119209-27-7				pos.	0.5			Sultan et al. (2001)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Dicofol	115-32-2	neg.							Otsuka Pharmaceutical Co. (2001)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Di-(2-ethylhexyl)phthalate	117-81-7	neg.							Otsuka Pharmaceutical Co. (2001)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Diethylstilbestrol	56-53-1	neg.							Otsuka Pharmaceutical Co. (2001)
CV-1 hAR(T)+Luc(T)	DHT	Diethylstilbestrol	56-53-1				pos.				Kelce et al. (1995)
LNCaP-FGC hAR(E)+CP	DHT	Diethylstilbestrol	56-53-1						neg.		Sonnenschein et al. (1989)
PALM hAR(S)+Luc(S)	DHT	Diethylstilbestrol	56-53-1	neg.			pos.	0.36			Schrader and Cooke (2000)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	Diethylstilbestrol	56-53-1	neg.							Gaido et al. (1997)
CHO hAR (S)+Luc (S)	DHT	5 -Dihydrotestosterone	521-18-6	pos.							Deckers et al. (2000)
CHO hAR(T)+CAT(T)+ gal(T)	DHT	5 -Dihydrotestosterone	521-18-6	pos.	100x (0.1nM)						Deslypere et al. (1992)
CHO-K1 hAR(S)+Luc(S)	DHT	5 -Dihydrotestosterone	521-18-6	pos.		0.000153					Otsuka Pharmaceutical Co. (2001)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	5 -Dihydrotestosterone	521-18-6	pos.		0.000153					Otsuka Pharmaceutical Co. (2001)
CV-1 hAR(T)+CAT(T)	R1881	5 -Dihydrotestosterone	521-18-6	pos.	7.0x (0.01nM)						Kemppainen et al. (1992)
CV-1 hAR(T)+Luc(T)	DHT	5 -Dihydrotestosterone	521-18-6	pos.							Kelce et al. (1995)
CV-1 hAR(T)+Luc(T)	DHT	5 -Dihydrotestosterone	521-18-6	pos.							Kemppainen and Wilson (1996)
CV-1 hAR(T)+Luc(T)	DHT	5 -Dihydrotestosterone	521-18-6	pos.	10x (0.001nM)						Kemppainen et al. (1999)
CV-1 hAR(T)+Luc(T)	DHT	5 -Dihydrotestosterone	521-18-6	pos.							Lambright et al. (2000)
CV-1 hAR(T)+Luc(T)*	DHT	5 -Dihydrotestosterone	521-18-6	pos.	45x (0.1nM)						Hartig et al. (2002)
CV-1 mAR(T)+CAT(T)	DHT	5 -Dihydrotestosterone	521-18-6	pos.							Van Dort et al. (2000)
EPC rtAR ( ) +CAT(T)	DHT	5 -Dihydrotestosterone	521-18-6	pos.							Takeo and Yamashita (2000)



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HeLa hAR(S)+DS-Luc(T)	DHT	5 -Dihydrotestosterone	521-18-6	pos.	7x (100nM)						Wang and Fondell (2001)
HeLa hAR(S)+M-Luc(T)	DHT	5 -Dihydrotestosterone	521-18-6	pos.	4.5x (100nM)						Wang and Fondell (2001)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	5 -Dihydrotestosterone	521-18-6	pos.							Gaido et al. (2000)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	5 -Dihydrotestosterone	521-18-6	pos.							Maness et al. (1998)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	5 -Dihydrotestosterone	521-18-6	pos.							Tamura et al. (2001)
LNCaP-FGC hAR(E)+CP	DHT	5 -Dihydrotestosterone	521-18-6						pos.	100	Sonnenschein et al. (1989)
MDA-MB-453 hAR(E)+Luc(T)*	DHT	5 -Dihydrotestosterone	521-18-6	pos.	23.7x (0.1nM)						Hartig et al. (2002)
MDA-MB-453-kb2 hAR(E)+Luc(S)	DHT	5 -Dihydrotestosterone	521-18-6								Lambright et al. (2000)
MDA-MB-453-kb2 hAR(E)+Luc(S)	DHT	5 -Dihydrotestosterone	521-18-6	pos.							Wilson et al. (2002)
PALM hAR(S)+Luc(S)	DHT	5 -Dihydrotestosterone	521-18-6	pos.							Schrader and Cooke (2000)
PALM hAR(S)+Luc(S)	R1881	5 -Dihydrotestosterone	521-18-6	pos.							Sultan et al. (2001)
PALM hAR(S)+Luc(S)	R1881	5 -Dihydrotestosterone	521-18-6	pos.		0.00004					Terouanne et al. (2000)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	5 -Dihydrotestosterone	521-18-6	pos.		0.0035					Gaido et al. (1997)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	5 -Dihydrotestosterone	521-18-6	pos.							O'Connor et al. (1998)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	5 -Dihydrotestosterone	521-18-6	pos.							O'Connor et al. (1999)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	5 -Dihydrotestosterone	521-18-6	pos.		0.0024					O'Connor et al. (2000)
Yeast( <i>S. cer</i> ) hAR(S)+ gal(S)	DHT	5 -Dihydrotestosterone	521-18-6	pos.		0.002		100			Moffat et al. (2001)
CHO hAR(T)+CAT(T)+ gal(T)	DHT	5 -Dihydrotestosterone	571-22-2	pos.	5x (1nM)						Deslypere et al. (1992)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	4,4'-Dihydroxybenzophenone	611-99-4				neg.				Gaido et al. (2000)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	Dihydroxy-DDE					pos.				Gaido et al. (2000)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	4,4-Dimethoxybenzhydrol	728-87-0				neg.				Gaido et al. (2000)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	Dimethoxy-DDE					pos.				Gaido et al. (2000)

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CHO hAR(T)+Luc(T)	R1881	Dimethylbenz[ <i>a</i> ]anthracene	57-97-6	pos.			pos.	10.4			Vinggaard et al. (2000)
PALM hAR(S)+Luc(S)	R1881	Diphenylphthalate	84-62-8				neg.				Sultan et al. (2001)
CV-1 mAR(T)+CAT(T)	DHT	DTIB		pos.							Van Dort et al. (2000)
CHO hAR(T)+CAT(T)+ gal(T)	DHT	Epitestosterone	481-30-1	pos.	15x (1nM)						Deslypere et al. (1992)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Equilin	474-86-2	neg.							Otsuka Pharmaceutical Co. (2001)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Equol	531-95-3	neg.							Otsuka Pharmaceutical Co. (2001)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	17 -Estradiol	57-91-0	neg.							Otsuka Pharmaceutical Co. (2001)
CHO hAR(T)+CAT(T)+ gal(T)	DHT	17 -Estradiol	50-28-2	pos.	40x (1nM)						Deslypere et al. (1992)
CHO hAR(T)+Luc(T)	R1881	17 -Estradiol	50-28-2	pos.			pos.	1			Vinggaard et al. (1999)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	17 -Estradiol	50-28-2	weak							Otsuka Pharmaceutical Co. (2001)
CV-1 hAR(T)+CAT(T)	R1881	17 -Estradiol	50-28-2	pos.	7.0x (10nM)						Kemppainen et al. (1992)
CV-1 hAR(T)+Luc(T)	DHT	17 -Estradiol	50-28-2				pos.				Kelce et al. (1995)
CV-1 hAR(T)+Luc(T)	DHT	17 -Estradiol	50-28-2	pos.							Kemppainen and Wilson (1996)
CV-1 hAR(T)+Luc(T)	DHT	17 -Estradiol	50-28-2	pos.	10x (0.01µM)		pos.	0.5			Kemppainen et al. (1999)
CV-1 hAR(T)+Luc(T)*	DHT	17 -Estradiol	50-28-2								Hartig et al. (2002)
EPC rtAR ( ) +CAT(T)	DHT	17 -Estradiol	50-28-2	neg.							Takeo and Yamashita (2000)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	17 -Estradiol	50-28-2	pos.			pos.				Maness et al. (1998)
LNCaP-FGC hAR(E)+CP	DHT	17 -Estradiol	50-28-2						pos.	1	Sonnenschein et al. (1989)
MDA-MB-453 hAR(E)+Luc(T)*	DHT	17 -Estradiol	50-28-2	pos.							Hartig et al. (2002)
MDA-MB-453-kb2 hAR(E)+Luc(S)	DHT	17 -Estradiol	50-28-2	pos.			pos.	0.05			Wilson et al. (2002)
PALM hAR(S)+Luc(S)	DHT	17 -Estradiol	50-28-2	pos.							Schrader and Cooke (2000)
PALM hAR(S)+Luc(S)	R1881	17 -Estradiol	50-28-2	neg.	2x (1µM)						Terouanne et al. (2000)

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Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	17 -Estradiol	50-28-2	pos.		0.0861					Gaido et al. (1997)
LNCaP-FGC hAR(E)+CP	DHT	Estriol	50-27-1						neg.		Sonnenschein et al. (1989)
CHO-K1 hAR(S)+Luc(S)	DHT	Estrone	53-16-7	pos.		0.0551					Otsuka Pharmaceutical Co. (2001)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Estrone	53-16-7	weak							Otsuka Pharmaceutical Co. (2001)
LNCaP-FGC hAR(E)+CP	DHT	Estrone	53-16-7						neg.		Sonnenschein et al. (1989)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	17 -Ethinyl estradiol	57-63-6	neg.							Otsuka Pharmaceutical Co. (2001)
LNCaP-FGC hAR(E)+CP	DHT	17 -Ethinyl estradiol	57-63-6						pos.	1	Sonnenschein et al. (1989)
CHO hAR (S)+Luc (S)	DHT	Etonogestrel	54048-10-1	pos.							Deckers et al. (2000)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Fenbuconazole	114369-43-6	neg.							Otsuka Pharmaceutical Co. (2001)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Fenitrothion	122-14-5	pos.			pos.				Otsuka Pharmaceutical Co. (2001)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	Fenitrothion	122-14-5	weak			pos.				Tamura et al. (2001)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	Fenvalerate	51630-58-1	neg.							Gaido et al. (1997)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	Finasteride	98319-26-7	neg.			neg.				O'Connor et al. (1998)
CHO hAR(T)+Luc(T)	R1881	Fluoranthene	206-44-0				pos.	4.6			Vinggaard et al. (2000)
CV-1 hAR(T)+Luc(T)	DHT	Fluoxymesterone	76-43-7	pos.	10x (0.001 $\mu$ M)		neg.				Kemppainen et al. (1999)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Flutamide	13311-84-7	neg.							Otsuka Pharmaceutical Co. (2001)
CV-1 hAR(T)+CAT(T)	R1881	Flutamide	13311-84-7	neg.			pos.				Kemppainen et al. (1992)
HeLa hAR(S)+DS-Luc(T)	DHT	Flutamide	13311-84-7	neg.	0.2x (100nM)						Wang and Fondell (2001)
HeLa hAR(S)+M-Luc(T)	DHT	Flutamide	13311-84-7	neg.	0.2x (100nM)						Wang and Fondell (2001)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	Flutamide	13311-84-7	neg.			pos.				Maness et al. (1998)
PALM hAR(S)+Luc(S)	DHT	Flutamide	13311-84-7				pos.				Schrader and Cooke (2000)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	Flutamide	13311-84-7	neg.							Gaido et al. (1997)

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Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	Flutamide	13311-84-7				pos.	220			O'Connor et al. (1999)
Yeast( <i>S. cer</i> ) hAR(S)+ gal(S)	DHT	Flutamide	13311-84-7	neg.			pos.				Moffat et al. (2001)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	Flutamide	13311-84-7	neg.			pos.	22			O'Connor et al. (1998)
CHO hAR (S)+Luc (S)	DHT	Gestodene	60282-87-3	pos.							Deckers et al. (2000)
Yeast( <i>S. cer</i> ) hAR(S)+ gal(S)	DHT	HEPES	7365-45-9	neg.			neg.				Moffat et al. (2001)
CHO hAR(T)+Luc(T)	R1881	2,2',3,4,4',5,5'-Heptachlorobiphenyl	35065-29-3	neg.			neg.				Bonefeld-Jorgenson et al. (2001)
CHO hAR(T)+Luc(T)	R1881	2,2',3,4,4',5'-Hexachlorobiphenyl	35065-28-2	neg.			pos.	1			Bonefeld-Jorgenson et al. (2001)
CHO hAR(T)+Luc(T)	R1881	2,2',4,4',5,5'-Hexachlorobiphenyl	35065-27-2	neg.			neg.				Bonefeld-Jorgenson et al. (2001)
PALM hAR(S)+Luc(S)	DHT	-Hexachlorocyclohexane	319-84-6	neg.			pos.	8.2			Schrader and Cooke (2000)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	-Hexachlorocyclohexane	319-85-7	neg.							Otsuka Pharmaceutical Co. (2001)
PALM hAR(S)+Luc(S)	DHT	-Hexachlorocyclohexane	319-85-7	neg.			neg.				Schrader and Cooke (2000)
PALM hAR(S)+Luc(S)	DHT	-Hexachlorocyclohexane	319-86-8	neg.			pos.	17.9			Schrader and Cooke (2000)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Hydramethylnone	67485-29-4	neg.			neg.				Otsuka Pharmaceutical Co. (2001)
CHO hAR(T)+Luc(T)	R1881	Hydroxyflutamide	52806-53-8				pos.	0.01			Vinggaard et al. (1999)
CV-1 hAR(T)+CAT(T)	R1881	Hydroxyflutamide	52806-53-8	neg.			pos.				Kemppainen et al. (1992)
CV-1 hAR(T)+Luc(T)	DHT	Hydroxyflutamide	52806-53-8				pos.				Kelce et al. (1995)
CV-1 hAR(T)+Luc(T)	DHT	Hydroxyflutamide	52806-53-8	pos.			pos.				Kemppainen and Wilson (1996)
CV-1 hAR(T)+Luc(T)	DHT	Hydroxyflutamide	52806-53-8	weak	10x (10µM)		pos.	0.1			Kemppainen et al. (1999)
CV-1 hAR(T)+Luc(T)*	DHT	Hydroxyflutamide	52806-53-8	pos.	2.1x (10µM)		pos.				Hartig et al. (2002)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	Hydroxyflutamide	52806-53-8	pos.			pos.				Maness et al. (1998)
MDA-MB-453-kb2 hAR(E)+Luc(S)	DHT	Hydroxyflutamide	52806-53-8				pos.	10			Wilson et al. (2002)
PALM hAR(S)+Luc(S)	R1881	Hydroxyflutamide	52806-53-8				pos.	0.1			Terouanne et al. (2000)

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PALM hAR(S)+Luc(S)	R1881	Hydroxyflutamide	52806-53-8	weak	5x (1μM)		pos.	10			Terouanne et al. (2000)
PC-3 hAR(T)+Luc(T)	R1881	Hydroxyflutamide	52806-53-8				pos.	0.02			Terouanne et al. (2000)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	Hydroxyflutamide	52806-53-8	pos.		8.21					Gaido et al. (1997)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	Hydroxyflutamide	52806-53-8	pos.		82	pos.				O'Connor et al. (1998)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	4-Hydroxytamoxifen	68047-06-3	neg.							Otsuka Pharmaceutical Co. (2001)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	ICI 182,780	129453-61-8	neg.			neg.				O'Connor et al. (1998)
PALM hAR(S)+Luc(S)	R1881	Inocoteron	83646-86-0	pos.	18x (1μM)		pos.	30			Terouanne et al. (2000)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Kepone	143-50-0	neg.							Otsuka Pharmaceutical Co. (2001)
CV-1 hAR(T)+Luc(T)	DHT	Kepone	143-50-0				neg.				Kelce et al. (1995)
PALM hAR(S)+Luc(S)	DHT	Kepone	143-50-0	neg.			pos.	6.9			Schrader and Cooke (2000)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	Ketoconazole	65277-42-1	weak			neg.				O'Connor et al. (1998)
CHO hAR (S)+Luc (S)	DHT	11-Keto-15-dehydronorethisterone		pos.							Deckers et al. (2000)
CHO hAR (S)+Luc (S)	DHT	11-Ketonorethisterone	5210-27-5	pos.							Deckers et al. (2000)
CHO-K1 hAR(S)+Luc(S)	DHT	11-Ketotestosterone	564-35-2	pos.		0.0058					Otsuka Pharmaceutical Co. (2001)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	11-Ketotestosterone	564-35-2	pos.		0.00151					Otsuka Pharmaceutical Co. (2001)
EPC rtAR ( ) +CAT(T)	DHT	11-Ketotestosterone	564-35-2	pos.							Takeo and Yamashita (2000)
CHO hAR (S)+Luc (S)	DHT	Levonorgestrel	797-63-7	pos.							Deckers et al. (2000)
CHO-K1 hAR(S)+Luc(S)	DHT	Levonorgestrel	797-63-7	pos.		0.0016					Otsuka Pharmaceutical Co. (2001)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Levonorgestrel	797-63-7	pos.		0.000373					Otsuka Pharmaceutical Co. (2001)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Lindane	58-89-9	neg.							Otsuka Pharmaceutical Co. (2001)
PALM hAR(S)+Luc(S)	DHT	Lindane	58-89-9	neg.			neg.				Schrader and Cooke (2000)
PALM hAR(S)+Luc(S)	R1881	Lindane	58-89-9				neg.				Sultan et al. (2001)

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CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Linuron	330-55-2	pos.							Otsuka Pharmaceutical Co. (2001)
CV-1 hAR(T)+Luc(T)	DHT	Linuron	330-55-2				pos.	10			Lambright et al. (2000)
MDA-MB-453-kb2 hAR(E)+Luc(S)	DHT	Linuron	330-55-2				pos.	5			Lambright et al. (2000)
MDA-MB-453-kb2 hAR(E)+Luc(S)	DHT	Linuron	330-55-2				pos.	5			Wilson et al. (2002)
CV-1 hAR(T)+Luc(T)	DHT	Medroxyprogesterone acetate	71-58-9	pos.	10x (0.1nM)		neg.				Kemppainen et al. (1999)
CV-1 hAR(T)+Luc(T)*	DHT	Medroxyprogesterone acetate	71-58-9	pos.	10x (0.01 μM)						Hartig et al. (2002)
MDA-MB-453 hAR(E)+Luc(T)*	DHT	Medroxyprogesterone acetate	71-58-9	pos.	64x (0.01 μM)						Hartig et al. (2002)
MDA-MB-453-kb2 hAR(E)+Luc(S)	DHT	Medroxyprogesterone acetate	71-58-9	pos.							Wilson et al. (2002)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Methoxychlor	72-43-5	neg.							Otsuka Pharmaceutical Co. (2001)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	Methoxychlor	72-43-5				pos.				Gaido et al. (2000)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	Methoxychlor	72-43-5				weak				Maness et al. (1998)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	Methoxychlor	72-43-5	neg.							Gaido et al. (1997)
CHO-K1 hAR(S)+Luc(S)	DHT	Methyltestosterone	58-18-4	pos.		0.000135					Otsuka Pharmaceutical Co. (2001)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Methyltestosterone	58-18-4	pos.		0.0000274					Otsuka Pharmaceutical Co. (2001)
EPC rAR ( ) +CAT(T)	DHT	Methyltestosterone	58-18-4	pos.							Takeo and Yamashita (2000)
CHO hAR(T)+Luc(T)	R1881	Methyltrienolone	965-93-5	pos.				0.0001			Bonefeld-Jorgenson et al. (2001)
CHO hAR(T)+Luc(T)	R1881	Methyltrienolone	965-93-5	pos.							Vinggaard et al. (1999)
CHO hAR(T)+Luc(T)	R1881	Methyltrienolone	965-93-5	pos.							Vinggaard et al. (2000)
CV-1 hAR(T)+CAT(T)	R1881	Methyltrienolone	965-93-5	pos.	7.5x (0.01nM)						Kemppainen et al. (1992)
CV-1 hAR(T)+Luc(T)	DHT	Methyltrienolone	965-93-5	pos.	10x (0.001nM)		neg.				Kemppainen et al. (1999)
LNCaP-FGC hAR(E)+CP	DHT	Methyltrienolone	965-93-5						pos.	10,000	Sonnenschein et al. (1989)
PALM hAR(S)+Luc(S)	R1881	Methyltrienolone	965-93-5	pos.							Sultan et al. (2001)

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PALM hAR(S)+Luc(S)	R 1881	Methyltrienolone	965-93-5	pos.							Terouanne et al. (2000)
PC-3 hAR(T)+Luc(T)	R 1881	Methyltrienolone	965-93-5	pos.							Terouanne et al. (2000)
CV-1 hAR(T)+Luc(T)	DHT	Mibolerone	3704-09-4	pos.	10x (0.001nM)		neg.				Kemppainen et al. (1999)
LNCaP-FGC hAR(E)+CP	DHT	Mibolerone	3704-09-4						pos.	10,000	Sonnenschein et al. (1989)
PALM hAR(S)+Luc(S)	R 1881	Mibolerone	3704-09-4	pos.		0.00003					Terouanne et al. (2000)
CHO-K1 hAR(S)+Luc(S)	DHT	Mifepristone	84371-65-3	pos.		0.0136					Otsuka Pharmaceutical Co. (2001)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Mifepristone	84371-65-3	neg.							Otsuka Pharmaceutical Co. (2001)
PALM hAR(S)+Luc(S)	R 1881	Mifepristone	84371-65-3	pos.	4x (1µM)		pos.	0.05			Terouanne et al. (2000)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	Mifepristone	84371-65-3	pos.		2100					O'Connor et al. (2000)
CV-1 hAR(T)+CAT(T)	R 1881	Mifepristone	84371-65-3	pos.	10x (10nM)		pos.				Kemppainen et al. (1992)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Mirex	2385-85-5	neg.							Otsuka Pharmaceutical Co. (2001)
PALM hAR(S)+Luc(S)	DHT	Mirex	2385-85-5	neg.			neg.				Schrader and Cooke (2000)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	Monohydroxy-DDE					pos.				Gaido et al. (2000)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	Monohydroxymethoxychlor	28463-03-8				pos.				Gaido et al. (2000)
LNCaP-FGC hAR(E)+CP	DHT	Moxestrol	34816-55-2						neg.		Sonnenschein et al. (1989)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Nafoxidine	1847-63-8	neg.							Otsuka Pharmaceutical Co. (2001)
MDA-MB-453-kb2 hAR(E)+Luc(S)	DHT	Neburon	555-37-3				pos.	10			Wilson et al. (2002)
PALM hAR(S)+Luc(S)	R 1881	Nilutamide	63612-50-0				pos.	0.3			Terouanne et al. (2000)
PALM hAR(S)+Luc(S)	R 1881	Nilutamide	63612-50-0	weak	5x (1µM)		pos.	10			Terouanne et al. (2000)
PC-3 hAR(T)+Luc(T)	R 1881	Nilutamide	63612-50-0				pos.	0.15			Terouanne et al. (2000)
PALM hAR(S)+Luc(S)	DHT	<i>cis</i> -Nonachlor	5103-73-1	neg.			neg.				Schrader and Cooke (2000)
PALM hAR(S)+Luc(S)	DHT	<i>trans</i> -Nonachlor	39765-80-5	neg.			neg.				Schrader and Cooke (2000)

## Information Sorted by Substance Name and Assay

Assay Type*	Reference Androgen**	Substance Name	CASRN†	AGONISM Qualitative††	AGONISM Maximum Fold x (conc. $\mu$ M)††	AGONISM EC50 ( $\mu$ M)††	ANTAG. <sup>a,b</sup> Qualitative††	ANTAG. <sup>a,b</sup> IC50 ( $\mu$ M)††	Prolif. Response <sup>a</sup> ††	RPP <sup>a,b,††</sup>	Reference
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	<i>p</i> - Nonylphenol	104-40-5	neg.							Otsuka Pharmaceutical Co. (2001)
Yeast( <i>S. cer</i> ) hAR(S)+ gal(S)	DHT	<i>p</i> -Nonylphenol	104-40-5	neg.							Gaido et al. (1997)
Yeast( <i>S. cer</i> ) hAR(S)+ gal(S)	DHT	<i>p</i> -Nonylphenol	104-40-5	pos.		2	neg.	0.001			Moffat et al. (2001)
Yeast( <i>S. cer</i> ) hAR(S)+ gal(S)	DHT	1-O-(Nonylphenyl)-a,b-D-glucopyranosiduric acid		neg.			neg.				Moffat et al. (2001)
CHO hAR (S)+Luc (S)	DHT	Norethisterone	68-22-4	pos.							Deckers et al. (2000)
CHO-K1 hAR(S)+Luc(S)	DHT	Norethisterone	68-22-4	pos.		0.00716					Otsuka Pharmaceutical Co. (2001)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Norethisterone	68-22-4	pos.		0.00371					Otsuka Pharmaceutical Co. (2001)
CHO-K1 hAR(S)+Luc(S)	DHT	Norgestrel	6533-00-2	pos.		0.00102					Otsuka Pharmaceutical Co. (2001)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Norgestrel	6533-00-2	pos.		0.000404					Otsuka Pharmaceutical Co. (2001)
CHO-K1 hAR(S)+Luc(S)	DHT	19-Nortestosterone	434-22-0	pos.		0.00022					Otsuka Pharmaceutical Co. (2001)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	19-Nortestosterone	434-22-0	pos.		0.0000923					Otsuka Pharmaceutical Co. (2001)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	<i>p</i> - <i>tert</i> -Octylphenol	140-66-9	neg.							Otsuka Pharmaceutical Co. (2001)
Yeast( <i>S. cer</i> ) hAR(S)+ gal(S)	DHT	<i>p</i> - <i>tert</i> -Octylphenol	140-66-9	neg.			neg.				Moffat et al. (2001)
Yeast( <i>S. cer</i> ) hAR(S)+ gal(S)	DHT	1-O-(Octylphenyl)-a,b-D-glucopyranosiduronic acid		neg.			neg.				Moffat et al. (2001)
CV-1 hAR(T)+Luc(T)	DHT	Oxandrolone	53-39-4	pos.	10x (0.001 $\mu$ M)		neg.				Kemppainen et al. (1999)
PALM hAR(S)+Luc(S)	DHT	Oxychlordane	27304-13-8	neg.			neg.				Schrader and Cooke (2000)
Yeast( <i>S. cer</i> ) hAR(S)+ gal(S)	DHT	Permethrin	52645-53-1	neg.							Gaido et al. (1997)
CHO hAR(T)+Luc(T)	R1881	Phenanthrene	85-01-8				neg.				Vinggaard et al. (2000)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Phenobarbital, sodium salt	57-30-7	neg.							Otsuka Pharmaceutical Co. (2001)
Yeast( <i>S. cer</i> ) hAR(S)+ gal(S)	DHT	Phenothrin	26002-80-2	neg.							Gaido et al. (1997)
PALM hAR(S)+Luc(S)	DHT	Photomirex	39801-14-4	neg.			neg.				Schrader and Cooke (2000)
LNCaP-FGC hAR(E)+CP	DHT	Pregnenolone	145-13-1						pos.	1	Sonnenschein et al. (1989)



## Information Sorted by Substance Name and Assay

Assay Type*	Reference Androgen**	Substance Name	CASRN†	AGONISM Qualitative††	AGONISM Maximum Fold x (conc. $\mu$ M)††	AGONISM EC50 ( $\mu$ M)††	ANTAG. Qualitative††	ANTAG. IC50 ( $\mu$ M)††	Prolif. Response ††	RPP <sup>a,b</sup> ††	Reference
CHO hAR(T)+Luc(T)	R1881	Procymidone	32809-16-8				pos.	5			Vinggaard et al. (1999)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Procymidone	32809-16-8	neg.							Otsuka Pharmaceutical Co. (2001)
MDA-MB-453-kb2 hAR(E)+Luc(S)	DHT	Procymidone	32809-16-8				pos.	10			Wilson et al. (2002)
CHO hAR(T)+CAT(T)+ gal(T)	DHT	Progesterone	57-83-0	neg.							Deslypere et al. (1992)
CHO hAR(T)+Luc(T)	R1881	Progesterone	57-83-0	pos.			pos.	0.1			Vinggaard et al. (1999)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Progesterone	57-83-0	neg.							Otsuka Pharmaceutical Co. (2001)
CV-1 hAR(T)+CAT(T)	R1881	Progesterone	57-83-0	pos.	7.0x (100nM)						Kemppainen et al. (1992)
CV-1 hAR(T)+Luc(T)	DHT	Progesterone	57-83-0	pos.							Kemppainen and Wilson (1996)
CV-1 hAR(T)+Luc(T)	DHT	Progesterone	57-83-0	pos.	10x (0.1 $\mu$ M)		weak	0.5			Kemppainen et al. (1999)
EPC $\alpha$ AR ( ) +CAT(T)	DHT	Progesterone	57-83-0	neg.							Takeo and Yamashita (2000)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	Progesterone	57-83-0	pos.			weak				Maness et al. (1998)
LNCaP-FGC hAR(E)+CP	DHT	Progesterone	57-83-0						pos.	100	Sonnenschein et al. (1989)
PALM hAR(S)+Luc(S)	DHT	Progesterone	57-83-0	pos.							Schrader and Cooke (2000)
PALM hAR(S)+Luc(S)	R1881	Progesterone	57-83-0	weak	10x (1 $\mu$ M)						Terouanne et al. (2000)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	Progesterone	57-83-0	pos.		0.0089					Gaido et al. (1997)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	Progesterone	57-83-0	pos.		5.2					O'Connor et al. (2000)
LNCaP-FGC hAR(E)+CP	DHT	Promegestone	34184-77-5						pos.	1	Sonnenschein et al. (1989)
PALM hAR(S)+Luc(S)	R1881	Promegestone	34184-77-5	weak	40x (1 $\mu$ M)		pos.	0.09			Terouanne et al. (2000)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Propylthiurea	927-67-3	neg.							Otsuka Pharmaceutical Co. (2001)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Prothiofos	34643-46-4	pos.			weak				Otsuka Pharmaceutical Co. (2001)
CHO hAR(T)+Luc(T)	R1881	Pyrene	129-00-0				neg.				Vinggaard et al. (2000)
PALM hAR(S)+Luc(S)	R1881	R2956	42438-88-0	pos.	45x (1 $\mu$ M)		pos.	45			Terouanne et al. (2000)

## Information Sorted by Substance Name and Assay

Assay Type*	Reference Androgen**	Substance Name	CASRN†	AGONISM Qualitative††	AGONISM Maximum Fold x (conc. µM)††	AGONISM EC50 (µM)††	ANTAG. <sup>a,b</sup> Qualitative††	ANTAG. <sup>a,b</sup> IC50 (µM)††	Prolif. Response <sup>a</sup> ††	RPP <sup>a,b,†</sup> ††	Reference
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Rimsulfuron	122931-48-0	neg.							Otsuka Pharmaceutical Co. (2001)
CV-1 hAR(T)+Luc(T)	DHT	RU56187	143782-25-6	pos.	10x (0.01µM)		weak	0.1nM			Kemppainen et al. (1999)
CV-1 mAR(T)+CAT(T)	DHT	RU 59063	155180-53-3	pos.							Van Dort et al. (2000)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	-Sitosterol	83-46-5	neg.							Otsuka Pharmaceutical Co. (2001)
CHO hAR(T)+Luc(T)	R1881	Spironolactone	52-01-7				pos.	0.5			Vinggaard et al. (1999)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Spironolactone	52-01-7	pos.			neg.				Otsuka Pharmaceutical Co. (2001)
PALM hAR(S)+Luc(S)	R1881	Spironolactone	52-01-7	pos.	40x (1µM)		pos.	0.09			Terouanne et al. (2000)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Tamoxifen	10540-29-1	neg.							Otsuka Pharmaceutical Co. (2001)
CHO hAR(T)+CAT(T)+ gal(T)	DHT	Testosterone	58-22-0	pos.	100x (1nM)						Deslypere et al. (1992)
CHO-K1 hAR(S)+Luc(S)	DHT	Testosterone	58-22-0	pos.		0.000527					Otsuka Pharmaceutical Co. (2001)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Testosterone	58-22-0	pos.		0.000107					Otsuka Pharmaceutical Co. (2001)
CV-1 hAR(T)+CAT(T)	R1881	Testosterone	58-22-0	pos.	7.5x (0.01nM)						Kemppainen et al. (1992)
CV-1 hAR(T)+Luc(T)	DHT	Testosterone	58-22-0	pos.	10x (0.01nM)		neg.				Kemppainen et al. (1999)
EPC rtAR ( ) +CAT(T)	DHT	Testosterone	58-22-0	pos.							Takeo and Yamashita (2000)
HeLa hAR(S)+DS-Luc(T)	DHT	Testosterone	58-22-0	pos.	6x (100nM)						Wang and Fondell (2001)
HeLa hAR(S)+M-Luc(T)	DHT	Testosterone	58-22-0	pos.	5x (100nM)						Wang and Fondell (2001)
HeLa hAR(S)+PB-Luc(T)	DHT	Testosterone	58-22-0	pos.	6x (100nM)						Wang and Fondell (2001)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	Testosterone	58-22-0	pos.							Maness et al. (1998)
LNCaP-FGC hAR(E)+CP	DHT	Testosterone	58-22-0						pos.	100	Sonnenschein et al. (1989)
PALM hAR(S)+Luc(S)	R1881	Testosterone	58-22-0	pos.							Sultan et al. (2001)
PALM hAR(S)+Luc(S)	R1881	Testosterone	58-22-0	pos.		0.0002					Terouanne et al. (2000)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	Testosterone	58-22-0	pos.		0.0047					Gaido et al. (1997)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	Testosterone	58-22-0	pos.		0.0099					O'Connor et al. (1999)
Yeast( <i>S. cer</i> YPH500) hAR(S)+ gal(S)	DHT	Testosterone	58-22-0	pos.		0.012					O'Connor et al. (2000)

## Information Sorted by Substance Name and Assay

Assay Type*	Reference Androgen**	Substance Name	CASRN†	AGONISM Qualitative††	AGONISM Maximum Fold x (conc. µM)††	AGONISM EC50 (µM)††	ANTAG. <sup>a,b</sup> Qualitative††	ANTAG. <sup>a,b</sup> IC50 (µM)††	Prolif. Response <sup>a</sup> ††	RPP <sup>a,b,†,†</sup>	Reference
PALM hAR(S)+Luc(S)	DHT	2,3,7,8-Tetrachlorodibenzo-p-dioxin	1746-01-6	neg.			pos.	6.5			Schrader and Cooke (2000)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Toxaphene	8001-35-2	neg.							Otsuka Pharmaceutical Co. (2001)
PALM hAR(S)+Luc(S)	DHT	Toxaphene	8001-35-2	pos.		10	weak	1935			Schrader and Cooke (2000)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Tralomethrin	66841-25-6	pos.			neg.				Otsuka Pharmaceutical Co. (2001)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	Trihydroxymethoxychlor					neg.				Gaido et al. (2000)
HepG2 hAR(T)+Luc(T)+ gal(T)	DHT	Trimethoxymethoxychlor					neg.				Gaido et al. (2000)
CHO hAR(T)+Luc(T)	R1881	Vinclozolin	50471-44-8				pos.	0.5			Vinggaard et al. (1999)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Vinclozolin	50471-44-8	neg.			weak				Otsuka Pharmaceutical Co. (2001)
MDA-MB-453-kb2 hAR(E)+Luc(S)	DHT	Vinclozolin	50471-44-8				pos.	0.05			Wilson et al. (2002)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Zearalenone	17924-92-4	neg.							Otsuka Pharmaceutical Co. (2001)
CHO-K1 hAR(T)+Luc(T)+EGFP(T)	DHT	Zearanol	26538-44-3	neg.							Otsuka Pharmaceutical Co. (2001)

\* Assays used for testing listed alphabetically.

\*\*R1881 = Methyltrienolone, DHT = 5 - Dihydrotestosterone, T=Testosterone, M=Mibolerone.

† Empty cells indicate that no CASRN could be found.

†† Empty cells indicate that no information was provided in the publication.

<sup>a</sup>ANTAG. = Antagonism; Prolif. Resp. = proliferative response; RPP = relative proliferative potency.

<sup>b</sup> Neg. and pos. indicate that a negative or positive response was reported for a specific substance in a specific assay.

hAR(E) indicates that the hAR is endogenous.

hAR(T) indicates that the hAR has been transiently transfected into the cell line.

hAR(S) indicates that the hAR has been stabilized in the cell by selection of a transiently transfected cell line.

Luc/CAT(T) indicates that the reporter gene was transiently transfected into the cell line.

Luc/CAT(S) indicates that the reporter gene has been stabilized in the cell by selection of a transiently transfected cell line.

CHO hAR(T)+CAT(T)+ gal(T) are Chinese hamster ovary cells transiently transfected with the human AR and the MMTV response element linked to the chloramphenicol acetyltransferase gene plus a plasmid harboring the -galactosidase gene.

CHO hAR(T)+Luc(T) are Chinese hamster ovary cells transiently transfected with the human AR and the MMTV response element linked to the luciferase gene.

CHO-K1 hAR(S)+Luc(S) are Chinese hamster ovary cells stably transfected with both the human AR and the IND response element linked to the luciferase gene.

CHO-K1-hAR(T)+IND-ARE-luc+EGFP are Chinese hamster ovary cells transiently transfected with the human AR and the IND response element linked to the luciferase gene plus a

plasmid harboring the green fluorescent protein gene to measure toxicity.

CV1 hAR(T)+CAT(T) are CV1 cells transiently transfected with the human AR and the MMTV response element linked to the chloramphenicol acetyltransferase gene.

CV1 hAR(T)+Luc(T) are CV1 cells transiently transfected with the human AR and the MMTV response element linked to the luciferase gene.

CV1 hAR(T)+Luc(T)\* are CV1 cells that are transduced with a virion carrying the genes for the human AR and the MMTV response element linked to the luciferase gene.

CV-1 mAR(T)+CAT(T) are CV1 cells transiently transfected with the mouse AR and the chloramphenicol acetyltransferase gene.

EPC rAR (T)+CAT(T) are fish cells transiently transfected with the rainbow trout AR and the tyrosine kinase response element linked to the chloramphenicol acetyltransferase gene.

HeLa hAR(S)+Luc(T) are HeLa cells with stable human AR gene and a reporter plasmid linked to three different response elements linked to the luciferase gene (M, DS and PB-Luc).

HepG2 hAR(T)+Luc(T)+ gal(T) are Hep G2 cells transiently transfected with the human AR and the MMTV response element linked to the luciferase gene plus a plasmid harboring the -galactosidase gene.

LNCaP-FGC hAR(E)+CP cells are cells from human supraclavicular lymph node from patient with prostatic adenocarcinoma used to measure cell proliferation.

MDA-MB-453 hAR(E)+Luc(T)\* are MDA-MB-453 cells with an endogenous human AR gene and a transduced virion containing the luciferase reporter gene.

MDA-MB-453-kb2 hAR(E)+Luc(S) are MDA cells with an endogenous human AR gene and the stably transfected MMTV response element linked to the luciferase gene.

PALM hAR(S)+Luc(S) are PC-3 cells stably transfected with both the human AR and the MMTV response element linked to the luciferase gene.

PC-3 hAR(T)+Luc(T) are PC-3 cells transiently with the human AR and the MMTV response element linked to the luciferase gene.

Yeast (*S. cere*) hAR(S)+ gal(S) are *Saccharomyces cerevisiae* cells stably transfected with both the human AR and the metallotheinin response element linked to the -galactosidase gene.

Yeast (*S. cer YPH500*) hAR(S)+ gal(S) are *Saccharomyces cerevisiae* YPH500 cells stably transfected with both the human AR and the metallotheinin response element linked to the -galactosidase gene.

Values in italics have been estimated from a graphical representation of the data.

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## **Appendix D2**

### **Substances Tested in the *In Vitro* AR TA Assays**

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# **Appendix E**

## **Assay Distribution of Substances Tested in *In Vitro* AR TA Assays**

**E1 Substances Tested for Agonism Activity**

**E2 Substances Tested for Antagonism Activity**

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## **Appendix E1**

### **Substances Tested in Two or More Selected *In Vitro* AR TA Assays**

### **Substances Tested for Agonism Activity**

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## Substances Tested for Agonism Activity

Substance	CHO hAR(S)+Luc(S)	CHO-K1 hAR(S)+Luc(S)	CHO hAR(T)+CAT(T)+ βgal(T)	CHO hAR(T)+Luc(T)	CHO-K1 hAR(T)+Luc(T) +EGFP(T)	CV-1 hAR(T)+CAT(T)	CV-1 hAR(T)+Luc(T)	CV-1 hAR(T)+Luc(T)*
Aldosterone					neg.			
Androstenedione		pos.	pos.		pos.			
Bicalutamide							pos.	
2,2-Bis-( <i>p</i> -hydroxyphenyl)-1,1,1-trichloroethane					neg.			pos.
Butyl benzyl phthalate					neg.			
Cortisol		pos.	pos.		neg.			
Coumestrol					neg.			
Cyproterone acetate					pos.	pos.	pos.	
<i>p,p'</i> -DDD					neg.			
<i>o,p'</i> -DDE					neg.			
<i>p,p'</i> -DDE					neg.			
<i>o,p'</i> -DDT					neg.			
Dexamethasone				neg.				pos.
3',5'-Dichloro-2-hydroxy-2-methylbut-3-enamide								pos.
(4-[2,2-Dichloro-1-(4-hydroxyphenyl)vinyl]phenol								pos.
2-[[3,5-(Dichlorophenyl)-carbamoyloxy]-2-methyl-3-butenic acid								pos.
Diethylstilbestrol					neg.			
5 -Dihydrotestosterone	pos.	pos.	pos.		pos.	pos.	pos. (4)	pos.
17 -Estradiol			pos.	pos.	pos.	pos.	pos. (3)	pos.
Estrone		pos.			pos.			
17 -Ethinyl estradiol					neg.			
Fenitrothion					pos.			
Flutamide					neg.	neg.		
-Hexachlorocyclohexane					neg.			
Hydroxyflutamide						neg.	pos. (2)	pos.
Kepone					neg.			
11-Ketotestosterone		pos.			pos.			
Levonorgestrel	pos.	pos.			pos.			
Lindane					neg.			
Medroxyprogesterone acetate							pos.	pos.
Methoxychlor					neg.			
Methyltestosterone		pos.			pos.			
Methyltrienolone				pos. (3)		pos.	pos.	
Mibolerone							pos.	
Mifepristone		pos.			neg.	pos.		
Mirex					neg.			
<i>p</i> -Nonylphenol					neg.			
Norethisterone	pos.	pos.			pos.			
Norgestrel		pos.			pos.			
19-Nortestosterone		pos.			pos.			
Progesterone			neg.	pos.	neg.	pos.	pos. (2)	
Promegestone								
Spirolactone					pos.			
Testosterone		pos.	pos.		pos.	pos.	pos.	
Toxaphene					neg.			

Abbreviations: neg. = negative call, pos. = positive call.

Numbers in parentheses refer to the number of times the substance was tested for agonism activity in that assay, if more than once. If the study calls within an assay were discordant, the substance was classified as positive and the number of positive calls among the number of times it was tested presented in parentheses. A response classified as weak by the investigator was classified as positive.

## Substances Tested for Agonism Activity

Substance	HepG2 hAR(T)+Luc(T)+βgal(T)	MDA-MB-453 hAR(E)+Luc(T)*	MDA-MB-453-kb2 hAR(E)+Luc(S)	PC-3 hAR(T)+Luc(T)	PALM hAR(S)+Luc(S)	Yeast( <i>S. cer</i> ) hAR(S)+βgal(S)
Aldosterone					neg.	
Androstenedione						
Bicalutamide					pos.	
2,2-Bis-( <i>p</i> -hydroxyphenyl)-1,1,1-trichloroethane		pos.				
Butyl benzyl phthalate						neg.
Cortisol					pos. (2)	neg.
Coumestrol						neg. (2)
Cyproterone acetate				pos.	pos. (2)	
<i>p,p'</i> -DDD						neg.
<i>o,p'</i> -DDE						neg.
<i>p,p'</i> -DDE	pos.				pos.	pos. (2/3)
<i>o,p'</i> -DDT						neg.
Dexamethasone		pos.			pos. (2)	
3',5'-Dichloro-2-hydroxy-2-methylbut-3-enamide		pos.				
(4-[2,2-Dichloro-1-(4-hydroxyphenyl)vinyl]phenol		pos.				
2-[3,5-(Dichlorophenyl)-carbamoyloxy]-2-methyl-3-butenic acid		pos.				
Diethylstilbestrol					neg.	neg.
5-α-Dihydrotestosterone	pos. (3)	pos.	pos. (2)		pos. (3)	pos. (5)
17-β-Estradiol	pos.	pos.	pos.		pos. (1/2)	pos.
Estrone						
17-β-Ethinyl estradiol						
Fenitrothion	pos.					
Flutamide	neg.					neg. (3)
1-Hexachlorocyclohexane					neg.	
Hydroxyflutamide	pos.				pos.	pos. (2)
Kepone					neg.	
11-Ketotestosterone						
Levonorgestrel						
Lindane					neg.	
Medroxyprogesterone acetate		pos.	pos.			
Methoxychlor						neg.
Methyltestosterone						
Methyltrienolone				pos.	pos. (2)	
Mibolerone					pos.	
Mifepristone					pos.	pos.
Mirex					neg.	
<i>p</i> -Nonylphenol						pos. (1/2)
Norethisterone						
Norgestrel						
19-Nortestosterone						
Progesterone	pos.				pos. (2)	pos. (2)
Promegestone					pos.	
Spirolactone					pos.	
Testosterone	pos.				pos. (2)	pos. (3)
Toxaphene					pos.	

Abbreviations: neg. = negative call, ¶Abbreviations: neg. = negative call, pos. = positive call.

Numbers in parentheses refer to the number of times the substance was tested for agonism activity in that assay, if more than once. If the study calls were discordant, the substance was classified as positive and the number of positive calls among the number of times it was tested classified as weak by the investigator presented in parentheses. A response classified as weak by the investigator was classified as positive.



## **Appendix E2**

### **Substances Tested in Two or More Selected *In Vitro* AR TA Assays**

### **Substances Tested for Antagonism Activity**

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## Substances Tested for Antagonism Activity

Substance	CHO hAR(T)+Luc(T)	CHO-K1 hAR(T)+Luc(T) +EGFP(T)	CV-1 hAR(T)+CAT(T)	CV-1 hAR(T)+Luc(T)	CV-1 hAR(T)+Luc(T)*	HepG2 hAR(T)+Luc(T) + βgal(T)	MDA-MB-453 hAR(E)+Luc(T)*	MDA-MB-453- kb2 hAR(E)+Luc(S)	PC-3 hAR(T)+Luc(T)	PALM hAR(S)+Luc(S)	Yeast( <i>S. cer</i> ) hAR(S)+β-gal(S)
Bicalutamide	pos.			pos.					pos.	pos. (2)	
2,2-Bis-( <i>p</i> -hydroxyphenyl)-1,1,1-trichloroethane					pos.	pos. (2)	pos.	pos.			
Bisphenol A						neg.				pos.	
Cyproterone acetate	pos.		pos.	pos.					pos.	pos. (2)	
<i>p,p'</i> -DDD				pos.		pos.					
<i>o,p'</i> -DDE						pos.				pos.	
<i>p,p'</i> -DDE	pos.			pos.		pos. (2)		pos.		pos. (2)	neg.
<i>o,p'</i> -DDT				pos.		pos.					
<i>p,p'</i> -DDT				pos.		pos.					
3',5'-Dichloro-2-hydroxy-2-methylbut-3-enanilide					pos.		pos.	pos.		pos.	
2-[[3,5-(Dichlorophenyl)-carbamoyloxy]-2-methyl-3-butenic acid					pos.		pos.	pos.		pos.	
Diethylstilbestrol				pos.						pos.	
17 -Estradiol	pos.			pos. (2)		pos.		pos.			
Fenitrothion		pos.				pos.					
Flutamide			pos.			pos.				pos.	pos. (3)
Hydroxyflutamide	pos.		pos.	pos. (3)	pos.	pos.		pos.	pos.	pos. (2)	pos.
Kepone				neg.						pos.	
Linuron				pos.				pos. (2)			
Mifepristone			pos.							pos.	
Nilutamide									pos.	pos. (2)	
Procymidone	pos.							pos.			
Progesterone	pos.			pos.		pos.					
Spironolactone	pos.	neg.								pos.	
Vinclozolin	pos.	pos.						pos.			

Abbreviations: neg. = negative call, pos. = positive call.

Numbers in parentheses refer to the number of times the substance was tested for antagonism activity in that assay, if more than once. If the study calls within an assay were discordant, the substance was classified as positive and the number of positive calls among the number of times it was tested presented in parentheses. A response classified as weak by the investigator was classified as positive.

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