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Background Review Document

Current Status of Test Methods for Detecting Endocrine Disruptors: *In Vitro* Androgen Receptor Binding 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

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Additional Information

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

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

Background Review Document

Prepared for
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Interagency Center for the Evaluation of Alternative Toxicological Methods
(NICEATM)

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LIST OF ACRONYMS AND ABBREVIATIONS

ACC	American Chemistry Council
AR	Androgen receptor
ARE	Androgen response element
B_{max}	Number of binding sites in a cytosolic preparation
BRD	Background Review Document
CASRN	Chemical Abstracts Service Registry Number
cDNA	Complementary DNA
Ci	Curies
CMA	Chemical Manufacturers Association
COS	Cell line derived from monkey kidney
CUC	Calf uterine cytosol
DDE	1,1-Dichloro-bis[4-chlorophenyl]ethylene
DDT	Dichlorodiphenyltrichloroethane
DHT	5α-Dihydrotestosterone
DMSO	Dimethyl sulfoxide
DPM	Disintegrations per minute
ED	Endocrine disruptor
EDSP	Endocrine Disruptor Screening Program
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
EDTA	Ethylenediamine tetraacetic acid
EPA	(U.S.) Environmental Protection Agency
ER	Estrogen receptor
FDA	(U.S.) Food and Drug Administration
FFDCA	Federal Food, Drug, & Cosmetic Act
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
fmol	Femtomole
FQPA	Food Quality Protection Act
g	Gravity

GLP	Good Laboratory Practices
HAP	Hydroxyapatite
hAR	Human androgen receptor
HDT	Highest dose tested
HGF	Human genital fibroblasts
HPTE	2,2-Bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane
IC_{50}	Concentration of test substance (inhibitor) that displaces 50% of radiolabeled reference androgen from the receptor in a competitive binding assay
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
K_d	Dissociation or binding constant
kDa	Kilodalton
K_i	Equilibrium dissociation constant of the receptor-inhibitor complex
LnCaP	Cell line derived from lymph node of a patient with metastatic prostatic adenocarcinoma
µg	Microgram
µL	Microliter
µM	Micromolar
M	Molar
MCF-7	Cell line from a human mammary adenocarcinoma
mg	Milligram
mL	Milliliter
mm	Millimeter
mM	Millimolar
mmol	Millimole
NAS	(U.S.) National Academy of Sciences
NCTR	National Center for Toxicological Research
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences

nM	Nanomolar
NSB	Nonspecific binding
NTP	(U.S.) National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
pM	Picomolar
QC	Quality control
QSAR	Quantitative structure-activity relationship
R1881	Methyltrienolone
RBA	Relative binding affinity
REC	Rat epididymal cytosol
RECNR	Rat epididymal cytosol nuclear fraction
rhAR	Recombinant human androgen receptor
RPC	Rat prostate cytosol
rtARα	Rainbow trout androgen receptor alpha
SAB	Science Advisory Board
SAP	Scientific Advisory Panel
SDWA	Safe Drinking Water Act
SE	Standard error
$t_{1/2}$	Time it takes for 50% of the ligand to dissociate from the receptor
TeBG	Testosterone-estradiol binding globulin
TEDG	Buffer containing Tris, EDTA, dithiothreitol, and glycerol
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 binding 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* ER transcriptional activation assays, and *in vitro* AR transcriptional activation 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 binding assay. It was envisioned that these criteria would be based on the performance of existing standardized *in vitro* AR binding 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 binding 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 binding 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 binding 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 binding assays.

This BRD reviews available *in vitro* AR binding assays and presents the data available for substances evaluated in these assays. The relative performance of various types of *in vitro* AR binding 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 binding 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 binding 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 consistsq 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.

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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 bind to the androgen receptor (AR); (2) assess the *in vitro* AR binding assays considered for their effectiveness in identifying endocrine-active substances; (3) identify and prioritize *in vitro* AR binding 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 binding assays; and (5) generate a list of recommended substances to be used in validation efforts.

The data summarized in this BRD are based on information obtained from the peer-reviewed scientific literature. An online literature search was conducted to retrieve records on publications reporting on the testing of substances for their endocrine disrupting effects *in vitro*. Of the 459 records obtained from the initial search, 105 contained information on AR binding. Additional citations were located while reviewing these publications. Ultimately, data from 23 publications were extracted for consideration during the preparation of this BRD. Some of the peer-reviewed publications that contained AR binding 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.

Data were abstracted on 108 substances tested in 11 different AR binding assays. These assays used:

- cytosol prepared from animal tissues containing the AR (rat prostate [RPC], rat epididymis [REC], calf uteri [CUC]), human cell lines (MCF-7, LnCaP) with an endogenous AR, and a mammalian cell line (COS-1) transfected with human (h) AR;
- primary human genital fibroblasts (HGF) with an endogenous AR;
- mammalian cell lines (COS-1) transfected with either hAR or rainbow trout (rt) ARα; and
- recombinant hAR produced in Sf 9 insect cells.

The assays measured the competitive displacement of one of four radiolabeled androgens from the AR. Two of the reference androgens are naturally occurring (testosterone, 5α-dihydrotestosterone [DHT]), while the other two are synthetic (mibolerone, 17β-hydroxy-estra-4,9,11-trien-3-one [methyltrienolone or R1881]); all were radiolabeled with tritium (^{3}H). Seventy-three substances were evaluated in competitive AR binding experiments that used DHT as the reference androgen; 47 were tested with R1881, 24 were tested with testosterone, and 16 were tested with mibolerone.

The chemical classes that have been tested most extensively in *in vitro* AR binding assays are nonphenolic steroids, organochlorines, and phenolic steroids, while the most common product classes are pharmaceuticals and pesticides. Not all substances could be assigned to a product class.

Of the 108 substances tested in the 11 different *in vitro* AR binding assays, only 34 (31.4%) had been tested in two or more assays, irrespective of the reference androgen used. No substance had been tested in all 11 assays. The assays for which the most substances had been tested are the HGF assay (38 substances, 35.2%), the RPC assay (34 substances, 31.5 %), and the COS-1+hAR assay (19 substances, 17.6%). A majority of the substances (74; 68.5%) were tested in only one assay.

The majority of the publications reported the data as IC_{50} values (the concentration that reduces the binding of the reference androgen by 50%) or as relative binding affinity (RBA) values, that is, the ratio of the IC_{50} of the reference androgen, divided by the IC_{50} of the test substance multiplied by 100.

As so few substances have been tested more than once in the same *in vitro* AR binding assay or in multiple assays using the same reference androgen, no quantitative or qualitative analyses of the comparative performance or the reliability of these assays were possible. However, based on general principles, recommendations were made in regard to the use of *in vitro* AR binding assays as a component of a Tier 1 endocrine disruptor screening battery:

- After consideration of factors such as a desire to eliminate animal use when feasible, and a possible advantage associated with the use of hAR transfected into a cell line free of other endocrine receptors (to avoid possible cross-reactivity) or the use of a recombinant hAR assay, the COS-1+hAR and hAR assays are recommended as the *in vitro* AR binding assays with the greatest priority for validation. If an assay chosen for validation requires the use of animals, efforts should be made to minimize the number of animals used, and animal pain and distress.
- In conducting future validation studies with these assays, the RPC assay, which is currently undergoing validation efforts sponsored by the U.S. EPA, should be used as the reference test method.
- Formal validation studies should be conducted using appropriate substances covering the range of expected RBA values to adequately demonstrate the performance characteristics of the *in vitro* AR binding assays recommended as possible screening assays.
- There is little information about the AR binding activity of metabolites of xenobiotics and it is not clear whether metabolic activation needs to be included in *in vitro* AR binding test methods used as screening assays. This issue should be considered prior to the implementation of future validation studies.

An important step towards acceptance of an *in vitro* AR binding 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 binding 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. In conducting these validation studies, all of the original data and documentation supporting the validation of a test method must be carefully documented, and include detailed protocols under which the data were produced.

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

assays are roughly equivalent, as would be the costs for the cell culture assays and assays using semi-purified AR.

Since only one guideline for conducting an *in vitro* AR binding assay has been published, and no formal validation studies have been performed to assess the reliability or performance of *in vitro* AR binding assays, the U.S. EPA requested that minimum procedural standards based on a comparative evaluation of *in vitro* AR binding assays be provided. In addition it was requested that a list of recommended test substances be provided for use in validation studies.

The minimum procedural standards include selection of the reference androgen, methods for determining the K_d of the reference androgen, methods for test substance preparation, the concentration range of the test substance (including the limit dose), the use of negative and positive controls, the number of replicates per test substance concentration, dose spacing, assay acceptance criteria, data analysis, evaluation and interpretation of results, minimal information to include in the test report, and the need for replicate studies.

Four *in vitro* AR binding assay protocols, including the RPC assay protocol being used in the U.S. EPA-sponsored validation study for AR competitive binding, were provided for consideration (**Appendix B**). Inspection of these protocols provides a perspective on how various assays are conducted by different investigators and for developing a more general protocol, one that takes into account the recommended minimum procedural standards.

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A number of factors were considered in developing a list of recommended substances to be used in validation efforts, including the number of times the substance had been tested in various assays, the median RBA value of the substance across assays and its extent of concordance. The selected substances were sorted according to their median RBA values, over six orders of magnitude, ranging from 100 to 0.0001. Weakly-binding substances (RBA values <0.001) were difficult to identify because they were not always consistently positive in tests within an assay or using different assays. Also included were substances classified as "negative" for AR binding based on the lack of a positive response in multiple assays when tested at doses of at least 1 mM. Where possible, five substances were selected for each RBA category and three for the negative

category group. To ensure that each RBA category contained a representative sampling of chemical classes, selection was based on the chemical class to which the substance belongs, whether it was representative of a chemical class used in commerce or found in the environment, and whether the substance is commercially available. The latter criterion was based on whether the substance could be located in a chemical supply catalogue.

The resulting list of 31 substances was compared with an U.S. EPA list of 19 substances that has been proposed for testing in an RPC assay procedure by Battelle Pacific Northwest National Laboratory. The U.S. EPA has fewer substances in the organochlorine chemical class. Two of the substances on the U.S. EPA list were not included in the list of recommended substances because of the absence of published data on their AR-binding activity.

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 BINDING 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 in exposed organisms (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 suggest 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), and to precocious puberty, 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 (Safe, 2000; Barlow et al., 1999).

In 1996, the U.S. Congress responded to societal concerns by enacting 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 in 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 only 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 assay
- Invertebrate reproduction

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 can measure if there is a 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 hormones) and an antagonist (a substance that binds to a receptor without eliciting 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 for 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 to prepare a series of 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 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 Binding Assays

Although there has been research conducted in the past few years to develop new or improved *in vitro* assays to identify substances with AR binding and transcriptional activation 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 binding assays, in concert with reviews of AR TA assays and *in vitro* ER binding and TA assays.

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

1.2.1 Purpose for Using *In Vitro* AR Binding Assays

The *in vitro* AR binding assays are designed to identify substances that bind to the AR and that might interfere with normal androgen activity *in vivo* by acting as an agonist or as an antagonist. The assays can be divided into two mechanistic categories: those that measure binding to the receptor, and those that measure transcriptional activation subsequent to binding to the receptor. Although receptor binding assays detect both agonists and antagonists, they do not distinguish between the two. In contrast, TA assays are capable of distinguishing between agonists and antagonists.

The binding affinity of a substance for the AR determines how well it will compete with the natural androgen, 5α-dihydrotestosterone (DHT). *In vitro* binding assays are generally performed by quantifying the ability of substances to compete with DHT or another reference androgen for binding. However, AR binding alone is not sufficient to indicate or predict subsequent cellular effects. For this reason, the *in vitro* AR binding assays will be used in conjunction with AR TA assays and other Tier 1 *in vivo* screening assays. Results from such assays will be used in a weight-of-evidence approach to select substances for Tier 2 testing.

1.2.2 Development of *In Vitro* AR Binding Assays: Historical Background

Current AR binding procedures can be traced back to the late 1960s when Anderson and Liao (1968), Bruchovsky and Wilson (1968), and Fang et al. (1969) demonstrated that DHT, a metabolite of testosterone, bound to a nuclear protein in rat ventral prostate tissue. Subsequently, other investigators (Mainwaring, 1969a,b; Unhjem et al., 1969) isolated a soluble, testosterone-binding protein complex from the rat ventral prostate gland, from prostate tissue slices *in vitro*, and from prostate cytosol. This androgen-protein complex associated with nuclear chromatin, and its size and physicochemical behavior suggested that it was similar to the ER (Noteboom and Gorski, 1965; Toft and Gorski, 1966). In addition to being present in male secondary sex tissues and genitalia, the AR has been identified in other tissues, including the pituitary and hypothalamus glands (Perez-Palacios et al., 1983), human male and female bone marrow (Mantalaris et al., 2001), human skin (Mowszowicz et al., 1981), and human mammary cancer cells in culture (Hackenberg et al, 1993; Schoonen et al., 1995; Deckers et al., 2000). Although male external genitalia lack ER, the female external genitalia contain AR (Kalloo et al., 1993).

Testosterone, which is produced by the Leydig cells of the testes, is the principal endogenous androgenic substance. It is metabolized to its more active metabolite, DHT, by steroid 5α-reductase (Δ^4-3-ketosteroid-5α-oxidoreductase), which is located in the microsomal and nuclear fractions of the cell. DHT appears to be the favored ligand *in vivo*, primarily as a result of its ability to stabilize the receptor complex more effectively than testosterone (E. Wilson, personal communication). *In vitro*, DHT and testosterone have similar equilibrium dissociation constants of approximately 2-5 x 10^{-10} M (Wilson and French, 1976; Lubahn et al., 1988).

The AR has a high degree of homology with members of the steroid hormone receptor family. There is a high degree of sequence conservation in the cysteine-rich DNA-binding domain, with less conservation in the carboxyl-terminal, androgen-binding domain. Based on these domains, the AR is closely related to the progesterone, glucocorticoid, and mineralocorticoid receptors (Tilley et al., 1989). Kelce et al. (1998) reported that there is 100% homology between the human and rat ligand-binding domains of the AR.

Androgen binds to the AR, which subsequently dimerizes (Wong et al., 1993). This hormone-receptor complex interacts with AR-associated transcriptional factors including activators, repressors, and modulators (Culig et al., 2000; Sharma et al., 2000; Haendler et al., 2001). The activated receptor complex binds to specific DNA regulatory sequences of androgen-responsive genes (androgen response elements; ARE) that are located upstream from or within the intron regions of the genes under androgen control.

Some researchers have reported that the AR protein is relatively unstable *in vitro*. It is highly sensitive to pH and temperature, and rapidly degrades in the absence of ligand. Wilson and French (1976) found that cytosolic AR from rat testis or epididymis degraded rapidly ($t_{1/2}$ = 15-25 minutes at 23°C) when not bound to a ligand. Kemppainen et al. (1992) also reported rapid degradation of the AR expressed in transiently transfected COS cells ($t_{1/2}$ = 1 hour at 37°C) in the absence of ligand. However, stability of the receptor was greatly enhanced at lower temperatures, at a basic pH (e.g., pH = 8 at 0°C), and in the presence of testosterone, DHT, or a synthetic androgen, such as 17β-hydroxy-estra-4,9,11-trien-3-one (methyltrienolone or R1881) (Wilson and French, 1976; Kemppainen et al., 1992).

AR binding assays are most often conducted with a cell-free AR preparation obtained from androgen-responsive tissues or cells (i.e., ventral prostate, foreskin fibroblasts). Traditional techniques to measure competitive binding are routinely used, including the use of dextran-coated charcoal and hydroxyapatite (HAP) to separate receptor-bound ligand from free ligand. Although AR binding assays have changed very little over their 30+ years of use, some of the more recent procedures have incorporated new technology, including the use of recombinant AR proteins in place of AR isolated from tissues or cells (Bauer et al., 2000). The AR binding assays, as currently performed, are described in detail in **Section 2.0**.

The procedures used to calculate the binding parameters are essentially variations on the method published by Scatchard (1949), who developed models for the binding of small molecules to proteins and for extrapolating binding data. In a "Scatchard plot", a straight line indicates that a single class of binding site is present; if competing binding sites are present, the line will deviate from linearity. The intercept on the abscissa indicates the number of binding sites available; the

association constant is the ratio of the intercepts on the abscissa and ordinate (Puca and Bresciani, 1969). Scatchard plots are widely used in receptor binding studies.

Baulieu and Raynaud (1970) proposed using an alternative procedure for approximating the binding parameters of small molecules in protein mixtures. They developed a nonlinear function by plotting the log of the bound fraction to the log of the total ligand, and demonstrated that this procedure was able to quantitatively distinguish between specific and nonspecific binding (i.e., to sites other than the AR) in a tissue extract that contained a mixture of specific and nonspecific receptors.

The AR binding assays measure the affinity of radiolabeled androgen for the AR (K_d), the affinity of unlabeled, reference androgen for the AR (K_i), and the concentration at which the unlabeled androgen displaces half the specific binding of radiolabeled androgen to the AR (IC_{50}). The K_d, which is measured in concentration units, is the equilibrium dissociation constant of the radiolabeled androgen-AR complex and represents the concentration of labeled reference androgen that will bind to half the binding sites at equilibrium in the absence of competitors. A low K_d represents high affinity and a high K_d represents low affinity. The K_i is the analogous constant for the unlabeled ligand. The IC_{50} values depend on a number of factors, such as the specific assay system used, binding affinity of the unlabeled ligand for the AR, androgen concentration, AR concentration, and experimental conditions (e.g., pH, exposure duration). In *in vitro* AR binding assays, there are substances that, because of biological inactivity, low solubility, or other considerations, do not decrease the binding of the radiolabeled, reference androgen by at least 50%. The IC_{50}s for these substances are often reported as being greater than the highest concentration tested or they are classified as "non-binders." In this BRD, such substances are classified as negative in the AR binding assay conducted.

Because of the potential for variation in IC_{50} values among AR binding assays, the generally accepted method for presenting and comparing the assay results is to compute the relative binding affinity (RBA) of the test substance against a reference androgen. The RBA is calculated as $IC_{50(\text{reference androgen})}/IC_{50(\text{test substance})} \times 100$. DHT is generally used as the reference androgen for calculating the RBA value, but testosterone and the synthetic androgens R1881 and

7α,17α-dimethyl-19-nortestosterone (mibolerone) have been used also. Because the RBA values cover approximately seven orders of magnitude, and there is no current guidance as to which levels of activity are biologically meaningful, there is no general agreement regarding the distinction between the values needed to distinguish endocrine disruptors from non-disruptors.

1.2.3 Mechanistic Basis of *In Vitro* AR Binding Assays

The AR is a transcriptional regulatory protein belonging to the nuclear hormone receptor superfamily. The human AR gene was cloned and sequenced by Lubahn et al. (1988a) 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. 1988a,b; Brown, et al., 1989; Tilley, et al., 1989). The AR contains 919 amino acids and is localized in the soluble nuclear fraction of androgen target cells; the protein plays a major role in controlling the transcriptional activation and/or repression of androgen-responsive genes (Culig et al., 2000). The AR contains two discrete domains that are necessary for its role as a transcription factor -- a ligand-binding domain in the *C*-terminal region, and a DNA-binding domain located approximately centrally in the receptor. 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).

Unlike the ER in which two subtypes with different binding characteristics have been identified (Kuiper et al., 1996, 1998; Gaido et al., 1999), there is only one known form of the AR in mammals. However, there is recent evidence for two subtypes in rainbow trout (Takeo and Yamashita, 1999) and Japanese eel (Ikeuchi et al., 1999, 2001).

As the primary receptor for endogenous androgens that initiate the transcription of messenger RNA and ultimately protein synthesis in androgen-target cells, the AR plays a pivotal role in the development and maintenance of the male and female reproductive systems. The interaction of androgens with the AR in a cell initiates conformational changes in the receptor that allow the binding of co-activator proteins. This interaction subsequently initiates or inhibits the transcription of androgen-controlled genes, which may lead to the initiation or inhibition of certain cellular processes.

The current hypothesis for AR-mediated endocrine disruption is that certain xenobiotic substances may mimic or block the action of DHT, the natural ligand for the AR. If the structure of a xenobiotic is similar to that of DHT, it may bind to the AR and displace the natural ligand or interfere with its binding. Any of these actions may produce an androgen-like effect or interfere with normal, physiological, androgen-mediated processes. Some xenobiotics may bind to the AR without initiating a biological response; in so doing, they could prevent the binding of DHT, thereby acting as an androgen antagonist.

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

Factors that affect ligand binding to the AR include:

- Affinity for the AR. This affinity depends on the rates of the association and disassociation of the ligand with the receptor. The natural ligand, DHT, has a low equilibrium constant because of its rapid association rate, about 5.3×10^{-7} M^{-1} h^{-1}, and slow disassociation rate, $t_{1/2}$ = 38 hours at 0ºC, for AR in rat prostate cytosol (Wilson and French, 1976).
- Systemic half-life of the ligand. This half-life will depend on its rate of metabolism to an active intermediate, or metabolic inactivation, and to the clearance of the ligand and its metabolites from the organism.
- Concentration of the ligand. Weakly binding ligands may produce a biological effect if they are administered at high enough concentrations, and strongly binding ligands would be ineffective if they do not reach androgen-sensitive tissues.

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

Although the AR ligand binding domain is highly conserved among vertebrate species, and substances that bind to AR derived from one species are expected to bind to the AR from another

vertebrate species, the relative binding affinities of these receptors for the same ligand may be different.

Due to a lack of information on interspecies comparisons, the present working hypothesis proposes that the biological effects resulting from androgen exposure in one vertebrate species is presumed to occur in other species. This hypothesis is the basis for the use of AR binding *in vitro* as a general screen for androgenic effects. The most widely used assay systems use human or rat AR-containing cells, or cytosolic AR derived from human or rat cells or tissues. Substances that bind the AR from these cells and tissues are presumed to be capable of producing androgenic effects in humans, rodents, fish, amphibians, and birds. However, there is insufficient evidence to demonstrate that an extrapolation among other vertebrate species and humans is appropriate, because there is little information about comparative binding of ligands to the AR of different species. It is not known whether *in vitro* differences in ligand binding affinity among species can be extrapolated to *in vivo* effects.

1.3 Intended Uses of the Proposed *In Vitro* AR Binding Assays

In vitro AR binding 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 that 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 humans.

1.3.1 Validation of *In Vitro* AR Binding Assays

The FQPA requires the U.S. EPA to base its endocrine disruptor screening program on validated test systems, and that the assays selected for inclusion in the program 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 the *in vitro* AR binding assays described in this BRD, relevance is restricted to how well an assay identifies substances that are capable, *in vitro*, of binding to the AR. 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 binding assays that have been commonly used to characterize substances as potential endocrine disruptors. Where appropriate data are available, the qualitative and quantitative performances 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., whole cell, cell cytosol, tissue cytosol, recombinant AR) 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 binding assays.

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

There are no *in vitro* AR binding or TA assays that are currently accepted by regulatory agencies as validated assays. The *in vitro* AR binding 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 binding activity *in vitro* is not currently required for regulatory decision-making. However, there are a number of *in vitro* assays available for measuring receptor binding. These assays are based on the same principles, but may use different sources of the AR and different protocols.

The most frequently used AR binding assays use cytosol from prostate or epididymal tissues of rats, intact human genital skin (usually foreskin) fibroblasts, cells containing the AR (including MCF-7 mammary tumor cells), or cells (usually COS-1 cells) transfected with a human or other AR-cDNA vector. The relative binding of a test substance with the AR is quantified by measuring the displacement of bound, radiolabeled DHT or other reference androgen.

1.3.4 Role of *In Vitro* AR Binding Assays in Hazard Assessment

The *in vitro* AR binding assays are proposed as a component of the EDSP Tier 1 screening battery that also includes estrogen and thyroid receptor binding assays, *in vitro* ER and AR 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 one or the other, or both assays, 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 transcriptional activation only, and may therefore produce false positive results due to limited absorption and distribution, or rapid metabolism and excretion of the substance *in vivo*. 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 the AR binding assay or in other Tier 1 screening assays would not be 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 Binding Assay and/or Limits of the *In Vitro* AR Binding Assay

The range of substances amenable to testing in *in vitro* AR binding assays has yet to be determined and will depend, in part, on the outcome of an independent peer review of the assays considered in this BRD. The AR binding 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 BRD

The *in vitro* AR binding data summarized in this BRD are based on information found in the peer-reviewed scientific literature. An online literature search of entries in MEDLINE, CANCERLIT, TOXLINE, AGRICOLA, NIOSHTIC, EMBASE, CABA, BIOSIS, and LifeSci was conducted to retrieve database records on publications reporting on *in vitro* testing of substances for their 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 estrogen- and androgen-receptor binding assays, and estrogen and androgen TA assays were

sought. The search strategy involved combining "*vitro*" with alternative terms for estrogens, androgens, receptors, binding, and testing. Each database record included authors, bibliographic citation, and indexing terms. Most records also included abstracts.

Of the 459 records obtained from the initial search conducted on December 12, 2000, 105 contained data from androgen-related assays and 354 contained data from estrogen-related assays. Abstracts of selected titles were reviewed, and the relevant articles were selected and retrieved from the literature for analysis. A database of the literature citations was established using relational database software. Subsequent to the initial search, additional articles with relevant information were identified and retrieved; many of these 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 this BRD, and recently published articles were added to the database as they became available. Identification of AR-related publications for data extraction was completed on September 30, 2001.

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 AR binding 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, 23 contained data that have been abstracted and included in this BRD.

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2.0 *IN VITRO* AR COMPETITIVE BINDING ASSAY METHODS

2.1 Introduction

The basic procedures to measure test substance binding to the AR were developed between 1970 and 1975. Cells containing an AR or cytosolic fractions from cells containing an AR, typically from the prostate or epididymis, are treated with sufficient amounts of the radiolabeled reference androgen (generally labeled with tritium, ^3H) to saturate all of the AR binding sites. Reference androgens traditionally used in AR binding studies have been DHT, testosterone, mibolerone, and R1881. Following this treatment, the cells or cellular extracts are challenged with the test substance, and the release of radiolabeled reference androgen is measured using scintillation counting. The amount of the radiolabeled reference androgen released is a function of the receptor-binding capacity of the test substance and the test substance concentration.

As discussed in **Section 1**, results from these competitive binding assays are expressed as the equilibrium dissociation constant of the receptor-inhibitor complex (K_i), or as the concentration of test substance causing displacement of 50% of the radiolabeled reference androgen from the receptor (IC_{50}). The K_i is a function of the affinity of the test substance and the radiolabeled reference androgen for the receptor. Despite the fact that the IC_{50} is very sensitive to experimental conditions while the K_i is less sensitive to these conditions, the majority of investigators present their data as IC_{50} values. This may have been due to the fact that the most commonly used approach for comparing data within and between laboratories is the RBA, which is based on relative IC_{50} values of the reference androgen and the test substance.

In the development of the competitive AR binding assays, an approach similar to that used in the development of ER binding assays was used. Cytosol from tissues containing the receptor, specifically the prostate and epididymis, were the initial sources of the AR. Subsequently, cells that intrinsically harbor the AR were adapted to measure binding. More recently, AR cDNA constructs from human and rainbow trout have been transfected into mammalian cells, and either the intact cells or cytosol derived from these cells have been used. For the purpose of summarizing the available AR binding assay data, the various protocols have been sorted according to whether they were performed with cytosolic preparations of animal tissue, intact

cultured cells, cytosolic preparations of cultured cells, or with constructs of human or rainbow trout AR proteins transfected into cells (**Appendix A**).

The first step in the performance of AR binding studies is to determine the equilibrium dissociation constant, K_d, of the reference androgen to the specific AR preparation used in the assay. The purpose of determining the K_d for each AR assay system is to demonstrate that the assay system is valid (e.g., a finite number of high affinity receptors are saturated with ligand) and to optimize the system with respect to receptor and ligand concentration. The K_d is

$$Specific\ binding = \frac{B_{max}\ x\ [Free\ radiolabeled\ reference\ androgen]}{K_d + [Free\ radiolabeled\ reference\ androgen]}$$

determined in a saturation binding experiment that involves adding increasing concentrations of the radiolabeled reference androgen (usually from 1×10^{-8} to 3.3×10^{-11} M) to the cells/cytosol and measuring the amount that binds to the AR (Motulsky, 1995). To calculate specific binding of the radiolabeled reference androgen to the AR preparation, nonspecific binding (i.e., to sites other than AR) is measured at each radioligand concentration by the addition of a nonlabeled androgen at a concentration that occupies all available receptors and then nonspecific binding is subtracted from the total binding (in the absence of nonlabeled compound) of the radiolabeled reference androgen (Motulsky, 1995). The amount of radioligand specifically bound depends on the number (concentration) of receptors in the preparation. Free and bound radiolabeled reference androgen is separated by the addition of a non-reactive absorbent, such as dextran-charcoal. The AR, the bound radiolabeled reference androgen, and other proteins in the reaction mix bind to the absorbent, while the displaced radiolabeled reference androgen remains in the supernatant. The mixture is centrifuged and the amount of AR-bound radiolabeled reference androgen in the pellet is eluted from the absorbent and its concentration is measured. The specific binding data from such saturation assays are analyzed to obtain the number of binding sites in a specific AR preparation, B_{max}, and the K_d by nonlinear regression using log concentration of radiolabeled reference androgen as the independent variable (Motulsky, 1995).

The saturation binding curve may also be analyzed using a linear Scatchard analysis (Scatchard, 1949) with specific binding on the abscissa (usually labeled "Bound") and the ratio of specific

binding of reference androgen to free reference androgen (usually labeled "Bound/Free") on the ordinate. In these plots, B_{max} is the x-intercept and K_d is the negative reciprocal of the slope. The K_d is used to determine the appropriate concentration of the labeled reference androgen to be used in the competitive binding assay.

The majority of the publications reporting on the binding of a radiolabeled reference androgen to the AR and its competition by other substances were conducted to investigate the nature of the binding process, the kinetics of the reaction, or to identify which molecular moieties enhanced or inhibited binding. These studies were generally not conducted to identify potential endocrine disrupting chemicals, and the methodologies presented in the publications frequently lacked detail.

Because the largest number of publications reporting results of AR binding used rat prostate cytosol, a general outline for this method is described first. This outline is followed with less-detailed descriptions of other assays used to measure AR binding. The major difference between these latter assays and those using prostate cytosol is the source of the AR (**Table 2-1**). Some assays used intact mammalian cells (including those derived from human foreskin explants, from an excised metastatic supraclavicular lymph node from a patient with prostate adenocarcinoma, and from monkey kidney [COS-1 cells]) transfected with either a human AR (hAR) or a rainbow trout AR (rtARα) construct. A semi-purified recombinant hAR protein produced in the baculovirus expression system was another source of the AR.

2.2 Protocols Used To Measure Competitive AR Binding *In Vitro*

In contrast to the ER binding assays, very few laboratories have used the same source of the receptor and/or the same protocol to measure AR binding (**Table 2-1**). Thus, the generalized methods described below are a composite of the methods described in the literature.

2.2.1 Sources of Cytosolic AR

2.2.1.1 Rat Prostate Cytosol

Rat prostate cytosol is prepared by homogenizing the prostate in cold buffer in a 1:5 ratio of tissue to buffer. Due to the instability of the AR, dithiothreitol is added to the homogenization

Table 2-1 Source of the AR and Corresponding Reference Androgens used in *In Vitro* AR Binding Assays

Source of Receptor	Reference Androgen	Reference
Calf uterine cytosol	DHT	Bauer et al., 1998
COS-1 cells + rtARα	Mibolerone	Takeo and Yamashita, 2000
COS-1 cells + hAR	R1881	Wong et al., 1995
		Kemppainen and Wilson, 1996
		Kemppainen et al., 1992
	DHT	Kemppainen et al., 1999
	R1881	Lambright et al., 2000
COS-1 cytosol + hAR	DHT	Tilley et al., 1989
Human genital fibroblasts	DHT, R1881, Mibolerone	Brown et al., 1981
	DHT	Eil and Edelson, 1984
Human genital fibroblast	DHT	Breiner et al., 1986
LnCaP cytosol	Testosterone	Sonnenschein et al., 1989
MCF-7 cytosol	DHT	Deckers et al., 2000
		Schoonen et al., 1995
Rat epididymal cell (nuclear fraction)	R1881	Kelce et al., 1994
Rat epididymal cytosol	R1881	Kelce et al., 1994
		Waller et al., 1996
Rat prostate cytosol	DHT	Danzo, 1997
		Wilson and French, 1976
	Mibolerone	Schilling and Liao, 1984
		Van Dort et al., 2000
	R1881	Kelce et al., 1995
	Testosterone	Teutsch et al., 1994
hAR	DHT	Bauer et al., 2000

Abbreviations: DHT = 5α-Dihydrotestosterone, R1881 = Methyltrienolone

buffer to protect sulfhydryl groups in the protein while phenylmethylsulfonyl fluoride is added as a protease inhibitor. The homogenate is centrifuged for 10 minutes at 2,500 x g at 4°C and the pellet containing cell debris is discarded. The supernatant is centrifuged at 105,000 x g for 30 minutes at 4°C to pellet organelles, and the supernatant consisting of cell cytosol containing the AR is stored at -70°C. The cytosolic protein concentration is determined using conventional methods.

Before chemical testing begins, the equilibrium dissociation constant of the radioactive reference androgen to the specific AR is determined, as described in **Section 2.1.** To measure competitive

binding, the radiolabeled reference androgen is added to the AR reaction mixture at a concentration that approximates the K_d. Next, a range of concentrations of the test substance in a solvent, usually absolute ethanol, is added; this step is also conducted with unlabeled reference androgen. Nonspecific binding of the radiolabeled reference androgen is determined using a 100-fold molar excess of the unlabeled reference androgen (Motulsky, 1995). Following incubation of the mixture, the displaced radioactive reference androgen is separated from the receptor-bound radioactive reference androgen using an absorbent, such as dextran-charcoal. The AR-radioactive reference androgen complex binds to the absorbent, and the unbound, free radioactive reference androgen is removed by extensive washing of the absorbent. After centrifugation, the pellet is extracted with ethanol to dissociate the radioactive reference androgen from the receptor, and the concentration of radioactive reference androgen is determined by scintillation counting. Specific binding is calculated by subtracting the amount of nonspecific binding from each sample evaluated in the assay. Data for the binding of the radiolabeled reference androgen and its displacement by the test substance or unlabeled reference androgen are plotted as the percentage of radiolabeled androgen bound versus the molar concentration of competing test substance. For a substance with high affinity for the receptor, the top of the curve correlates with maximal receptor binding in the absence of the unlabeled reference or test substance, and the bottom of the curve is the nonspecific binding (Motulsky, 1995). The concentration of the test substance or unlabeled reference androgen that produces radiolabeled androgen binding half way between the upper and lower plateaus is the IC_{50}. Estimates of the IC_{50} can be determined using appropriate statistical software. The RBA value for each competing test substance is calculated by using the following equation:

$$\text{RBA} = \frac{IC_{50} \text{ for reference androgen}}{IC_{50} \text{ for test substance}} \times 100$$

The K_i, which reflects the affinity of the test substance for the AR, can be calculated from the IC_{50} using the equation of Cheng and Prusoff (1973):

$$K_i = \frac{IC_{50}}{1 + \frac{[\text{Radiolabeled androgen}]}{K_d}}$$

2.2.1.2 Rat Epididymal Cytosol

Preparation of the epididymal cytosol and the measurement of AR binding are similar to that used for prostate cytosol. As with the isolation of the AR from the prostate cytosol, dithiothreitol and phenylmethylsulfonyl fluoride are added to the homogenization buffer to protect sulfhydryl groups in the protein and to inhibit protease, respectively.

2.2.1.3 MCF-7 Cytosol

A number of cell lines contain endogenous AR and ER proteins. The human breast adenocarcinoma cell line, MCF-7, which has most frequently been used for evaluating ER binding, has also been used to measure AR binding. The cells are maintained in standard growth medium. Prior to their use in competitive-binding assays, the cells are grown for one to two days in medium containing charcoal-stripped serum to remove residual androgenic steroids that may competitively interfere with the binding of reference androgens and test substances to the receptor.

MCF-7 cytosol is obtained from MCF-7 cells that have been grown to confluency. The cells are washed and removed from the flask with trypsin. The cell suspension is centrifuged, the supernatant removed, and the pellet homogenized in Tris buffer containing dithiothreitol and sodium molybdate. The homogenate is centrifuged at 100,000x g and the cytosol is used in the AR binding assay, as described above for prostate cytosol.

2.2.1.4 LnCaP Cytosol

The LnCaP cell line is derived from an excised human supraclavicular lymph node from a patient with prostatic adenocarcinoma. The AR in LnCaP cells has a point mutation in the ligand binding domain of the receptor. This mutation alters the binding of the receptor. Preparation of LnCaP cytosol for measurement of AR binding is similar to that used for MCF-7 cytosol.

2.2.2 Source of AR from Intact Cells

2.2.2.1 Human Genital Fibroblasts

Two sources of human genital fibroblasts have been used to measure binding to the AR: those derived from the genital skin of neonates at circumcision, and those derived from adult males

who have undergone surgery for phimosis (Breiner et al., 1986). The explants are allowed to grow out and the established cells are used to measure AR binding. The equilibrium dissociation constant of the radiolabeled reference androgen is determined, and the cells are exposed to the test substance as described for tissue and cell cytosols. Following exposure, the cells are incubated at room temperature or 37°C, and then washed to remove the unbound radiolabeled reference androgen. The cells are lysed and the bound radiolabeled reference androgen is separated, as described for cell cytosols, and measured by scintillation counting. IC_{50} and RBA values are calculated as described in **Section 2.2.1.1**.

2.2.3 Semi-Purified AR

Seven publications described the use of semi-purified cDNA of AR proteins as the receptor for AR binding assays (**Table 2-1**). Four publications described the transient transfection of COS-1 cells with the entire hAR cDNA (Tilley et al., 1989; Wong et al., 1995; Kemppainen and Wilson, 1996; Kemppainen et al., 1999), and one publication transfected the cDNA of ARα from rainbow trout (Takeo and Yamashita, 2000). In all cases, COS-1 cells were transiently transfected with an expression vector containing the AR gene. For mammalian and fish transfections, the pCMV3 and pCMV expression vectors were used, respectively. After transfection and subsequent expression of the protein, the intact cells are used or lysed and the cytosol containing the AR protein is used in a typical competitive binding assay. After incubation of the intact cells with the radiolabeled reference androgen and the test substance, the cells are washed, and then lysed for measurement of the radiolabeled reference androgen. One publication described the use of a semi-purified hAR that was expressed in Sf9 insect cells infected with baculovirus (Bauer et al., 2000). The pSG5-HAOa construct containing the complete human AR gene was the source of the AR. Following restriction of the plasmid, cDNA coding for an 880 amino acid fragment starting with amino acid 38 to the end of the gene was inserted into the baculovirus transfer vector, pAcSG-His NT-C. Sf9 insect cells were transfected with this vector. Following growth of the cells, AR-containing virus was isolated and recombinant protein was produced in a heterologous expression system. The resultant recombinant protein differed from the hAR by the absence of the first 37 amino acids and the addition of a histidine tag and a protein kinase A domain. Nevertheless, the binding properties of this protein were similar to the intact AR (Bauer et al., 2000). The hAR was not purified from

Sf9 cytosol. The protocol used to measure AR binding of a test substance in the presence of radiolabeled DHT is similar to that used for cytosols (**Appendix A**).

2.2.4 Permutations of the *In Vitro* AR Binding Assays as Described in the Literature

Irrespective of the source of the AR used in a particular study, the protocols vary from laboratory to laboratory. Some of these variations are in response to the differing properties of the AR preparations used, or because of the specific questions the studies were designed to address. Other variations are in the exposure time and test conditions. The permutations in the protocols used by each laboratory for each source of AR are summarized in **Appendix A**.

3.0 CHARACTERIZATION OF SUBSTANCES TESTED IN *IN VITRO* AR BINDING ASSAYS

3.1 Introduction

AR binding data were collected for a total of 108 substances from 23 publications that reported results from studies in which the competitive binding of a substance to the AR was measured. The data for these substances were grouped into four different categories, depending on which one of four reference androgens was used. As shown in **Table 3-1**, 10 AR binding studies used DHT as the reference androgen, eleven used R1881, three used mibolerone, and three used testosterone. In addition, one publication (Brown et al., 1981) conducted three different AR binding experiments that evaluated the same test compounds using DHT, R1881, and testosterone as reference androgens.

Relevant information on the substances tested (i.e., chemical name, Chemical Abstract Service Registry Numbers [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. If not provided in the publication, 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.

Seventy-three substances were evaluated in competitive AR binding experiments that used DHT as the reference androgen. Of these, only nine substances were evaluated in more than one of the AR binding assays addressed in this BRD and/or in more than one study (i.e., publication) (**Table 3-2**).

Table 3-1 Reference Androgens Used in *In Vitro* AR Binding Studies

Publication	Reference Androgen	Number of Substances Abstracted
Bauer et al., 1998	DHT	7
Bauer et al., 2000	DHT	18
Breiner et al., 1986	DHT	25
Brown et al., 1981*	DHT	6
Danzo, 1997	DHT	12
Deckers et al., 2000	DHT	9
Kemppainen et al., 1999	DHT	12
Schoonen et al., 1995	DHT	10
Tilley et al., 1989	DHT	6
Wilson and French, 1976	DHT	8
Brown et al., 1981*	R1881	6
Eil and Edelson, 1984	R1881	22
Kelce et al., 1994** (Cytosolic AR)	R1881	7
Kelce et al., 1994** (Nuclear AR)	R1881	11
Kelce et al., 1995	R1881	9
Kemppainen and Wilson, 1996	R1881	6
Kemppainen et al., 1992	R1881	7
Lambright et al., 2000** (COS-1 cells + hAR)	R1881	3
Lambright et al., 2000** (Rat Prostate Cytosol)	R1881	3
Waller et al., 1996	R1881	28
Wong et al., 1995	R1881	7
Schilling and Liao, 1984	Mibolerone	12
Takeo and Yamashita, 2000	Mibolerone	5
Van Dort et al., 2000	Mibolerone	5
Brown et al., 1981*	Testosterone	6
Sonnenschein et al., 1989	Testosterone	16
Teutsch et al., 1994	Testosterone	10

Abbreviations: DHT = 5α-Dihydrotestosterone; R1881 = Methyltrienolone
*All Brown et al. (1981) entries are from the same publication.
**Kelce et al. (1994) and Lambright et al. (2000) tested substances using two different AR binding assays.

Forty-seven substances were tested with R1881 as the reference ligand. Of these, 20 substances were evaluated in more than one type of AR binding assay and/or in more than one study (**Table 3-3**).

Table 3-2 Substances Tested in Two or More *In Vitro* AR Binding Assays with DHT as the Reference Androgen

Substance	Number of Assays	Number of Publications
Progesterone	6	6
17β-Estradiol	5	6
Testosterone	5	6
Medroxyprogesterone acetate	4	4
Cyproterone acetate	3	4
R1881	3	4
Gestodene	2	3
Cortisol	2	2
Mibolerone	2	2

Abbreviations: R1881 = Methyltrienolone.

Twenty-four substances were tested with testosterone as the reference androgen. Of these, only four substances were evaluated in more than one type of AR binding assay and/or in more than one study (**Table 3-4**).

Sixteen substances were tested with mibolerone as the reference androgen. Of these, only two were evaluated in more than one type of AR binding assay and/or in more than one publication (**Table 3-5**).

As these tables demonstrate, few substances have been evaluated in multiple AR binding assays and/or in multiple studies using the same reference androgen.

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

Many of the substances tested in AR binding assays were selected to address basic research questions regarding the nature of the AR and the kinetics of its interactions. For example, a number of the naturally-occurring phenolic and nonphenolic steroids (e.g.,

Table 3-3 Substances Tested in Two or More *In Vitro* AR Binding Assays with R1881 as the Reference Androgen

Substance	Number of Assays	Number of Publications
17β-Estradiol	5	6
Cyproterone acetate	4	5
DHT	4	8
Flutamide	4	3
Hydroxyflutamide	4	7
2-[[3,5-(Dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid *(Vinclozolin metabolite M1)*	3	3
3',5'-Dichloro-2-hydroxy-2-methylbut-3-enanilide *(Vinclozolin metabolite M2)*	3	3
Linuron	3	2
Progesterone	3	3
Testosterone	3	4
Vinclozolin	3	3
17α-Hydroxyprogesterone	2	2
4-Androstenedione	2	2
Androstanediol	2	2
Kepone	2	2
Diethylstilbestrol	2	2
o,p′-DDT	2	2
p,p′-DDE	2	2
p,p′-DDT	2	2
R 2956	1	2

Abbreviations: DHT = 5α-Dihydrotestosterone; DDT = Dichlorodiphenyltrichloroethane; DDE = 1,1 Dichloro-2,2-bis[*p*-chlorophenyl]ethylene.

Table 3-4 Substances Tested in Two or More *In Vitro* AR Binding Assays with Testosterone as the Reference Androgen

Substance	Number of Assays	Number of Publications
DHT	3	3
R1881	3	3
Cyproterone acetate	2	2
Nilutamide	2	2

Abbreviations: DHT = 5α-Dihydrotestosterone; R1881 = Methyltrienolone.

Table 3-5 Substances Tested in Two or More *In Vitro* AR Binding Assays with Mibolerone as the Reference Androgen

Substance	Number of Assays	Number of Publications
DHT	2	3
Testosterone	2	3

Abbreviations: DHT = 5α-Dihydrotestosterone.

DHT, testosterone, androstanediol, estradiol, progesterone, and cortisol), were studied to obtain a better understanding of AR binding processes. Some of the synthetic anti-androgens (e.g., cyproterone acetate and nilutamide) were investigated in AR binding studies to evaluate their mechanism of action as therapeutic agents. In addition, some substances were investigated to determine which metabolite or derivative of a molecule enhanced or inhibited binding to the AR, or to determine structure-activity relationships for the development of quantitative structure-activity relationship (QSAR) models.

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, chlordecone, dieldrin, and linuron) were tested in AR binding assays to identify substances that may act as androgen agonists or antagonists in humans and wildlife.

3.3 Chemical and Product Classes Tested

Chemical and product class information for the substances tested in AR binding 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 3-6**, the chemical classes that have been tested most extensively in AR binding assays are nonphenolic steroids, organochlorines, and phenolic steroids. Of the 108 substances included in this BRD, six were classified in two chemical classes.

Table 3-6 Chemical Classes Tested in *In Vitro* AR Binding Assays

Chemical Classes	Number of Substances
Amidine	1
Anilide	4
Aniline	1
Benzothiadiazine	1
Imidazole	7
Imide	1
Nitrile	5
Organochlorine	15
Phenol	1
Polychlorinated biphenyl	2
Steroid, nonphenolic	61
Steroid, phenolic	9
Stilbene	1
Triazine	1
Urea	4

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 3-7.** The most common product classes tested in AR binding assays are pharmaceuticals and pesticides. Of the substances included in this BRD, 15 had no known commercial use, so were not classified within a product class.

Table 3-7 Product Classes Tested in *In Vitro* AR Binding Assays

Product Classes	Number of Substances
Aromatase inhibitor	1
Chemical intermediate	2
Dielectric fluid	1
Natural product	3
Pesticide/pesticide metabolite	22
Pharmaceutical/pharmaceutical metabolite	64
Not classified	15

4.0 REFERENCE DATA

The ability of a test substance to bind to the AR *in vitro*, whether to an isolated protein receptor molecule or to ARs in cultured cells, suggests, but does not demonstrate, the ability of the substance to act as an androgen agonist or antagonist. A commonly used *in vitro* approach to measure such biological effects is the TA assay. In these assays, the ability of a test substance to initiate or block transcription of a reporter gene, or to initiate cell proliferation in an appropriate cell line is measured.

The purpose of this BRD is to assess the performance of various *in vitro* AR binding assays with regard to their sensitivity for detecting weak AR binding compounds 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 vitro*, such as transcriptional activation, or *in vivo*, such as the promotion of growth in male reproductive tissues. Such comparisons will be addressed elsewhere. Therefore, no reference data are included for measuring the biological relevance of the AR binding assays.

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5.0 DATA ON *IN VITRO* AR BINDING ASSAYS

5.1 Introduction

AR binding data and methods were collected from 23 publications reporting studies in which the competitive binding of a substance to the AR was measured and IC_{50} and/or RBA values were included or could be calculated. When provided, the specific information extracted for each substance included its name, source, purity, methodological details, relevant data (K_i, IC_{50}, and/or RBA values for positive studies, and highest dose tested [HDT] for negative studies), and the citation. For studies in which only chemical structures were provided, every effort was made to identify the name of each substance tested. No attempt was made to identify the source and purity of a substance if the investigators did not provide such information. If available, a CASRN was entered for each substance. This identifier was obtained from various sources, including the publication, the National Library of Medicine's ChemID database, and *The Merck Index*. Chemical name synonyms were entered for substances that were identified in the literature by more than one name, and for substances where the literature name 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 used in the original publication. **Appendix C** provides information on the names, synonyms, CASRN, and chemical/product class, if available, for each substance, while **Appendix D** contains the *in vitro* AR binding data, organized by chemical name, CASRN, assay, and reference androgen.

5.2 Availability of Detailed *In Vitro* AR Binding Protocols

The scientific methods presented in the publications containing data from *in vitro* AR competitive binding studies provided various levels of detail. To the extent possible, the most important method parameters were extracted from each publication and summarized in **Appendix A**. Details about the following method parameters are included in the Appendix to the extent this information was available:

- *Preparation of the receptor* (e.g., species or cell line, buffer used for preparation of cytosol, protein concentration of cytosol).
- *Competitive binding assay* (e.g., reference androgen, concentration of radiolabeled androgen, solvent used to dissolve competing ligand, concentration range of competing ligand, number of replicates, number of times assay was repeated).

- *Separation of ligand* (e.g., type of slurry used, incubation time and temperature).
- *Data calculations* (e.g., program or method used for calculating data, data format).

5.3 Availability of *In Vitro* AR Binding Data

AR binding data were collected for a total of 108 substances tested in competitive binding studies with AR obtained from the following sources:

1. Intact COS-1 cells containing human AR (COS-1 cells + hAR);
2. Intact COS-1 cells containing the AR from rainbow trout (COS-1 cells + rtARα);
3. Cytosol from COS-1 cells containing human AR (COS-1 cytosol + hAR);
4. Calf uterine cytosol (CUC);
5. Human genital fibroblasts (HGF);
6. Cytosol from a cell line derived from an excised human supraclavicular lymph node from a patient with prostatic adenocarcinoma (LnCaP cytosol) in which the AR has a point mutation in the ligand binding domain of the receptor;
7. Cytosol from human adenocarcinoma MCF-7 cells (MCF-7 cytosol);
8. Rat epididymal cytosol (REC);
9. Rat epididymal cytosol, nuclear receptors (RECNR);
10. Semi-purified recombinant human AR (hAR);
11. Rat prostate cytosol (RPC).

In all studies, competitive binding was measured by the displacement of the radiolabeled reference androgen (DHT, R1881, testosterone, mibolerone) from the AR-androgen complex by a competing ligand. **Appendix D** presents the extracted and compiled data sorted first by substance name, then by assay, and then by the reference androgen. In those cases in which the RBA value was not provided in the citation, this value was calculated, when possible, from the IC_{50} values. Not all of these values were reported in all publications. In some publications, neither the IC_{50} value nor the RBA value was presented. In many of these cases, the binding of the test substance to the AR over a range of concentrations was presented graphically, so that the IC_{50} values of the reference androgen and the test substance could be estimated. These estimated IC_{50} values and the corresponding calculated RBA value are italicized in **Appendix D**. For substances that did not bind sufficiently well to the AR under the conditions of the test to

displace the reference androgen (i.e., an IC_{50} value could not be calculated), the only parameter that could be entered into the database was the highest dose at which the substances were tested. This information is entered in the spreadsheet as the HDT.

5.4 *In Vitro* AR Binding Results for Individual Substances

Of the 108 substances tested in the 11 different *in vitro* AR binding assays, only 34 (31.4%) had been tested in two or more assays, irrespective of the reference androgen used (**Table 5-1**). Of these, no substance had been tested in all 11 assays. The most frequently tested substances were 17β-estradiol, progesterone, and testosterone, which were each tested in eight assays. One substance was tested in seven assays, three substances were tested in five assays, six were tested in four assays, three were tested in three assays, and 18 were tested in two assays.

The assays for which the most substances had been tested are the HGF assay (38 substances, 35.2%), the RPC assay (34 substances, 31.5 %), and the COS-1 + hAR assay (19 substances, 17.6%).

A majority of the substances (66; 61.1%) were tested in one study only.

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

Based on the available information in the scientific literature, it appears that the published *in vitro* AR binding studies neither used coded chemicals nor complied with GLP guidelines.

Table 5-1 Substances Tested in Two or More *In Vitro* AR Binding Assays Irrespective of Reference Ligand

Substance	Number of Assays	Number of Studies
17β-Estradiol	8	14
Testosterone*	8	12
Progesterone	8	11
DHT*	7	13
Cyproterone acetate	5	9
R1881*	5	7
Flutamide	5	4
Hydroxyflutamide	4	9
Androstanediol	4	5
Diethylstilbestrol	4	5
Medroxyprogesterone acetate	4	5
Mibolerone*	4	4
Linuron	4	3
2-[[3,5-Dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid	3	3
3',5'-Dichloro-2-hydroxy-2-methylbut-3-enanilide	3	3
Vinclozolin	3	3
17α-Hydroxyprogesterone	2	3
Cortisol	2	3
o,p′-DDT	2	3
p,p′-DDE	2	3
p,p′-DDT	2	3
17α-Ethinyl estradiol	2	2
4-Androstenedione	2	2
Bicalutimide	2	2
Kepone	2	2
Dexamethasone	2	2
Gestodene	2	3
Methoxychlor	2	2
Methyltestosterone	2	2
Nilutamide	2	2
Pregnenolone	2	2
Promegestone	2	2
RU 56187	2	2
Triamcinolone acetonide	2	2

Abbreviations: DHT = 5α-Dihydrotestosterone; R1881 = Methyltrienolone; DDE = 1,1 Dichloro-2,2-bis[*p*-chlorophenyl]ethylene; DDT = Dichlorodiphenyltrichloroethane.
*Count excludes assays and studies in which these substances were used as the reference ligand.

6.0 *IN VITRO* AR BINDING TEST METHOD PERFORMANCE ASSESSMENT

6.1 Introduction

The ICCVAM Submission Guidelines (ICCVAM, 1999) request that an assessment be conducted of the performance (i.e., accuracy, sensitivity, specificity, positive and negative predictivity, and false positive and false negative rates[1]) 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 binding ability, and data on endocrine disruption in humans or wildlife are too limited to be used for this purpose. Thus, ICCVAM concluded that a traditional performance assessment of *in vitro* AR binding assays was not feasible, and that one approach to evaluate the performance of AR binding assays BRD would be to compare the data from existing *in vitro* AR binding assays against each other with regard to their ability to detect substances capable of binding to the AR.

6.2 Quantitative Assessment of Assay Performance

Due to the very limited nature of the published *in vitro* AR binding assay database in terms of replicate data obtained for the same substances tested using the same reference androgen within and among assays, quantitative analyses of the relative performance of the 11 *in vitro* AR binding assays considered in this BRD could not be conducted (**see *In Vitro* ER Binding Assay BRD, Section 6**). **Table 6-1** demonstrates the limited nature of *in vitro* AR binding assay data available in the published literature. The type of reference androgen used in a study was not considered in compiling the number of assays in which the RBA value of a substance was determined. The four reference androgens (DHT, mibolerone, R1881, testosterone) were used as

[1] 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 (NIEHS, 1997).

test substances in many of these assays and their RBA values were determined as for any other substrate.

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

Number of Assays	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**Total**
Number of Substances	74	18	3	6	3	0	1	3	0	0	0	108
Percentage of Substances	68.5	16.6	2.8	5.6	2.8	0	0.9	2.8	0	0	0	100

6.3 Qualitative Assessment of Assay Performance

For the same reasons that a quantitative assessment of assay performance was not feasible, a qualitative assessment of the relative performance of the 11 *in vitro* AR binding assays based on RBA values of substances tested in pairs of assays using the same reference androgen was not possible. The majority of studies conducted to measure the ability of substances to bind to the AR *in vitro* used R1881 or DHT as the reference androgen (**Table 6-2**). However, even for these reference androgens, the number of substances tested in any two of the 11 *in vitro* AR binding assays was too small to be informative (**Appendix D**).

6.4 Performance of *In Vitro* AR Binding Assays

The *in vitro* AR binding 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 greatest ability to detect weak AR binding substances) and the most reliable (i.e., exhibit the lowest variance) (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. Finally, when taking animal welfare and human health and safety issues into consideration, assays that do not use AR obtained directly from experimental animals or assays that do not use radioactivity, respectively, might have the greatest utility.

Table 6-2 Number of Tests Conducted in Each Assay Sorted by Reference Androgen

Assay	Reference Androgen				
	R1881	**DHT**	**Mibolerone**	**Testosterone**	**Totals**
COS-1 cells+hAR	23	12			35
COS-1 cells+rtARα			5		5
COS-1 cytosol+hAR		6			6
CUC		7			7
HGF	28	31		6	65
LnCaP cytosol				16	16
MCF-7 cytosol		18			18
REC	35				35
RECNR	11				11
rhAR		18			18
RPC	12	20	17	10	59
TOTALS	109	112	22	32	275

R1881 = Methyltrienolone; DHT = 5α-Dihydrotestosterone.

Based on the very limited data available, there is no single assay that can be said to perform better than any other assay. However, the RPC, REC, COS-1+hAR and the HGF assays might offer some advantages over the other seven *in vitro* AR binding assays. The RPC and REC assays are similar; both assays involve the direct use of animals with the only difference being the use of the prostate cytosol in the former and epididymal cytosol in the latter. Among all of the assays, the RPC assay has been used by the most investigators (7) to identify substances with AR binding activity, suggesting that the assay has demonstrated transferability between laboratories. Only two laboratories have generated REC assay data extracted for this BRD. In contrast to the animal-based assays, the COS-1+hAR and the HGF assays use cultured cells. An advantage of using HGF cells is that they are normal human cells containing an endogenous AR receptor. However, the number of AR receptors per cell in HGF cells is lower than can be attained in COS-1 cells transfected with the hAR (Wilson, personal communication). This difference in AR density per cell presumably can be adjusted during testing. A consideration in recommending the use of the COS-1 cells +hAR is whether an expression vector for hAR is commercially available.

The results of an assessment of the utility (source of AR, absence of animal use) of the various assays are summarized in **Table 6-3**. All of the assays require the use of radiolabeled reference

androgen and thus, the handling and disposal of radioactivity would be similar for all assays.

Table 6-3 Summary of Utility of *In Vitro* AR Binding Assays

Assay	Lack of Need for Animal Tissues[a]	AR from Species of Interest[b]
COS-1 cells+hAR	+	+
COS-1 cells+rtARα	+	++
COS-1 cytosol+hAR	+	+
CUC		
HGF	+	+
LnCaP cytosol[c]	+	-
MCF-7 cytosol	+	+
REC		
RECNR		
hAR	+	+
RPC		

[a](+) Based on the lack of need for tissues from experimental animals.
[b]Based on the use of AR from a species of direct interest (i.e., + for human AR for human health, ++ for ecological effects, - for mutated form of human AR).
[c] The AR in LnCaP cells contains a point mutation that alters the binding of the receptor.

6.5 Strengths and Limitations of *In Vitro* AR Binding Assays

Competitive binding assays indicate whether a substance can interact with the target receptor in such a way that it can displace the natural ligand. These assays, by themselves, do not provide sufficient evidence to conclude that a substance is an androgenic agonist or an antagonist, or take into consideration other mechanisms of action that may lead to endocrine disruption (Zacharewski, 1998). However, AR binding assays can be important components of a battery of screening tests because they:

- 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);
- Can be performed using small amounts of test substances;
- Can be used to test multiple substances simultaneously; and
- Can be easily standardized among laboratories.

These assays have limitations also, including:

- Instability of the AR at temperatures above 4°C;
- Inability to distinguish agonists from antagonists; and
- Potential generation of false positive and false negative results.

With regard to generating false positive results, high concentrations of a test substance might disrupt the binding of the radioactive ligand to the AR by deactivating the receptor or decrease binding via noncompetitive inhibition (Kupfer, 1988). Alternatively, it may be difficult to accurately measure the binding of rapidly dissociating, low affinity ligands because they are more likely to dissociate when the unbound ligand is washed away from the receptor. This dissociation is a concern when the receptor or ligand is bound to a solid support such as dextran-charcoal or HAP (National Research Council, 1999). Other reasons for obtaining a false negative response are a requirement for metabolic activation to an active AR binding intermediate; incomplete solubility of the test substance in the assay buffer; and incompatibility of the substance with the assay conditions. Because traditional AR binding assays using tissue or cell cytosols do not include the enzymes and cofactors required for metabolic activation, some potential AR binding substances will be missed. A possible solution to this limitation is to develop *in vitro* AR binding assays that include a metabolic activation system, as has been done with some ER TA assays (Charles et al., 2000; Sumida et al., 2001).

6.6 Conclusions and Recommendations

Relatively very few substances have been tested more than once in the same *in vitro* AR binding assay or in multiple assays. Furthermore, as the primary focus of many of the investigations using *in vitro* AR binding assays has been to understand mechanisms of binding and transcriptional activation and not to identify substances with AR binding activity, much of the published data are of limited value in terms of an analysis of performance.

After taking into account the lack of comparative performance information on the 11 *in vitro* AR binding assays considered in this BRD, only general principles can be used to prioritize these assays for possible validation as screening test methods within a battery of Tier 1 endocrine disruptor tests.

- Based on a consideration of such factors such as the elimination of animal use and a focus on the AR from the species of interest, assays that utilize mammalian cells lines carrying an endogenous AR (HGF, LnCaP, MCF-7) or have been transfected with the hAR (COS-1), or ones that utilize a semi-purified/purified hAR should be considered for validation as screening assays for human health-related issues.
- There are difficulties recommending any of the cell lines with an endogenous AR (HGF, LnCaP, MCF-7). The LnCaP cells have a point mutation in the AR (Kempppainen and Wilson, 1996), the MCF-7 cells have high levels of the ER, and the HGF cells have low levels of the AR (Wilson, personal communication).
- Among the transfected cell lines, stably transfected cell lines might be more reliable than transiently transfected ones and would involve fewer experimental manipulations.
- It might be expected that assays that use semi-purified or purified AR proteins would be more reliable than those based on extracts of AR from animal tissues. Thus, the COS-1+hAR and hAR assays are recommended as the *in vitro* AR binding assays with the greatest priority for validation.
- In conducting future validation studies with these assays, the RPC assay should be used as the reference test method. The RPC assay is currently undergoing validation efforts sponsored by the U.S. EPA and the resulting performance and reliability information could be used to establish minimal performance standards for other assays.
- Formal validation studies should be conducted using appropriate substances covering the range of expected RBA values to adequately demonstrate the performance characteristics of the *in vitro* AR binding assays recommended as possible screening assays.
- There is little information about the AR binding activity of metabolites of xenobiotics and it is not clear whether metabolic activation needs to be included in *in vitro* AR binding test methods used as screening assay. This issue should be considered prior to the implementation of future validation studies.

7.0 *IN VITRO* AR BINDING TEST METHOD RELIABILITY ASSESSMENT

7.1 Introduction

The ICCVAM Submission Guidelines (ICCVAM, 1999) request that an assessment of test method reliability[1] 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 *in vitro* AR binding assay reliability have been conducted and the nature of the current database for these assays precludes a formal analysis. Historically, four different reference androgens have been used to measure AR binding in 11 different assays. However, there is data on only twelve compounds that have been tested more than once in the same assay using the same reference androgen (**Section 7.2**). Historically, investigators have used these assays primarily to gain insight into the mechanisms of the binding of a ligand to the AR, to compare the binding of different ligands to AR isolated from different tissues and/or species, and to understand the process of AR-induced transcriptional activation. Only relatively recently have AR studies been conducted to investigate the biological activities of putative endocrine disruptors.

7.2 Assessment of Assay Reliability

7.2.1 Variability in RBA and IC_{50} Values Among *In Vitro* AR Binding Assays

Because of the limitations discussed above, neither a quantitative nor a qualitative assessment of IC_{50} and RBA values could be conducted to assess the inter- and intra-laboratory reproducibility of the 11 *in vitro* AR binding assays considered in this BRD. Based on the available published literature, very few substances have been tested more than once using the same reference androgen within the same laboratory and only five of the assays (MCF-7 cytosol, RPC, REC,

[1] 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.

HGF, COS-1+hAR) have been performed in more than one laboratory (**Appendix D**). Thus, it is not possible to identify which *in vitro* assay or assays exhibit the greatest reliability in identifying substances with AR binding activity.

7.2.2 Variability in the IC_{50} Value for the Four Reference Androgens

An attempt was made to evaluate the variability in the IC_{50} values for the four reference androgens (DHT, mibolerone, R1881, and testosterone) used historically in *in vitro* AR binding studies. However, a total of only 50 IC_{50} values for these reference androgens could be located among four of the most frequently used *in vitro* AR binding assays (COS-1+hAR, HGF, REC, and RPC), and four IC_{50} values were the maximum number of replicate data points for any single combination of reference androgen and assay. No conclusion can be made based on the extent of available data.

7.3 Conclusions and Recommendations

The *in vitro* AR binding 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 AR binding substances) (see **Section 6**) and the most reliable (i.e., exhibit the least variability within and across laboratories). Based on the available data, no assessment of assay reliability was possible. However, it might be expected that assays that use semi-purified or purified AR proteins would be more reliable than those based on extracts of AR from animal tissues.

It is essential that validation studies be conducted to assess assay reliability and that these validation studies use appropriate substances covering the range of expected RBA values. A list of potential test substances for use in such a validation effort is provided in **Section 12**.

8.0 QUALITY OF DATA REVIEWED

8.1 Extent of Adherence to Good Laboratory Practice (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 report and archive 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 available information, none of the published *in vitro* AR binding studies identified for this BRD appear to have been 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 for a study. No attempt was made to formally assess the quality of the *in vitro* AR binding data included in this BRD. The published data on the competitive binding of substances to the AR were limited to RBA and, to a lesser extent, IC_{50} and K_i values. Auditing these reported values would require obtaining the original data for each study, which is not readily available.

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

- *Data reporting*: Some of the data reported in the publications were RBA values only, with no accompanying error term provided to assess the quality of the estimate. Thus, the variability of the experimental data could not be assessed.
- *Large number of substances tested in only one laboratory*: The majority of the substances included in this BRD were tested in one laboratory only. Therefore, the interlaboratory 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 test results is not known.

- *Insufficient methodology information:* Some publications contained limited details about the test methods 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, and some supplied neither names nor CASRNs, which in some cases made unequivocal identification difficult.

8.3 Quality Control Audit

NICEATM staff conducted a quality control (QC) audit of the AR binding 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.

8.4 Need for Data Quality

Data quality is a critical component of the test method validation process. To ensure data quality, ICCVAM recommends that all of the data supporting validation of a test method be available with the detailed protocols under which the data were produced. Original data should be available for examination, as should supporting documentation, such as laboratory notebooks. Ideally, the data should adhere to national or international GLP guidelines (ICCVAM, 1997).

All of the *in vitro* AR binding assay data included in this BRD were obtained from peer-reviewed scientific articles reporting the results of studies conducted at facilities that do not typically perform studies in compliance with GLP guidelines. It should be noted that a majority of these studies were performed in response to basic research questions and/or to evaluate the binding affinities of androgen analogs, not to support prevalidation or validation of the test method, or the formal submission of data to regulatory agencies. Because these studies span

three decades and a multitude of laboratories, verifying the integrity of the data via a formal audit process was not possible.

An informal assessment of the *in vitro* AR binding assay data showed that the test substances and data were not consistently represented in the same format. In addition, the methods were presented in varying levels of detail and completeness. Since the published data were not verified for their accuracy against the original experimental data, caution must be exercised when evaluating the *in vitro* AR binding data in **Appendix D**.

An important step towards acceptance of *in vitro* AR binding assay methods 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 binding assays be conducted with coded substances and in compliance with national and international GLP guidelines. Ideally, the substances should be obtained from a common source, and distributed from a central location. Laboratories not able to perform studies in compliance with GLP guidelines should perform studies in the spirit of GLP. At a minimum, this would require detailed, accurate documentation of laboratory protocols, experiment-related notes, and data entries.

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9.0 OTHER SCIENTIFIC REPORTS AND REVIEWS

9.1 Availability of Other *In Vitro* AR Binding Data

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

- The studies lacked either appropriate quantitative data (i.e., RBA or IC_{50} values) or the necessary information to calculate or estimate IC_{50} or RBA values;
- The test substances were not adequately identified, or were undefined mixtures; and,
- 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 binding data and/or information from completed studies using or evaluating AR binding assays. No information was received in response to this request.

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

The U.S. EPA has an interagency agreement with the U.S. Food and Drug Administration (FDA) National Center for Toxicological Research (NCTR) to develop, refine, and validate AR binding models for identifying substances that bind to the human AR. As part of this agreement, a number of substances are being tested for AR binding *in vitro*. However, the NCTR test results are not available at this time.

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

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

To date, no independent peer reviews of *in vitro* AR binding 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 binding 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 binding 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).

The major strengths of *in vitro* cell-free AR binding assays cited by the authors include:

- Ease of use;
- Relatively inexpensive; and
- Potential to standardize.

The major limitations cited by the authors include:

- Use of radiolabeled ligands;
- Do not distinguish between androgen agonists and antagonists; and
- Provide no information about degradation of the AR, or rates of association and dissociation of the test substance from the AR.

In addition, Gray et al. (1997) discussed the major advantages and disadvantages of an AR whole-cell binding assay that uses monkey COS cells transfected with a human AR cDNA.

The major advantages of the assay cited by the authors include:

- Does not require the use of laboratory animals;
- Relatively easy to perform;
- Reproducible between laboratories;
- Rapid separation of bound and free ligand;
- Incubations are performed at physiological temperatures, which can aid solubilization of test substances;
- Use of human AR; and

- Monkey COS cells may metabolize test substances via similar metabolic pathways to those found in human cells.

The major disadvantages of this whole cell AR binding assay include:

- Requires an AR expression vector;
- Requires transient cell transfections;
- Requires tissue cultures that can be expensive to maintain; and
- Duration of assay, which is about four days.

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 AR binding assays as screening methods for detecting potential (anti-)androgenic substances in wildlife. Ankley et al. (1998) edited the proceedings of this workshop.

The major advantages cited by the authors for using AR binding assays as endocrine disruptor screens for wildlife include:

- Widespread acceptance and use; and
- Can be conducted using AR from various mammalian and nonmammalian species, including fish, reptiles and birds.

The major disadvantages include:

- Do not distinguish between agonists and antagonists; and
- Uncertainties regarding extrapolation across species.

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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 binding 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 Binding Assays

Of the 11 *in vitro* AR binding assays addressed in this BRD, four require the use of mammalian tissues: RPC, REC, RECNR, and CUC. The first three assays entail the humane killing of male rats for the purpose of obtaining reproductive tissues specific to the assay. Typically, the prostate and epididymal tissues required for these assays are obtained from mature male rats that are castrated 18 to 24 hours prior to tissue collection. The CUC assay entails the humane killing of female prepubertal calves for their uteri. Because the animals are not treated with a test substance, treatment-related pain and suffering are avoided.

With respect to refining the cytosol assays, procedures that are the least invasive and distressful to the animals should be used. As for reducing the number of animals used in these assays, protocols should maximize the number of substances that can be tested per gram of tissue, for

example, by optimizing the protocol to use the lowest possible concentration of AR per assay tube.

The seven other *in vitro* AR binding assays considered in this BRD do not entail animal use, but utilize either cultured whole cells or cell-free preparations. Of these assays, the HGF assay uses human genital skin fibroblast cells that naturally express human AR. Two other whole cell assays use COS-1 monkey kidney cells that have been transfected with cDNA encoding either hAR or rtAR. A variation of the assay that uses COS-1 cells is conducted with a cytosolic preparation from the cells. Two other cell-free assays use cytosolic preparations from human cancer cell lines, one of which is derived from the lymph node of a man with prostatic adenocarcinoma (LnCaP), while the other is derived from a human breast cancer cell line (MCF-7). The seventh assay uses semi-purified human receptors derived from cDNA expressed in a baculovirus expression system (hAR).

Although none of the *in vitro* AR binding assays has been extensively used for the routine testing of substances, a few general statements can be made regarding the assays that would be advantageous for animal welfare. The assays using whole cells or cell-free systems could potentially eliminate the use of animals for *in vitro* AR binding experiments; however, further development and validation of these assays is required. In comparison to the assays requiring rodent or bovine tissues, one major advantage of the whole cell and cell-free assays that use human AR is that they are directly relevant to humans. In addition, the whole cell and cell-free assays would be expected to be more economical to perform than assays requiring animal care and surgical costs.

11.0 PRACTICAL CONSIDERATIONS

11.1 Test Method Transferability

Test method transferability describes the ability of a new method to be adopted and routinely performed by laboratories with experience in the particular type of procedure, as well as by laboratories with little or no experience. It also implies that the necessary facilities, equipment, and trained staff to perform the method are readily obtained and that the cost or the level of expertise or training needed is not prohibitive. The issue of transferability essentially addresses the ability of the test method to be performed by competent laboratories and its resulting inter-laboratory reproducibility. ICCVAM defines test method transferability as the ability of a test method to be accurately and reliably performed in different, competent laboratories (ICCVAM, 1997).

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

- Availability of the facilities and the major fixed equipment needed to perform the test method;
- The training requirements for technicians to demonstrate proficiency with the test method;
- Cost involved in conducting the test; and
- Amount of time needed to conduct the test.

11.1.1 Facilities and Major Fixed Equipment

The facilities needed to conduct AR binding assays are widely available, and the necessary equipment is readily available from major suppliers. Specific needs as related to the various *in vitro* AR binding procedures are described below. To ensure personnel and community safety, pertinent State or Federal regulations for the handling of hazardous and radioactive substances/wastes must be strictly adhered to.

Prostate and epididymal cytosol *in vitro* AR binding assays

Facilities: Standard toxicology, biochemistry, or molecular biology laboratory; an animal facility with temperature, humidity, and light controls; and a small animal surgical facility.

Fixed Major Equipment: Refrigerated centrifuge; ultracentrifuge; and liquid scintillation counter.

Whole cell and cell-free *in vitro* AR binding assays (e.g., HGF, COS-1 based, and MCF-7 based assays)

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

Fixed Major Equipment: Liquid scintillation counter; sterile biohazard hoods; and incubators.

11.2 Training Considerations

Prostate and epididymal cytosol *in vitro* AR binding assays

Basic laboratory skills; and training in the handling and use of radioactive substances and in small animal handling and surgery.

HGF assays, MCF-7 cytosolic assay, and LnCaP cytosol assay

Basic laboratory skills; and training in the handling and use of radioactive substances and cell culture techniques.

Semi-purified recombinant human AR assay, and COS-1 cell and cytosolic assays

Basic laboratory skills; and training in the handling and use of radioactive substances, cell culture techniques, transient transfections, and protein purification.

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 for the assays. One laboratory that conducts the RPC assay provided cost information. It can be assumed that the costs for all of the prostate and epididymal cytosol assays (RPC, REC,

RECNR) are roughly equivalent. Similarly, it would be expected that the costs for the cell culture assays and assays using semi-purified AR would be roughly equivalent.

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

Assay	Cost/Test Substance	Duration	Special Equipment	Other Considerations
COS-1 Cells+hAR	n.a.	96-108 h	Liquid scintillation counter ($15K - $30K)	Patented cDNA
COS-1 Cells+rtARα	n.a.	96-108 h	Liquid scintillation counter ($15K - $30K)	
COS-1 Cytosol+hAR	n.a.	96-108 h	Liquid scintillation counter ($15K - $30K)	Patented cDNA
HGF	n.a.	96-108 h	Liquid scintillation counter ($15K - $30K)	Low number of AR in cells
LnCaP Cytosol	n.a.	~48 – 72 h	Liquid scintillation counter ($15K - $30K)	Mutant AR
MCF-7 Cytosol	n.a.	~48 – 72 h	Liquid scintillation counter ($15K - $30K)	High level of estrogen receptor
RPC	$85 - $175	~48 – 72 h	Liquid scintillation counter ($15K - $30K)	
REC and RECNR	n.a.	~48 – 72 h	Liquid scintillation counter ($15K - $30K)	
hAR	n.a.	~24 h	Liquid scintillation counter ($15K - $30K)	Patented cDNA

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 BINDING ASSAYS AND RECOMMENDED SUBSTANCES FOR USE IN VALIDATION STUDIES

12.1 Introduction

Relatively few studies have been published on the ability of substances to bind *in vitro* to the AR, and most of these studies have reported on the binding of only 10 to 20 substances. There is only one published guideline for conducting such studies, an RPC assay protocol, which is provided in the EDSTAC Final Report (1998). No formal validation studies have been conducted to assess the reliability or the performance of *in vitro* AR binding assays. To assist in the development and characterization of *in vitro* AR binding assays, minimum procedural standards for such assays and a recommended list of test substances for use in validation studies are provided below. The minimal procedural standards and recommended test substances are based on an evaluation of the 11 *in vitro* AR binding assays considered in this BRD (**Sections 6** and **7**). The RPC assay, which is one of the more frequently used methods for identifying substances with AR binding activity, is undergoing validation by the U.S. EPA. For this reason, this assay is proposed as the standard against which new tests should be evaluated.

12.2 Minimum Procedural Standards

12.2.1 Animal Studies

All studies utilizing animals should be approved by the Institutional Animal Care and Use Committee (IACUC) or its equivalent.

12.2.2 Dissociation Constant (K_d) of the Reference Androgen

Irrespective of the source of the AR used, the dissociation constant (K_d) of the reference androgen (**Section 12.2.3**) must be determined. The purpose of determining K_d is to demonstrate that the assay system is valid (e.g., a finite number of high affinity receptors are saturated with ligand) and to optimize the system with respect to receptor and ligand concentration. The K_d is determined in a saturation binding experiment that involves adding increasing concentrations of the radiolabeled reference androgen to the AR preparation and measuring binding to the AR (Motulsky, 1995). To calculate specific binding of the radiolabeled reference androgen to the AR, nonspecific binding is measured at each radioligand concentration by the addition of a

nonlabeled androgen at a concentration that occupies all available receptors. The nonspecific binding is then subtracted from the total binding (in the absence of nonlabeled compound) of the radiolabeled reference androgen (Motulsky, 1995). The K_d of the reference androgen, which reflects its affinity for the specific AR preparation, can then be calculated, and is used to determine the appropriate concentration of reference androgen to be used in competitive binding assays. To determine the K_d, the AR must be exposed to the reference androgen at concentrations spanning five to six orders of magnitude.

12.2.3 Reference Androgen

In contrast to *in vitro* ER binding assays where 17β-estradiol is the reference estrogen of choice, four different androgens have been used as reference ligands in *in vitro* AR competitive binding studies. Initially, the two endogenous androgens, testosterone and DHT, were used as the reference ligand. Subsequently, it was found that testosterone could be metabolized to DHT by cells or cell extracts, which is problematic in a competitive binding assay, since DHT binds the AR and dissociates at a slower rate than testosterone (Wilson and French, 1976). In addition, it was shown that DHT bound to a testosterone-estradiol binding globulin (TeBG) found in tissues of the male reproductive system (Bonne and Raynaud, 1975, 1976). As a result, R1881, a potent synthetic androgen, has been used by a number of investigators as the reference androgen in place of DHT. R1881 is not metabolized nor does it bind to the TeBG, but it does bind to the progesterone receptor. However, addition of triamcinolone acetonide suppresses the binding of R1881 to the progesterone receptor without interfering with its binding or that of test substances to the AR (Zava et al., 1979). Mibolerone, another synthetic androgen, has been used in several recent studies. This substance is a more selective AR ligand than R1881 since it does not bind to the progesterone receptor and only binds with low affinity to TeBG; it is also metabolically stable. Although potentially the reference androgen of choice, it is relatively expensive and difficult to obtain. Given that the *in vitro* AR binding assays are to be used in a screening mode only and not for the purpose of risk assessment, a natural ligand was not selected as the reference androgen because of the potential for cross-reactivity with other receptors in some assay systems. For these reasons, R1881 is proposed as the reference androgen.

12.2.4 Preparation of Test Substances

Test substances must be dissolved in water or in a solvent that is miscible with water. For substances not sufficiently water soluble, ethanol or dimethyl sulfoxide (DMSO) are proposed as solvents. Preference is given to 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 (or vehicle) control substance must be included in each assay. It might be necessary to characterize the solubility of the test substance in several solvents to identify the optimal solvent to use in the AR binding assay.

12.2.5 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 binding screening assays has not been established. Historically, the highest dose tested in such assays has ranged from 1 to 100 μM, with some tests conducted at dose levels as high as 1 mM. The RBA values reported for substances tested in various *in vitro* AR binding assays cover six orders of magnitude. In the RPC assay, the median IC_{50} values for three of the reference androgens (DHT, R1881, mibolerone) range from 1 to 8.5 nM (IC_{50} values for testosterone with this assay were not located). Thus, if *in vitro* AR binding assays are required to detect substances with IC_{50} values that are at least six orders of magnitude higher than that of R1881, then the limit dose (unless precluded by chemical properties such as solubility) should be 1 mM. However, if five orders of magnitude are sufficient for detecting AR-binding substances, then the limit dose could be 100 μM. Decreasing the limit dose to 10 μM would limit the sensitivity of the assay to RBA values that cover approximately four orders of magnitude.

For the purpose of screening, it is proposed that the limit dose be 1 mM and that a concentration range from 1 mM to 1 nM, in ten-fold increments, be used. However, if it is suspected that the test substance might bind more strongly to the AR than R1881, the dose range should extend from 10 pM to 10 μM, in ten-fold increments.

For relatively insoluble substances, the highest dose should be at the limit of solubility; the concentration range should then decrease in ten-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).

12.2.6 Solvent and Positive Controls

Concurrent negative, solvent, and positive controls must be included in each experiment. The negative control, which consists of buffer and cytosol only, provides assurance that the solvent does not interact with the test system. The negative control contains all the reagents of the test system, except the assay solvent, which is replaced with a known nonreactive material, such as water. This sample is processed with treated samples and other control samples to ensure the solvent does not interact with the test system. The solvent control consists of all the reagents of the test system, including the solvent, and should be tested at the highest concentration that is added with the test substance. The volume of materials in AR assay control tubes should equal that of AR assay tubes containing test substance and reference androgen. Since the RBA for the reference androgen, R1881, is set at 100, it is recommended that a substance (e.g., cyproterone acetate, or 17β-estradiol) that induces an RBA value between two and three orders of magnitude lower be used as the positive control. The median RBA values of cyproterone acetate and 17β-estradiol in all the assays in which they have been tested are 2.75 and 1.65, respectively (**Appendix D**). In general, the RBA values for these two substances were consistent among the assays using different reference androgens. If metabolic activation is included in the experimental protocol, then a positive control requiring metabolic activation will need to be included in each experiment to demonstrate the adequacy of the exposure conditions. An appropriate positive control for such studies has not yet been identified.

12.2.7 Within-Test Replicates

The IC_{50} value of the reference androgen (e.g., R1881), the solvent and positive controls, and each test substance should be based on triplicate measurements at each dose level.

12.2.8 Dose Spacing

Generally, to obtain a binding curve, 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 nM to 1 mM). This results in the testing of seven concentrations of the test substance or reference androgen in each test. If the range of doses is reduced due to, for example, insolubility of the substance at the limit dose, then equivalent spacing (e.g., half-log doses) of the seven doses over the smaller dose range should be used.

12.2.9 Data Analysis

Following the measurement of saturation binding of radiolabeled R1881 (or another reference androgen) to the AR and after correcting for nonspecific binding, the binding of radiolabeled R1881 to the AR is plotted against the log concentration of radiolabeled R1881. The curve is analyzed with nonlinear regression techniques to determine B_{max} and K_d. Although a Scatchard analysis (Scatchard, 1949) is frequently used to obtain the K_d, this method has many disadvantages and is not recommended as the primary method (see **Section 2**). Competitive binding experiments use a constant concentration of radiolabeled R1881 to measure its displacement from the AR by varying concentrations of reference androgen or test substance. These data are analyzed by nonlinear regression analysis to determine the IC_{50} of the test substance or the reference androgen. The RBA value for the test substance is calculated by dividing the IC_{50} for R1881 (or other reference androgen) by the IC_{50} of the test substance and multiplying the result by 100. The K_i is calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973) as a means of assessing the reproducibility of the data from experiment to experiment.

$$K_i = \frac{IC_{50}}{1 + \frac{[\text{Radiolabeled reference androgen}]}{K_d}}$$

12.2.10 Assay Acceptance Criteria

An assay will be considered acceptable for evaluation if the following conditions are met:

- The unlabeled R1881 standard curve demonstrates that increasing concentrations of unlabeled R1881 can displace radiolabeled R1881, and that the IC_{50} value for R1881 is

approximately equal to the molar concentration of radiolabeled R1881 plus the K_d (determined by nonlinear regression and viewed by a Scatchard plot);

- The K_d and IC_{50} values for the unlabeled R1881 standard curve are within the confidence limits for historical data;
- The ratio of total binding in the absence of competitor to the amount of radiolabeled R1881 added per assay tube is not greater than 10%;
- The K_i, IC_{50}, and RBA values for the concurrent positive control are within the confidence limits for historical data; and
- The solvent control, at the concentration used, did not alter the sensitivity or reliability of the assay.

12.2.11 Evaluation and Interpretation of Results

A substance is classified as positive for binding to the AR if an IC_{50} value can be obtained and an RBA can be calculated. If an IC_{50} cannot be obtained after testing to the limit dose or the highest dose possible, the test substance is usually classified as being "negative" for *in vitro* AR binding. However, due to solubility constraints (for example), some test substances might induce a significant reduction in binding but without achieving at least a 50% reduction in the binding of the reference androgen to the AR. Until additional information becomes available about the significance of this category of dose response curves, such responses should be noted and the substances classified appropriately (e.g., "equivocal") for the test.

12.2.12 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; and
- Physicochemical properties relevant to the study (e.g., solubility, stability, volatility).

Solvent:

- Justification for choice of solvent if other than water or ethanol; and
- Information to demonstrate that the solvent, if other than an established solvent, does not bind to, or otherwise affect, the AR.

Androgen receptor:

- Type and source of AR (if from a commercial source, the supplier must be identified);
- Isolation procedure or method for making construct if isolated protein used;
- Protein concentration of AR preparation; and
- Method for storage of AR, if applicable.

Test conditions:

- K_d of the reference androgen;
- Rationale for the concentration of the reference androgen;
- Composition of buffer(s) used;
- Concentration range of test substance, with justification;
- Volume of vehicle used to dissolve the test substance and the volume of test substance added;
- Incubation time and temperature;
- Type and composition of metabolic activation system, if added;
- Concentration range of positive and solvent/vehicle controls;
- Method used to separate free reference androgen, if applicable;
- Method for analyzing bound reference substance;
- Methods used to determine K_i and IC_{50} values; and
- Statistical methods used, if any.

Results:

- Extent of precipitation of test substance;
- The solvent control response compared to the negative control;
- IC data for each replicate at each dose level for all substances, including confidence levels or other measure of intradose repeatability;

- Calculated K_i and IC_{50} values and confidence limits for the reference androgen, the positive control, and the test substance; and
- Calculated RBA values for the positive control and the test substance.

Discussion of the results:

- Historical K_i and IC_{50} values for the reference androgen, including ranges, means, and standard deviations;
- Reproducibility of the K_i and IC_{50} values of the reference androgen, compared to historical data;
- Historical solvent and positive control data with ranges, means, and standard deviations;
- Reproducibility of the K_i and IC_{50}/RBA values for the positive control substance, compared to historical data; and
- The nature of the binding dose response relationship for the test substance.

Conclusion:

- Classification of test substance with regard to *in vitro* AR binding activity.

12.2.13 Replicate Studies

Generally, replicate studies are not mandated for screening assays. However, in situations where questionable data are obtained (i.e., the IC_{50} value is not well defined, "equivocal" results are obtained), additional testing using a more narrow range of test substance concentrations to clarify the results of the primary test would be prudent.

12.3 Standardization of AR Binding Assays for Validation

Appendix B provides *in vitro* AR binding assay protocols submitted by four investigators. These assay protocols (as titled by the investigator) are:

- Protocol for Androgen Receptor Competitive Binding Assay Using Rat Prostate Cytosol, as provided by Dr. Vickie Wilson, U.S. EPA, NHEERL, Research Triangle Park, NC and Mr. Gary Timm, U.S. EPA, Washington, DC, USA.

- Protocol for COS Cell Binding 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 Measuring Androgen-Binding Sites on Androgen Receptors or Binding Proteins, as provided by Dr. Benjamin Danzo, Department of Obstetrics and Gynecology, Vanderbilt University School of Medicine, Nashville, TN, USA.
- Technical Perspective on the U.S. EPA Endocrine Disruptor Screening Program: *In Vitro* EDSTAC Guideline Protocols, as 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.

The RPC assay protocol provided by Dr. Wilson and Mr. Timm is being used in the U.S. EPA-sponsored validation study for androgen receptor competitive binding. Inspection of these protocols provides a perspective on how various *in vitro* AR binding 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.

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

Table 12-1 provides a list of recommended substances to be used in the assessment of the reliability and comparative performance of existing or new *in vitro* AR binding assays. A number of factors were considered in developing this list, including the number of times the substance had been tested in multiple assays, and the median RBA value of the substance across all assays in which it was tested, regardless of the reference androgen used. Selection of the substances was based on the availability and concordance of multiple test results, to the extent possible, among the 11 *in vitro* AR binding assays considered in this BRD. The selected substances were sorted according to their median RBA values. Because the published spread of RBA values was six orders of magnitude below the reference androgen (from 100 to 0.0001), the substances were sorted into six categories in log decrements: ≥10, <10-1; <1-0.1; <0.01-0.1,

<0.01-0.001; <0.001. Weakly-binding substances (RBA values <0.001) were difficult to identify because they were not consistently positive within an assay or among different assays. Also included in the list were substances classified as "negative" for AR binding based on the lack of a positive response when tested at dose levels of at least 1 mM. Due to its selection as the reference androgen, R1881 is not included in this list. Due to intrinsic differences in the binding of the various reference androgens to the AR, this approach for classifying substances by median RBA value provides only a relative approximation of the anticipated level of AR binding activity.

Where possible, five substances were selected for each of those RBA dose-range categories in which a sufficient number of compounds were commercially available, and three for the negative chemical group. Within the constraints of the small database, an effort was made to select substances within each RBA category that were representative of different chemical classes. Consideration was also given as to the substance's commercial use, whether it was representative of a chemical class found in the environment, and whether the substance is commercially available. The latter criterion was based on whether it could be located in a chemical supply catalogue. Due to the sparseness of the data, some of the substances in this list have only been tested in one assay and in a few instances have only been tested once. Although vinclozolin and methoxychlor are less active in binding than their respective metabolites, both of these substances have been included in the recommended list for validation since they are being tested by Battelle.

Table 12-1 Recommended Substances for Validation of *In Vitro* AR Binding Assays

Classification RBA Range	Substance	CASRN	Median RBA Value	Chemical Class	No. Assays in which Tested[a]	No. Assays with a Positive[a]
≥10	17β-Trenbolone	10161-33-8	108.9	Steroid, nonphenolic	1	1
	5α-DHT**	521-18-6	96.5	Steroid, nonphenolic	7	7
	Spironolactone	52-01-7	33.8	Steroid, nonphenolic	1	1
	Testosterone	58-22-0	29.2	Steroid, phenolic	8	8
	Medroxyprogesterone acetate	71-58-9	11.6	Steroid, nonphenolic	4	4
<10 to 1	Levonorgestrel	797-63-7	9.25	Steroid, nonphenolic	1	1
	Progesterone	57-83-0	2.75	Steroid, nonphenolic	8	8
	Cyproterone acetate	427-51-0	2.8	Steroid, nonphenolic	5	5
	17β-Estradiol	50-28-2	1.65	Steroid, phenolic	8	8
	4-Androstenedione	63-05-8	1.03	Steroid, nonphenolic	2	2
<1 to 0.1	17α-Ethinyl estradiol	57-63-6	0.85	Steroid, phenolic	2	2
	4-Hydroxyandrostenedione	566-48-3	0.79	Steroid, nonphenolic	1	1
	Melengestrol acetate	2919-66-6	0.31	Steroid, nonphenolic	1	1
	Fluoxymestrone	76-43-7	0.3	Steroid, nonphenolic	1	1
	Estrone	53-16-7	0.1	Steroid, phenolic	1	1
<0.1 to 0.01	17α-Hydroxyprogesterone	68-96-2	0.087	Steroid, phenolic	2	2
	Vinclozolin	50471-44-8	0.018	Organochlorine	3	2
	p,p'-DDE	72-55-9	0.016	Organochlorine	2	2
	Diethylstilbestrol	56-53-1	0.010	Stilbene	4	3

Classification RBA Range	Substance	CASRN	Median RBA Value	Chemical Class	No. Assays in which Tested[a]	No. Assays with a Positive[a]
<0.01 to 0.001	Linuron	330-55-2	0.0055	Urea	4	4
	o,p'-DDT	789-02-6	0.00105	Organochlorine	2	2
	Atrazine	1912-24-9	0.0018	Triazine	1	1
	p,p'-DDT	50-29-3	0.0013	Organochlorine	2	2
<0.001	Kepone	143-50-0	0.00072	Organochlorine	2	2
	Methoxychlor	72-43-5	0.00053	Organochlorine	2	2
	Corticosterone	50-22-6	0.000068	Steroid, nonphenolic	1	1
	Pregnenolone	145-13-1	0.000068	Steroid, nonphenolic	2	1
	Procymidone	32809-16-8	0.000068	Imide	1	1
Negative	Cortisol	50-23-7	HDT-10 µM	Steroid, phenolic	2	0
	Cyanoketone	4248-66-2	HDT-10 µM	Steroid, nonphenolic	1	0
	Dexamethasone	50-02-2	HDT-10 µM	Steroid, nonphenolic	2	0

Abbreviations: DHT = Dihydrotestosterone; DDE = 1,1-Dichloro-bis[4-chlorophenyl]ethylene; DDT = Dichlorodiphenyltrichloroethane; HDT= highest dose tested.
*Median RBA value across assays.
**The median RBA values for DHT were determined from tests where these substances were not used as the reference androgen.

The chemical classes of the substances and the number of substances in each class in **Table 12-1** include nonphenolic steroids (15), phenolic steroids (6), organochlorines (6), a stilbene (1), an imide (1), an urea (1) and a triazine (1).

In January 2002, the U.S. EPA provided a list of 19 substances proposed for testing by Battelle Pacific Northwest (Richland, Washington) in an RPC assay procedure. Data generated by the U.S. EPA-sponsored study will be used to validate two QSAR models presently being developed by scientists at the FDA NCTR and by Dr. Mekenyan in Bulgaria. The 19 substances were chosen based on the availability of historical data demonstrating the *in vitro* AR binding affinity, ease of purchase at a purity of >98%, and the lack of extensive health and safety requirements for use (S. Laws, personal communication). Representation of all chemical classes was not a high priority. The substances on the U.S. EPA list (**Table 7-2**) were compared to those recommended in this BRD for use in validation studies. All of the substances on the U.S. EPA list are included, except for 4-*tert*-octylphenol and bis(2-ethylhexyl)phthalate. These two substances were omitted since no published RBA values for them could be found.

In a validation study, it is important to include substances that cover the range of possible responses and, therefore, the list of recommended substances includes approximately equal numbers of substances in each RBA category. When available, the results from the Battelle study might be used to modify the NICEATM list.

Table 12-2 List of 19 Substances Being Tested in the RPC Assay by Battelle

Classification RBA Range	Substances	Median RBA Value	No. Times Tested Among All Assays	Included in Recommended List in BRD
≥ 10	R1881*	137	8	No
	DHT*	96.5	14	Yes
	Spironolactone	33.8	2	Yes
	Testosterone	29.2	13	Yes
	Medroxyprogesterone acetate	11.6	5	Yes
<10 to 1	Cyproterone acetate	2.8	12	Yes
	Progesterone	2.75	11	Yes

Classification RBA Range	Substances	Median RBA Value	No. Times Tested Among All Assays	Included in Recommended List in BRD
	17β-Estradiol	1.65	14	Yes
	4-Androstenedione	1.03	2	Yes
<0.1 to 0.01	Vinclozolin	0.018	4	Yes
	p,p'-DDE	0.016	3	Yes
<0.01 to 0.001	Atrazine	0.0018	1	Yes
	Linuron	0.0055	4	Yes
<0.001	Methoxychlor	0.00053	2	Yes
	Corticosterone	0.00007	1	Yes
	Procymidone	0.00007	1	Yes
Negative	Dexamethasone	Negative	2	Yes
Unknown	4-*tert*-Octylphenol	?	?	No
	Bis-(2-ethylhexyl) phthalate	?	?	No

Abbreviations: R1881 = Methyltrienolone; DHT = 5α-Dihydrotestosterone; DDE = 1,1-Dichloro-bis[*p*-chlorophenyl]ethylene.
*The median RBA values for R1881 and DHT were determined from tests where these substances were not used as the reference androgen.

12.5 Conclusions and Recommendations

Currently, there are no published guidelines for conducting *in vitro* AR binding studies, and no formal validation studies to assess the reliability or performance of AR binding assays have been performed. To support the further development and characterization of *in vitro* AR binding 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 a comparative evaluation of the 11 *in vitro* AR binding assays summarized and evaluated in this BRD. The RPC assay, one of the more widely used methods for identifying substances with AR binding activity and an assay undergoing current validation efforts by the U.S. EPA, is proposed as the standard against which new tests should be evaluated.

The minimum procedural standards consider methods for determining the K_d of the reference estrogen, methods for test substance preparation, the concentration range of the test substance to evaluate (including the limit dose), the use of solvent and positive controls, the number of replicates to use per test substance concentration, 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 binding studies will be conducted to the same minimal standards.

The RPC assay protocol being used in the U.S. EPA-sponsored study for AR competitive binding is provided in **Appendix B**, along with three other protocols developed by experts in the field. Inspection of these protocols provides a perspective on how various 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, these protocols need to be evaluated for completeness and adequacy for their intended purpose.

A number of factors were considered in developing a list of recommended substances to be used in validation efforts, including the number of times the substance had been tested in various assays, the median RBA value of the substance across assays and its extent of concordance. The selected substances were sorted according to their median RBA values, over six orders of magnitude, ranging from 100 to 0.0001. Weakly-binding substances (RBA values <0.001) were difficult to identify because they were not always consistently positive in tests within an assay or using different assays. Also included were substances classified as "negative" for AR binding based on the lack of a positive response in multiple assays when tested at doses of at least 10 μM. Where possible, five substances were selected for each RBA category and three for the negative category group. To ensure that each RBA category contained a representative sampling of chemical classes, selection was based on the chemical class to which the substance belongs, whether it was representative of a chemical class used in commerce or found in the environment, and whether the substance is commercially available. The latter criterion was based on whether the substance could be located in a chemical supply catalogue.

The resulting list of 31 substances was compared with an U.S. EPA list of 19 substances that has been proposed for testing in an RPC assay procedure by Battelle Pacific Northwest. The U.S. EPA has fewer substances in the organochlorine chemical class. Two of the substances on the U.S. EPA list were not included in the list of recommended substances because of the absence of published data on their AR binding activity.

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14.0 GLOSSARY[1]

Accuracy[2]: 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 (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 5α-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**.

Cell-free: Not containing intact cells. May contain cell or tissue homogenates or artificial mixtures of cellular components.

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[1] The definitions in this Glossary are restricted to their uses with respect to endocrine mechanisms and actions.

[2] Definition used by the Interagency Coordinating Committee on the Validation of Alternative Methods.

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

Concordance[2]: 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.

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

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.

Dissociation constant: A measure of the ability of a molecule to be released from binding to a receptor.

DNA-regulatory activity: Refers to a DNA-binding molecule or complex that causes a change in DNA-related activities.

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 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.

Estrogen: A class of steroid hormones, which includes 17β-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[2]: An active substance incorrectly identified as negative by a test.

False negative rate[2]: The proportion of all positive (active) substances falsely identified as negative. A measure of test performance.

False positive[2]: An inactive substance incorrectly identified as positive by a test.

False positive rate[2]: 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.

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.

Homology (DNA): Similarity in DNA sequence of segments or genes from different strains or species of organisms.

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

Hydroxyapatite (HAP): A form of calcium phosphate with the ability to bind to some classes of organic molecules.

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[2]: A measure of whether different laboratories using the same protocol and test chemicals can produce qualitatively and quantitatively similar results. See **reliability**.

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

Intraperitoneal: Administration by injection directly into the peritoneal cavity.

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.

K_d: Equilibrium dissociation constant of a reference compound in a specific receptor preparation. A measure of the strength of binding between a receptor and ligand.

K_i: Equilibrium dissociation constant of an inhibitor in a competitive receptor binding experiment.

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

Ligand-binding domain: The area within a receptor molecule that noncovalently, but stereospecifically binds the cognate hormone, or other ligands, for the receptor of interest.

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

Negative control: An untreated sample containing all reagents of a test system, except the assay solvent, which is replaced with a known nonreactive material, such as water. This sample is processed with treated samples and other control samples to assess whether the solvent interacts with the test system.

Negative predictivity[2]: 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.

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 control: A sample containing all components of a test system and treated with a substance known to induce a positive response, that is processed with other samples to demonstrate the sensitivity of each experiment and to allow for an assessment of variability in the conduct of the assay over time.

Positive predictivity[2]: The proportion of correct positive responses among substances testing positive.

Protocol[2]: 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.

Radiolabel: A radioactive isotope of an atom that is added to a molecule to allow the molecule to be identified by **scintillation counting**.

Receptor: A protein or protein complex that binds to specific molecules for the purpose of initiating a specific cellular response or process, such as transcriptional activation.

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.

Relevance (of an assay)[2]: 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)[2]: The intra- and inter-laboratory **reproducibility** of the assay.

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

Scintillation counting: The measurement of radioactivity using a scintillation counter.

Screen/Screening Test[2]: 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[2]: The proportion of all positive substances that are correctly classified as positive in a test.

Specificity[2]: 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*.

Sulfhydryl: A functional group on a molecule containing sulfur in the form of -SH.

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 estrogen-estrogen 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[2]: A method determined to be acceptable for a specific use.

Validated method[2]: A method for which the reliability and relevance for a specific purpose has been established.

Validation[2]: The process by which the reliability and relevance of a procedure 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 foreign to the organism of interest.

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

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Appendix A
Methods for *In Vitro* AR Binding Assays

A1 Assays Using Rat Prostate or Epididymal Cytosol

A2 Assays Using Human Genital Fibroblast Cells

A3 Assays Using COS Cells Transfected with AR

A4 Assays Using MCF-7 Cells

A5 Miscellaneous AR Binding Assays

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Appendix A1

Assays Using Rat Prostate or Epididymal Cytosol

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Assays Using Rat Prostate or Epididymal Cytosol

Reference	Danzo (1997)	Kelce et al. (1994)	Kelce et al. (1995)
Preparation of receptor			
Source of receptor	Rat (otherwise unspecified)	Sprague Dawley rat	Rat (otherwise unspecified)
Tissue	prostate	epididymis	ventral prostate
Age of animals	n.p.	120 -150 days	n.p.
When castrated	n.p.	24 hours before sacrifice	24 hours before sacrifice
Diet of animals	n.p.	Purina Lab Chow - 5001	n.p.
Environment	n.p.	22° C, 40-50% humidity	n.p.
Lighting	n.p.	14 hours light:10 hours dark	n.p.
Buffer for preparation of cytosol	n.p.	TEDG, pH 7.4	n.p.
Dilution of tissue with buffer	n.p.	5 ml/gm	n.p.
Homogenization	n.p.	Polytron	n.p.
Centrifugation	n.p.	30000xg, 4° C, 10 min	n.p.
Protein concentration of cytosol	n.p.	n.p.	n.p.
Competitive binding assay			
Reference ligand	5α-Dihydrotestosterone	R1881	R1881
Volume and concentration of reference ligand	n.p.	volume n.p.; 0.5 to 20.0 nM	volume n.p.; 3 nM
Specific activity of labelled reference ligand	n.p.	86 Ci/mmol	n.p.
Volume and concentration of cold ligand	n.p.	volume n.p.; .01 - 1000 nM or .01 - 400 nM	volume n.p.; 100 nM
Final concentration of reference ligand	n.p.	n.p.	103 nM
Concentration range of competing ligand	n.p.	n.p.	0-100; 0-50; 0-1.2 µM
Volume of cytosol	n.p.	300 µl	n.p.
Volume of buffer	n.p.	n.p.	n.p.
Type of buffer used	n.p.	n.p.	n.p.
Replicates	2	n.p.	n.p.
Time of incubation	n.p.	20 hours	20 hours
Temperature of incubation	n.p.	4° C	4° C

Assays Using Rat Prostate or Epididymal Cytosol

Reference	Danzo (1997)	Kelce et al. (1994)	Kelce et al. (1995)
Separation of ligand			
Type of slurry	dextran-charcoal	hydroxylapatite	n.p.
Buffer for slurry	n.p.	Tris, pH 7.4	n.p.
Incubation time and temperature	n.p.	20 min, temp. n.p.	n.p.
Centrifugation speed	n.p.	600xg	n.p.
Centrifugation time and temperature	n.p.	2 min; 4º C	n.p.
Resuspension volume and buffer for pellet	n.p.	Tris, pH 7.4	n.p.
No. of washes	n.p.	3	n.p.
Extraction of label	n.p.	2 ml ethanol	n.p.
Incubation time and temperature	n.p.	10 min	n.p.
Vortexing during incubation time	n.p.	yes	n.p.
Centrifugation time and temperature	n.p.	n.p.	n.p.
Measurement of Binding			
Volume added for reading	n.p.	n.p.	n.p.
Volume of fluor	n.p.	15 ml scintillation fluid	n.p.
Type of fluor	n.p.	n.p.	n.p.
Instrumentation	n.p.	n.p.	n.p.
Measurement	n.p.	n.p.	n.p.
Blank without competitor	n.p.	n.p.	n.p.
Reading of blank	n.p.	n.p.	n.p.
Blank subtracted?	n.p.	n.p.	n.p.
Range of standard curve of reference ligand	n.p.	.01-1000 nM; .01-400 nM	n.p.
Nonspecific binding measured?	n.p.	yes	n.p.
Subtraction of nonspecific binding	n.p.	yes	n.p.
Data calculations			
Data plotted as	% Inhibition	Scatchard plots	Scatchard plots
Data calculated	n.p.	n.p.	n.p.
Calculation of RBA	from bar graph	n.p.	n.p.
Test substances			
Solvent used	n.p.	n.p.	n.p.
No. of samples/ dose	n.p.	2	n.p.
No. of times assay repeated	varies from 3 to 8 depending on substance	n.p.	n.p.

Abbreviations: n.a. = not applicable; No. = number; n.p. = not provided; RBA = relative binding affinity

Assays Using Rat Prostate or Epididymal Cytosol

Reference	Lambright et al. (2000)	Schilling and Liao (1984)	Teutsch et al. (1994)
Preparation of receptor			
Source of receptor	Sprague Dawley rat	Sprague Dawley rat	Sprague Dawley rat
Tissue	ventral prostate	ventral prostate	prostate
Age of animals	90 days	n.p.	n.p.
When castrated	24 hours before sacrifice	18 hours before sacrifice	24 hours before sacrifice
Diet of animals	n.p.	n.p.	n.p.
Environment	n.p.	n.p.	n.p.
Lighting	n.p.	n.p.	n.p.
Buffer for preparation of cytosol	TEDG	Dulbecco's MEM, Hepes, pH 7.5	Tris, DTT, phenylmethylsulfonyl fluoride, molybdate, pH 7.4
Dilution of tissue with buffer	10 ml/gm	n.p.	n.p.
Homogenization	Polytron	Potter-Elvejhem	n.p.
Centrifugation	30000xg	220,000xg, 45 min	209,000xg, 30 min, 0-4° C
Protein concentration of cytosol	n.p.	n.p.	n.p.
Competitive binding assay			
Reference ligand	R1881	Mibolerone	Testosterone
Volume and concentration of reference ligand	volume n.p.; 5 nM	volume n.p.; 10 - 30 nM	volume n.p.; 2.5 or 5 mM
Specific activity of labelled reference ligand	n.p.	80.9 Ci/mmol	54 Ci/mM
Volume and concentration of cold ligand	n.p.	n.p.	n.p.
Final concentration of reference ligand	n.p.	10 nM	0.1 - 20 nM
Concentration range of competing ligand	n.p.	50, 100, 300 nM	n.p.
Volume of cytosol	n.p.	n.p.	0.1 ml
Volume of buffer	n.p.	n.p.	n.p.
Type of buffer used	n.p.	n.p.	n.p.
Replicates	n.p.	n.p.	n.p.
Time of incubation	2 hours	n.p.	24 hours
Temperature of incubation	37° C	n.p.	0° C

Assays Using Rat Prostate or Epididymal Cytosol

Reference	Lambright et al. (2000)	Schilling and Liao (1984)	Teutsch et al. (1994)
Separation of ligand			
Type of slurry	n.p.	hydroxyapatite	dextran-charcoal
Buffer for slurry	n.p.	Tris, PO_4, pH 7.2	Tris, DTT, phenylmethylsulfonyl fluoride, molybdate, pH 7.4
Incubation time and temperature	n.p.	10 min, 0º C	10 min, 0-4º C
Centrifugation speed	n.p.	filtered	800xg
Centrifugation time and temperature	n.p.	n.a.	10 min, 0-4º C
Resuspension volume and buffer for pellet	n.p.	scintillation fluid	n.p.
No. of washes	n.p.	5	n.p.
Extraction of label	n.p.	scintillation fluid	n.p.
Incubation time and temperature	n.p.	n.p.	n.p.
Vortexing during incubation time	n.p.	n.p.	n.p.
Centrifugation time and temperature	n.p.	n.p.	n.p.
Measurement of Binding			
Volume added for reading	n.p.	n.p.	0.1 ml
Volume of fluor	n.p.	n.p.	n.p.
Type of fluor	n.p.	toluene, Triton-X100	n.p.
Instrumentation	n.p.	n.p.	n.p.
Measurement	n.p.	n.p.	n.p.
Blank without competitor	n.p.	n.p.	n.p.
Reading of blank	n.p.	n.p.	n.p.
Blank subtracted?	n.p.	no	n.p.
Range of standard curve of reference ligand	n.p.	10 nM	n.p.
Nonspecific binding measured?	n.p.	n.p.	n.p.
Subtraction of nonspecific binding	n.p.	n.p.	n.p.
Data calculations			
Data plotted as	n.p.	n.p.	Scatchard analysis
Data calculated	n.p.	% binding	n.p.
Calculation of RBA	n.p.	calculated from % binding	yes
Test substances			
Solvent used	n.p.	ethanol	n.p.
No. of samples/ dose	n.p.	n.p.	n.p.
No. of times assay repeated	n.p.	n.p.	n.p.

Abbreviations: n.a. = not applicable; No. = number; n.p. = not provided; RBA = relative binding affinity

Assays Using Rat Prostate or Epididymal Cytosol

Reference	Van Dort et al. (2000)	Waller et al. (1996)	Wilson and French (1976)
Preparation of receptor			
Source of receptor	Wistar rat	Sprague Dawley rat	Sprague Dawley or Osborne-Mendel rat
Tissue	ventral prostate	epididymis	prostate
Age of animals	n.p.	120 -150 days	n.p.
When castrated	n.p.	24 hours before sacrifice	24 hours before sacrifice
Diet of animals	n.p.	Purina Lab chow - 5001	n.p.
Environment	n.p.	22° C, 40-50% humidity	n.p.
Lighting	n.p.	14 hours light:10 hours dark	n.p.
Buffer for preparation of cytosol	PO_4 with protease inhibitor and triamcinolone acetate, pH 7.2	TEDG, pH 7.4	Tris-EDTA-glycerol, pH 7.5
Dilution of tissue with buffer	n.p.	5 ml/gm	n.p.
Homogenization	n.p.	Polytron	Ultra turrax, set at 7
Centrifugation	n.p.	30000xg, 4° C, 10 min	105000xg, 75 min
Protein concentration of cytosol	n.p.	n.p.	n.p.
Competitive binding assay			
Reference ligand	Mibolerone	R1881	5α-Dihydrotestosterone
Volume and concentration of reference ligand	volume n.p.; 2 nM	n.p.	volume n.p.; 15-20 nM
Specific activity of labelled reference ligand	n.p.	n.p.	80 Ci/mmol
Volume and concentration of cold ligand	volume n.p.; 0.3 - 100 nM	n.p.	volume n.p.; 2000 nM
Final concentration of reference ligand	2 nM	n.p.	2020 nM
Concentration range of competing ligand	0.3 - 100 nM	n.p.	20 - 2000 nM
Volume of cytosol	n.p.	300 µl	n.p.
Volume of buffer	n.p.	n.p.	n.p.
Type of buffer used	PO_4 with protease inhibitor and triamcinolone acetate, pH 7.2	n.p.	n.p.
Replicates	duplicate	n.p.	n.p.
Time of incubation	18 hours	20 hours	18 - 20 hours
Temperature of incubation	4° C	4° C	0° C

Assays Using Rat Prostate or Epididymal Cytosol

Reference	Van Dort et al. (2000)	Waller et al. (1996)	Wilson and French (1976)
Separation of ligand			
Type of slurry	hydroxyapatite	hydroxylapatite	charcoal-dextran
Buffer for slurry	n.p.	Tris, pH 7.4	Tris-EDTA, pH 7.5
Incubation time and temperature	15 min, temp. n.p.	20 min, temp. n.p.	20 min, 0º C
Centrifugation speed	n.p.	600xg	2000xg
Centrifugation time and temperature	n.p.	2 min; 4 C	15 min
Resuspension volume and buffer for pellet	n.p.	Tris, pH 7.4	n.p.
No. of washes	n.p.	3	n.p.
Extraction of label	centrifugation	2 ml ethanol	n.p.
Incubation time and temperature	n.p.	10 min	n.p.
Vortexing during incubation time	n.p.	yes	n.p.
Centrifugation time and temperature	n.p.	n.p.	n.p.
Measurement of Binding			
Volume added for reading	n.p.	n.p.	0.5 ml
Volume of fluor	n.p.	15 ml scintillation fluid	5 ml
Type of fluor	n.p.	n.p.	Aquasol/toluene, 1:1
Instrumentation	n.p.	n.p.	n.p.
Measurement	n.p.	n.p.	n.p.
Blank without competitor	n.p.	n.p.	n.p.
Reading of blank	n.p.	n.p.	n.p.
Blank subtracted?	n.p.	n.p.	n.p.
Range of standard curve of reference ligand	n.p.	.01-1000 nM; .01-400 nM	n.p.
Nonspecific binding measured?	n.p.	yes	n.p.
Subtraction of nonspecific binding	n.p.	yes	n.p.
Data calculations			
Data plotted as	n.p.	Scatchard plots	n.p.
Data calculated	Ki	n.p.	from binding graph
Calculation of RBA	n.p.	n.p.	from binding graph
Test substances			
Solvent used	n.p.	n.p.	n.p.
No. of samples/ dose	2	2	n.p.
No. of times assay repeated	3	n.p.	n.p.

Abbreviations: n.a. = not applicable; No. = number; n.p. = not provided; RBA = relative binding affinity

Appendix A2

Assays Using Human Genital Fibroblast Cells

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Assays Using Human Genital Fibroblast Cells

Reference	Breiner et al. (1986)	Brown et al. (1981)
Preparation of receptor		
Source of receptor	Human primary genital skin fibroblasts	Human penile fibroblast explants
Whole cells/ cell homogenate	whole cells	whole cells
Serum source	Fetal calf (10%)	Fetal bovine
Serum stripping method	none	n.p.
Residual androgen in serum	n.p.	n.p.
No. of treated cells/No. or weight of cells homogenized	monolayer	confluent
Treatment vessel used	60 x 15 mm falcon	culture plates
Competitive binding assay		
Reference ligand	5α-Dihydrotestosterone	5α-Dihydrotestosterone
Volume and concentration of reference ligand	2 nM	2 nM
Specific activity of labelled reference ligand	123 - 153 Ci/mmol	131 Ci/mmol
Volume and concentration of cold ligand	n.p.	2-1000 nM
Final concentration of reference ligand	2 nM	n.p.
Volume of competing ligand	n.p.	n.p.
Concentration range of competing ligand	n.p.	1 - 1000 nM
Volume of cytosol	n.a.	n.a.
Volume of buffer	3 ml	n.p.
Type of buffer used	Eagle's minimal essential medium	serum-free MEM
Replicates	duplicate	single
Time of incubation	60 min	45 min
Temperature of incubation	37° C	37° C
Separation of ligand		
Volume and type of slurry	dextran-charcoal	dextran-charcoal
Buffer for slurry	Tris-EDTA-KCl, pH 7.4	Tris-EDTA, pH 7.4
Incubation time and temperature	10 min; temp n.p.	10 min, 0-4° C
Time of vortexing	10 min	10 min
Centrifugation speed	2500 x g	2000xg
Centrifugation time and temperature	15 min; time n.p.	5 min, 0-4° C
Resuspension volume and buffer for pellet	n.p.	n.p.
No. of washes	n.p.	n.p.
Extraction of label	supernatant counted	n.p.
Incubation time and temperature	n.a.	n.p.
Volume of fluor	n.p.	n.p.
Type of fluor	n.p.	n.p.
Instrumentation	n.p.	n.p.
Measurement	n.p.	n.p.
Blank without competitor	n.p.	n.p.
Reading of blank	n.p.	n.p.
Blank subtracted?	n.p.	n.p.
Range of standard curve of reference ligand	n.p.	n.p.
Nonspecific binding measured?	n.p.	yes
Subtraction of nonspecific binding	n.p.	yes

Assays Using Human Genital Fibroblast Cells

Reference	Breiner et al. (1986)	Brown et al. (1981)
Data calculations		
Data plotted as	%[^{3}H]-DHT bound vs. Competitor concentration (M)	n.p.
Data calculated	Ki	IC_{50} (data not presented)
Calculation of RBA	from IC_{50} (data not presented)	yes
Test substances		
Solvent used	n.p.	n.p.
No. of samples/dose	2	1
No. of times assay repeated	n.p.	n.p.
Abbreviations: n.a. = not applicable; No. = number; n.p. = not provided; RBA = relative binding affinity		

Assays Using Human Genital Fibroblast Cells

Reference	Eil and Edelson (1984)
Preparation of receptor	
Source of receptor	Human newborn foreskin fibroblasts
Whole cells/ cell homogenate	whole cells
Serum source	Fetal calf
Serum stripping method	n.p.
Residual androgen in serum	n.p.
No. of treated cells/No. or weight of cells homogenized	0.5 - 2.0x10^{-6} cells/tube
Treatment vessel used	tissue culture flasks
Competitive binding assay	
Reference ligand	R1881; occasionally 5α-Dihydrotestosterone
Volume and concentration of reference ligand	0.5 μM R1881; 1.0 - 1.2 nM DHT
Specific activity of labelled reference ligand	n.p.
Volume and concentration of cold ligand	n.p.
Final concentration of reference ligand	n.p.
Volume of competing ligand	n.p.
Concentration range of competing ligand	n.p.
Volume of cytosol	n.a.
Volume of buffer	n.p.
Type of buffer used	EMEM medium
Replicates	n.p.
Time of incubation	60 min
Temperature of incubation	22° C
Separation of ligand	
Volume and type of slurry	n.p.
Buffer for slurry	n.p.
Incubation time and temperature	n.p.
Time of vortexing	n.p.
Centrifugation speed	n.p.
Centrifugation time and temperature	n.p.
Resuspension volume and buffer for pellet	n.p.
No. of washes	n.p.
Extraction of label	n.p.
Incubation time and temperature	n.p.
Volume of fluor	n.p.
Type of fluor	n.p.
Instrumentation	n.p.
Measurement	n.p.
Blank without competitor	n.p.
Reading of blank	n.p.
Blank subtracted?	n.p.
Range of standard curve of reference ligand	1.0 - 1.2 nM
Nonspecific binding measured?	n.p.
Subtraction of nonspecific binding	n.p.

Assays Using Human Genital Fibroblast Cells

Reference	Eil and Edelson (1984)
Data calculations	
Data plotted as	Scatchard plots
Data calculated	Ki
Calculation of RBA	from Ki
Test substances	
Solvent used	ethanol
No. of samples/dose	n.p.
No. of times assay repeated	n.p.

Abbreviations: n.a. = not applicable; No. = number; n.p. = not provided; RBA = relative binding affinity

Appendix A3

Assays Using COS Cells Transfected with AR

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Assays Using COS Cells Transfected with AR

Reference	Kemppainen and Wilson (1996)	Kemppainen et al. (1992)
Characteristics of Cells		
Cell line	COS-1	COS-7
Cell source	monkey kidney	monkey kidney
Source of receptor	pCMVhAR	pCMVhAR
AR source	human	human
Transfection of AR	Transient	Transient
Whole cells/cell homogenate/cytosol	whole cells	whole cells
Preparation of Cells for Assay		
Serum source	Fetal calf serum	Fetal calf serum
Serum stripping method	n.p.	n.p.
Residual androgen in serum	n.p.	n.p.
No. of treated cells/No. of cells homogenized	$2x10^5$ cells/well	$1x10^5$ cells/well
Treatment vessel used	12-well plates	24-well culture dishes
Preparation of cell homogenate	n.a.	n.a.
volume	n.a.	n.a.
buffer	n.a.	n.a.
method	n.a.	n.a.
time; temperature	n.a.	n.a.
Centrifugation of homogenate	n.a.	n.a.
Protein concentration of cytosol	n.a.	n.a.
Storage	n.a.	n.a.
Final protein concentration	n.a.	n.a.
Separation of bound hormone	Phosphate buffer saline wash	Phosphate buffer saline wash
Competitive Binding Assay		
Reference ligand	R1881	R1881
Volume and concentration of reference ligand	5 nM	5 nM
Specific activity of labelled reference ligand	80 Ci/mmol	80 Ci/mmol
Volume and concentration of cold ligand	100-fold molar excess	100-fold molar excess
Final concentration of reference ligand	5 nM	5 nM
Volume of competing ligand	n.p.	n.p.
Concentration range of competing ligand	5-500 nM	5-500 nM
Volume of cytosol	n.a.	n.a.
Volume of buffer	n.p.	n.p.
Type of buffer used	n.p.	n.p.
Replicates	n.p.	n.p.
Time of incubation	2 hr	2 hr
Temperature of incubation	37° C	37° C

Assays Using COS Cells Transfected with AR

Reference	Kemppainen and Wilson (1996)	Kemppainen et al. (1992)
Separation of ligand		
Volume and type of slurry	n.p.	n.p.
Buffer for slurry	n.p.	n.p.
Incubation time and temp	n.p.	n.p.
Time of vortexing	n.p.	n.p.
Centrifugation speed	n.p.	n.p.
Centrifugation time and temp	n.p.	n.p.
Resuspension volume and buffer for pellet	n.p.	n.p.
No. of washes	2	2
Extraction of label	n.p.	n.p.
Incubation time and temperature	n.p.	n.p.
Vortexing during incubation time	n.p.	n.p.
Centrifugation time and temperature	n.p.	n.p.
Volume added for reading	n.p.	n.p.
Volume of fluor	n.p.	n.p.
Type of fluor	n.p.	n.p.
Instrumentation	n.p.	n.p.
Measurement	n.p.	n.p.
Blank without competitor	n.p.	n.p.
Reading of blank	n.p.	n.p.
Blank subtracted?	n.p.	n.p.
Range of standard curve of reference ligand	n.p.	n.p.
Nonspecific binding measured?	n.p.	n.p.
Subtraction of nonspecific binding	n.p.	n.p.
Data calculations		
Data plotted as	% [^{3}H]-R1881 vs. Unlabeled ligand (μM)	% [^{3}H]-R1881 vs. Unlabeled hormone (nM)
Data calculated	n.p.	n.p.
Calculation of RBA	Estimated from competitive binding graph	Estimated from competitive binding graph
Test substances		
Solvent used	n.p.	n.p.
No. of samples/ dose	n.p.	n.p.
No. of times assay repeated	n.p.	n.p.
Abbreviations: n.a. = not applicable; n.p. = not provided; RBA = relative binding affinity		

Assays Using COS Cells Transfected with AR

Reference	Kemppainen et al. (1999)	Lambright et al. (2000)
Characteristics of Cells		
Cell line	COS (otherwise undefined)	COS (otherwise undefined)
Cell source	monkey kidney	monkey kidney
Source of receptor	pCMVhAR	pCMVhAR
AR source	human	human
Transfection of AR	Transient	Transient
Whole cells/cell homogenate/cytosol	whole cells	whole cells
Preparation of Cells for Assay		
Serum source	Fetal calf serum	n.p.
Serum stripping method	n.p.	n.p.
Residual androgen in serum	n.p.	n.p.
No. of treated cells/No. of cells homogenized	3.5×10^5 cells/well	n.p.
Treatment vessel used	6-well plates	n.p.
Preparation of cell homogenate	n.a.	n.a.
volume	n.a.	n.a.
buffer	n.a.	n.a.
method	n.a.	n.a.
time; temperature	n.a.	n.a.
Centrifugation of homogenate	n.a.	n.a.
Protein concentration of cytosol	n.a.	n.a.
Storage	n.a.	n.a.
Final protein concentration	n.a.	n.a.
Separation of bound hormone	Phosphate buffer saline wash	n.p.
Competitive Binding Assay		
Reference ligand	R1881	R1881
Volume and concentration of reference ligand	5 nM	5 nM
Specific activity of labelled reference ligand	n.p.	n.p.
Volume and concentration of cold ligand	10,000-fold molar excess	n.p.
Final concentration of reference ligand	5 nM	n.p.
Volume of competing ligand	n.p.	n.p.
Concentration range of competing ligand	n.p.	n.p.
Volume of cytosol	n.a.	n.p.
Volume of buffer	n.p.	n.p.
Type of buffer used	n.p.	n.p.
Replicates	n.p.	n.p.
Time of incubation	2 hr	2 hr
Temperature of incubation	37° C	37° C

Assays Using COS Cells Transfected with AR

Reference	Kemppainen et al. (1999)	Lambright et al. (2000)
Separation of ligand		
Volume and type of slurry	n.p.	n.p.
Buffer for slurry	n.p.	n.p.
Incubation time and temp	n.p.	n.p.
Time of vortexing	n.p.	n.p.
Centrifugation speed	n.p.	n.p.
Centrifugation time and temp	n.p.	n.p.
Resuspension volume and buffer for pellet	n.p.	n.p.
No. of washes	1	n.p.
Extraction of label	n.p.	n.p.
Incubation time and temperature	n.p.	n.p.
Vortexing during incubation time	n.p.	n.p.
Centrifugation time and temperature	n.p.	n.p.
Volume added for reading	n.p.	n.p.
Volume of fluor	n.p.	n.p.
Type of fluor	n.p.	n.p.
Instrumentation	n.p.	n.p.
Measurement	n.p.	n.p.
Blank without competitor	n.p.	n.p.
Reading of blank	n.p.	n.p.
Blank subtracted?	n.p.	n.p.
Range of standard curve of reference ligand	n.p.	n.p.
Nonspecific binding measured?	n.p.	n.p.
Subtraction of nonspecific binding	n.p.	n.p.
Data calculations		
Data plotted as	Scatchard plots	n.p.
Data calculated	Inhibition constant (K_i) and IC_{50}	n.p.
Calculation of RBA	From IC_{50} values	n.p.
Test substances		
Solvent used	n.p.	n.p.
No. of samples/ dose	n.p.	n.p.
No. of times assay repeated	n.p.	n.p.
Abbreviations: n.a. = not applicable; n.p. = not provided; RBA = relative binding affinity		

Assays Using COS Cells Transfected with AR

Reference	Takeo and Yamashita (2000)	Tilley et al. (1989)
Characteristics of Cells		
Cell line	COS-1	COS-1
Cell source	monkey kidney	monkey kidney
Source of receptor	rtARα expression vector	pCMVhAR
AR source	rainbow trout	human
Transfection of AR	Transient	Transient
Whole cells/cell homogenate/cytosol	cytosol	cell homogenate
Preparation of Cells for Assay		
Serum source	n.p.	n.p.
Serum stripping method	n.p.	n.p.
Residual androgen in serum	n.p.	n.p.
No. of treated cells/No. of cells homogenized	n.p.	n.p.
Treatment vessel used	n.p.	n.p.
Preparation of cell homogenate	n.p.	n.p.
volume	n.p.	2-3:1
buffer	n.p.	Tris-EDTA, pH 7.2
method	n.p.	aspiration thru 25 Ga needle
time; temperature	n.p.	n.p.
Centrifugation of homogenate	n.p.	250,000xg, 30 min
Protein concentration of cytosol	n.p.	1.5 mg/ml
Storage	n.p.	n.p.
Final protein concentration	n.p.	0.3 mg
Separation of bound hormone	n.p.	Dextran-charcoal
Competitive Binding Assay		
Reference ligand	Mibolerone	5α-Dihydrotestosterone
Volume and concentration of reference ligand	1 nM	3 nM
Specific activity of labelled reference ligand	n.p.	n.p.
Volume and concentration of cold ligand	n.p.	n.p.
Final concentration of reference ligand	n.p.	3 nM
Volume of competing ligand	n.p.	n.p.
Concentration range of competing ligand	1-1000 nM	3 - 300 nM
Volume of cytosol	n.a.	0.2 ml
Volume of buffer	n.p.	n.p.
Type of buffer used	n.p.	TEGM, pH 7.2
Replicates	n.p.	n.p.
Time of incubation	5 hr	5 hr
Temperature of incubation	4° C	4° C

Assays Using COS Cells Transfected with AR

Reference	Takeo and Yamashita (2000)	Tilley et al. (1989)
Separation of ligand		
Volume and type of slurry	dextran-charcoal, 50 µl	dextran-charcoal, 50 µl
Buffer for slurry	Tris, pH 7.2	Tris, pH 7.2
Incubation time and temp	5 min, 0° C	5 min, 0° C
Time of vortexing	n.p.	n.p.
Centrifugation speed	2000xg	2000xg
Centrifugation time and temp	10 min, 0° C	10 min, 0° C
Resuspension volume and buffer for pellet	5 ml	5 ml
No. of washes	1	1
Extraction of label	n.p.	n.p.
Incubation time and temperature	n.p.	n.p.
Vortexing during incubation time	n.p.	n.p.
Centrifugation time and temperature	n.p.	2000xg, 10 min
Volume added for reading	n.p.	n.p.
Volume of fluor	5 ml	5 ml
Type of fluor	n.p.	n.p.
Instrumentation	n.p.	n.p.
Measurement	n.p.	n.p.
Blank without competitor	n.p.	n.p.
Reading of blank	n.p.	n.p.
Blank subtracted?	n.p.	n.p.
Range of standard curve of reference ligand	n.p.	n.p.
Nonspecific binding measured?	n.p.	n.p.
Subtraction of nonspecific binding	n.p.	n.p.
Data calculations		
Data plotted as	Graphpad prism software	% DHT binding
Data calculated	n.p.	n.p.
Calculation of RBA	Estimated from competitive binding graph	Estimated from competitive binding graph
Test substances		
Solvent used	n.p.	n.p.
No. of samples/ dose	n.p.	n.p.
No. of times assay repeated	n.p.	n.p.
Abbreviations: n.a. = not applicable; n.p. = not provided; RBA = relative binding affinity		

Assays Using COS Cells Transfected with AR

Reference	Wong et al. (1995)
Characteristics of Cells	
Cell line	COS-1
Cell source	monkey kidney
Source of receptor	pCMVhAR
AR source	human
Transfection of AR	Transient
Whole cells/cell homogenate/cytosol	whole cells
Preparation of Cells for Assay	
Serum source	Fetal calf serum
Serum stripping method	n.p.
Residual androgen in serum	n.p.
No. of treated cells/No. of cells homogenized	$1x10^5$ cells/well
Treatment vessel used	12-well plates
Preparation of cell homogenate	n.a.
volume	n.a.
buffer	n.a.
method	n.a.
time; temperature	n.a.
Centrifugation of homogenate	n.a.
Protein concentration of cytosol	n.a.
Storage	n.a.
Final protein concentration	n.a.
Separation of bound hormone	Phosphate buffer saline wash
Competitive Binding Assay	
Reference ligand	R1881
Volume and concentration of reference ligand	5 nM
Specific activity of labelled reference ligand	85.5 Ci/mmol
Volume and concentration of cold ligand	100-fold molar excess
Final concentration of reference ligand	5 nM
Volume of competing ligand	n.p.
Concentration range of competing ligand	.005 -50 µM
Volume of cytosol	n.a.
Volume of buffer	n.p.
Type of buffer used	n.p.
Replicates	3
Time of incubation	2 hr
Temperature of incubation	37° C

Assays Using COS Cells Transfected with AR

Reference	Wong et al. (1995)
Separation of ligand	
Volume and type of slurry	n.p.
Buffer for slurry	n.p.
Incubation time and temp	n.p.
Time of vortexing	n.p.
Centrifugation speed	n.p.
Centrifugation time and temp	n.p.
Resuspension volume and buffer for pellet	n.p.
No. of washes	2
Extraction of label	n.p.
Incubation time and temperature	n.p.
Vortexing during incubation time	n.p.
Centrifugation time and temperature	n.p.
Volume added for reading	n.p.
Volume of fluor	n.p.
Type of fluor	n.p.
Instrumentation	n.p.
Measurement	n.p.
Blank without competitor	n.p.
Reading of blank	n.p.
Blank subtracted?	n.p.
Range of standard curve of reference ligand	n.p.
Nonspecific binding measured?	n.p.
Subtraction of nonspecific binding	n.p.
Data calculations	
Data plotted as	% [^{3}H]-R1881vs. Unlabeled ligand (µM)
Data calculated	n.p.
Calculation of RBA	Estimated from competitive binding graph
Test substances	
Solvent used	n.p.
No. of samples/ dose	n.p.
No. of times assay repeated	3
Abbreviations: n.a. = not applicable; n.p. = not provided; RBA = relative binding affinity	

Appendix A4

Assays Using MCF-7 Cells

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Assays Using MCF-7 Cells

Reference	Deckers et al. (2000)	Schoonen et al. (1995)
Characteristics of Cells		
Cell line	MCF-7	MCF-7
Source of cell line	human mammary tumor	human mammary tumor
Whole cells/cytosol	cytosol	cytosol
Preparation of Cells for Assay		
Serum source	fetal calf serum	fetal calf serum
Serum stripping method	charcoal treated serum	charcoal treated serum
Residual androgen in serum	n.p.	n.p.
No. treated cells/No. or weight of cells homogenized	1 gm cells	1 gm cells
Treatment vessel used	n.p.	n.p.
Preparation of cell homogenate		
volume	5 ml	5 ml
buffer	TrisHCl pH 7.4 + EDTA, dithioerythritol, molybdate	TrisHCl pH 7.4 + EDTA, dithioerythritol, molybdate
method	Dounce homogenizer	Dounce homogenizer
time; temperature	n.p.	n.p.
Centrifugation of homogenate	1,000,000N/kg	1,000,000N/kg
Protein concentration of cytosol	n.p.	n.p.
Storage	n.p.	n.p.
Final protein concentration	n.p.	n.p.
Competitive binding assay		
Reference ligand	5α-Dihydrotestosterone	5α-Dihydrotestosterone
Volume and concentration of reference ligand	1.9 nM	1.9 nM
Specific activity of labelled reference ligand	5.3 TBq/mmol	4070 GBq/mmol
Volume and concentration of cold ligand	n.p.	n.p.
Final concentration of reference ligand	1.9 nM	1.9 nM
Volume of competing ligand	n.p.	n.p.
Concentration range of competing ligand	0.1 - 10000 nM	0.1 - 10000 nM
Volume of cytosol	1:5 dilution	1:5 dilution
Volume of buffer	n.p.	n.p.
Type of buffer used	n.p.	n.p.
Replicates	6 or more	2
Time of incubation	overnight	overnight
Temperature of incubation	4° C	4° C

Assays Using MCF-7 Cells

Reference	Deckers et al. (2000)	Schoonen et al. (1995)
Separation of ligand		
Volume and type of slurry	dextran-charcoal	dextran-charcoal
Buffer for slurry	TrisHCl pH 7.4 + EDTA, dithioerythritol, molybdate	TrisHCl pH 7.4 + EDTA, dithioerythritol, molybdate
Incubation time and temperature	10 min, 4º C	10 min, 4º C
Time of vortexing	n.a.	n.a.
Centrifugation speed	8000N/kg	8000N/kg
Centrifugation time and temperature	5 min	5 min
Resuspension volume and buffer for pellet	n.p.	n.p.
No. of washes	n.p.	n.p.
Extraction of label	centrifugation	centrifugation
Incubation time and temperature	n.p.	n.p.
Vortexing during incubation time	n.p.	n.p.
Centrifugation time and temperature	n.p.	n.p.
Volume added for reading	n.p.	n.p.
Volume of fluor	n.p.	n.p.
Type of fluor	n.p.	n.p.
Instrumentation	Topcount microplate scintillation counter	n.p.
Measurement	n.p.	n.p.
Blank without competitor	n.p.	n.p.
Reading of blank	n.p.	n.p.
Blank subtracted?	n.p.	n.p.
Range of standard curve of reference ligand	n.p.	n.p.
Nonspecific binding measured?	yes	n.p.
Subtraction of nonspecific binding	yes	n.p.
Data calculations		
Data plotted as	n.p.	n.p.
Data calculated	specific binding	IC_{50}
Calculation of RBA	yes	yes
Test substances		
Solvent used	ethanol	ethanol
No. of samples/ dose	n.p.	n.p.
No. of times assay repeated	from 6 to 34	2
Abbreviations: n.a. = not applicable; n.p. = not provided; RBA = relative binding affinity		

Appendix A5

Miscellaneous AR Binding Assays

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Miscellaneous AR Binding Assays

Reference	Bauer et al. (1998)	Bauer et al. (2000)
Preparation of Receptor		
Animal or cell line	Prepubertal calves	Sf9 insect cells transfected with recombinant baculovirus
Source of receptor	Uterus	Human recombinant AR
Age of animals	n.p.	n.a.
When castrated	n.a.	n.a.
Diet of animals	n.p.	n.a.
Environment	n.p.	n.a.
Lighting	n.p.	n.a.
Buffer for preparation of cytosol	Tris-EDTA-glycerol-protease inhibitor, pH 7.4	n.a.
Dilution of tissue with buffer	1 to 4	n.a.
Homogenization	Ultraturrax	n.a.
Centifugation	285,000xg, 1 hr, 4° C	n.a.
Storage	-60° C	n.p.
Protein concentration of cytosol	n.p.	n.a.
Preparation of Cells for Assay		
Whole cells/ cell homogenate	n.a.	semi-purified recombinant protein
Serum source	n.a.	n.a.
Serum stripping method	n.a.	n.a.
Residual androgen in serum	n.a.	n.a.
No. treated cells/No. or weight of cells homogenized	n.a.	n.a.
Treatment vessel used	n.a.	n.a.
Preparation of cell homogenate	n.a.	n.a.
volume	n.a.	n.a.
buffer	n.a.	n.a.
method	n.a.	n.a.
time; temperature	n.a.	n.a.
Centrifugation of homogenate (time, speed, temperature)	n.a.	n.a.
Protein concentration of cytosol	n.a.	n.a.
Storage	n.a.	n.a.
Final protein concentration	n.a.	n.a.
Test chemical solvent	n.a.	n.a.
Separation of bound hormone	n.a.	n.a.
Competitive binding assay		
Reference ligand	5α-Dihydrotestosterone	5α-Dihydrotestosterone
Volume and concentration of reference ligand	4 nM	0.4 nM
Specific activity of labelled reference ligand	n.p.	4.70 TBq/mmol
ligand	n.p.	n.p.
ligand	4 nM	0.4 nM
Volume of competing ligand	n.p.	

Miscellaneous AR Binding Assays

Reference	Bauer et al. (1998)	Bauer et al. (2000)
Concentration range of competing ligand	n.p.	n.p.
Volume of cytosol	0.5 ml	0.5 ml
Volume of buffer	n.p.	n.p.
Type of buffer used	n.p.	phosphate, pH 7.2 + protease inhibitor
Replicates	n.p.	triplicate
Time of incubation	16 hr	16 hr
Temperature of incubation	0-4 C	0-4 C
Separation of ligand		
Volume and type of slurry	100 ul dextran-charcoal	dextran-charcoal
Buffer for slurry	Tris-EDTA-glycerol-protease inhibitor, pH 7.4	phosphate, pH 7.2 + protease inhibitor
Incubation time and temp	5 min, 4º C	5 min, 4º C
Time of vortexing	n.p.	n.p.
Centrifugation speed	2000xg	2000xg
Centrifugation time and temperature	15 min, 4º C	15 min, 4º C
Resuspension volume and buffer for pellet	n.p.	3 ml
No. of washes	1	n.p.
Extraction of label	n.a.	
Incubation time and temperature	n.a.	n.p.
Vortexing during incubation time	n.a.	n.p.
Centrifugation time and temperature	n.a.	n.p.
Volume added for reading	0.4 ml	n.p.
Volume of fluor	3 ml	3 ml
Type of fluor	Xylofluor	Xyloflour
Instrumentation	n.p.	n.p.
Measurement	n.p.	n.p.
Blank without competitor	n.p.	n.p.
Reading of blank	n.p.	n.p.
Blank subtracted?	n.p.	n.p.
Range of standard curve of reference ligand	n.a.	n.p.
Nonspecific binding measured?		n.p.
Subtraction of nonspecific binding	n.p.	n.p.
Data calculations		
Data plotted as	nonlinear, log progression, 4 parameters	Scatchard Plots; Sigma plot
Data calculated	Ki	Ki
Calculation of RBA	from Scatchard plot	yes
Test chemicals		
Solvent used	n.p.	n.p.
No. of samples/ dose	n.p.	3
No. of times assay repeated	n.p.	n.p.
Abbreviations: n.a. = not applicable; n.p. = not provided; RBA = relative binding affinity		

Miscellaneous AR Binding Assays

Reference	Sonnenschein et al. (1989)	Takeo and Yamashita (2000)
Preparation of Receptor		
Animal or cell line	LnCaP-FGC cells	Transfected COS-1 cells
Source of receptor	Human mutant AR from metastatic lymph node of a primary prostate adenocarcinoma	Rainbow trout ARα expression vector
Age of animals	n.a.	n.a.
When castrated	n.a.	n.a.
Diet of animals	n.a.	n.a.
Environment	n.a.	n.a.
Lighting	n.a.	n.a.
Buffer for preparation of cytosol	n.a.	n.a.
Dilution of tissue with buffer	n.a.	n.a.
Homogenization	n.a.	n.a.
Centifugation	n.a.	n.a.
Storage	n.a.	n.p.
Protein concentration of cytosol	n.a.	n.a.
Preparation of Cells for Assay		
Whole cells/ cell homogenate	cytosol	cytosol
Serum source	fetal bovine serum (5%)	n.p.
Serum stripping method	n.p.	n.p.
Residual androgen in serum	n.p.	n.p.
No. treated cells/No. or weight of cells homogenized	n.p.	n.p.
Treatment vessel used	n.p.	n.p.
Preparation of cell homogenate		n.p.
volume	n.p.	n.p.
buffer	Tris-EDTA-KCl, pH 7.4	n.p.
method	sonication	n.p.
time; temperature	n.p.	n.p.
Centrifugation of homogenate (time, speed, temperature)	105,000 x g, 45 min	n.p.
Protein concentration of cytosol	n.p.	n.p.
Storage	n.p.	n.p.
Final protein concentration	n.p.	n.p.
Test chemical solvent	n.p.	n.p.
Separation of bound hormone	n.p.	n.p.
Competitive binding assay		
Reference ligand	Testosterone	Mibolerone
Volume and concentration of reference ligand	6 nM	1 nM
Specific activity of labelled reference ligand	3.1 TBq/mmol	n.p.
ligand	n.p.	n.p.
ligand	6 nM	n.p.
Volume of competing ligand	n.p.	n.p.

Miscellaneous AR Binding Assays

Reference	Sonnenschein et al. (1989)	Takeo and Yamashita (2000)
Concentration range of competing ligand	0.5 - 5000 nM	1-1000 nM
Volume of cytosol	n.p.	n.a.
Volume of buffer	n.p.	n.p.
Type of buffer used	n.p.	n.p.
Replicates	n.p.	n.p.
Time of incubation	n.p.	5 hr
Temperature of incubation	n.p.	4 C
Separation of ligand		
Volume and type of slurry	n.p.	dextran-charcoal, 50 µl
Buffer for slurry	n.p.	Tris, pH 7.2
Incubation time and temp	n.p.	5 min, 0° C
Time of vortexing	n.p.	n.p.
Centrifugation speed	n.p.	2000xg
Centrifugation time and temperature	n.p.	10 min, 0° C
Resuspension volume and buffer for pellet	n.p.	5 ml
No. of washes	n.p.	1
Extraction of label	n.p.	n.p.
Incubation time and temperature	n.p.	n.p.
Vortexing during incubation time	n.p.	n.p.
Centrifugation time and temperature	n.p.	n.p.
Volume added for reading	n.p.	n.p.
Volume of fluor	n.p.	5 ml
Type of fluor	n.p.	n.p.
Instrumentation	n.p.	n.p.
Measurement	n.p.	n.p.
Blank without competitor	n.p.	n.p.
Reading of blank	n.p.	n.p.
Blank subtracted?	n.p.	n.p.
Range of standard curve of reference ligand	n.p.	n.p.
Nonspecific binding measured?	n.p.	n.p.
Subtraction of nonspecific binding	n.p.	n.p.
Data calculations		
Data plotted as	Cell number(10^5)/well vs. Steroid concentration (M)	Graphpad prism software
Data calculated	I_{50}	n.p.
Calculation of RBA	from I_{50} (data not presented)	Estimated from competitor binding graph
Test chemicals		
Solvent used	n.p.	n.p.
No. of samples/ dose	n.p.	n.p.
No. of times assay repeated	n.p.	n.p.
Abbreviations: n.a. = not applicable; n.p. = not provided; RBA = relative binding affinity		

Appendix B

In Vitro AR Binding Assay Protocols

B1 Protocol for Androgen Receptor Competitive Binding Assay Using Rat Prostate Cytosol
(Provided by Dr. Vickie Wilson, U.S. EPA, NHEERL, Research Triangle Park, NC and Mr. Gary Timm, U.S. EPA, Washington, DC, USA)

B2 Protocol for COS Cell Binding Assay
(Provided by Dr. Elizabeth M. Wilson, Departments of Pediatrics and of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC, USA)

B3 Protocol for Measuring Androgen-Binding Sites on Androgen Receptors or Binding Proteins
(Provided by Dr. Benjamin Danzo, Department of Obstetrics and Gynecology, Vanderbilt University School of Medicine, Nashville, TN, USA)

B4 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, and Dr. William Kelce, Pharmacia Corporation, Kalamazoo, MI, USA)

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Appendix B1

Protocol for Androgen Receptor Competitive Binding Assay Using Rat Prostate Cytosol

(Provided by Dr. Vickie Wilson, U.S. EPA, NHEERL, Research Triangle Park, NC and Mr. Gary Timm, U.S. EPA, Washington, DC, USA)

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ANDROGEN RECEPTOR COMPETITIVE BINDING PROTOCOL

RAT PROSTATE CYTOSOL*

Section 5.5 revised June 1, 2001

Final version of EPA Work Assignment 2-19 Appendix B. This slightly reformatted version incorporates editorial changes (typographic error corrections, pagination, etc.). It also contains several clarifications and standardization. For example, earlier versions contained permissive statements such as "Add 10 to 20 ml of scintillation cocktail". In this version, that step reads "Add 14 ml of scintillation cocktail". The prototype worksheet included as an example at the end of the protocol has been replaced with an example of the implemented worksheet.

This protocol was followed throughout Task 3

***** This protocol was provided in November, 2000 by the EPA as an attachment to the Statement of Work for Contract Number: 68-W-99-033 Work Assignment 2-19, " Development of Estrogen Receptor and Androgen Receptor Binding Data". The protocol has been reformatted, edited, and slightly revised as discussed and approved by the EPA.

Androgen Receptor Competitive Binding Protocol-Rat Prostate Cytosol

1. **Purpose and Applicability**-Determine ability of unknown compounds to compete with ^{3}H-ligand for binding to rat prostate homogenate.

2. **Safety and Operating Precautions**-All procedures with radioisotopes will follow the regulations and procedures as described in the Radiation Work Permit (RWP) and the Integrated Operations System (IOPS) hazard assessment summaries for the tracer laboratory. All staff working the tracer laboratory shall be DOE certified at the Radiation Worker II level.

3. **Animal Use**-The Battelle Animal Use Protocol for this assay is O-40. It and all appropriate Animal Resource Center protocols will be followed.

4. **Equipment and Materials**

4.1. Equipment

-Corning Stir/hot Plates

-Digital Pipettes

-Balance

-Polytron PT 35/10 Tissue Homogenizer

-Vacuum Concentrator

-Refrigerated General Laboratory Centrifuge

-High-Speed Refrigerated Centrifuge (up to 30,000 x *g*)

-pH Meter with Tris Compatible Electrode

-Scintillation Counter

4.2. Chemicals

-Negative Control (Corticosterone)

-Tris HCL & Tris Base

-Phenylmethylsulfonyl Fluoride (PMSF)

-Glycerol 99%+

-Sodium Molybdate

-Ethylenediaminetetraacetic acid (EDTA); Disodium salt

-Dithiothreitol (DTT)

-Potassium Chloride

-Hydroxylapatite (BIO-RAD)

-Scintillation Cocktail (Optifluor)

-Ethyl Alcohol, anhydrous

-^{3}H-R1881 (NEN)

- Radioinert R1881 (NEN)

-Triamcinolone Acetonide

-Steroids (Steraloids - recrystallized)

4.3. Supplies

-20 ml Polypropylene Scintillation Vials

-12 x 75 mm Borosilicate Glass Test Tubes

-1000 ml graduated cylinders

-500 ml Erlenmeyer flasks

-yellow (0-200 µl) pipette tips

5. Stock Preparations

5.1. Preparation of TEDG Stock Solutions

5.1.1. Add 7.444g disodium EDTA to 100 ml ddH_2O = 200mM. Store at 4ºC. Use 750 µl/100ml TEDG buffer = 1.5 mM.

5.1.2. Add 1.742 g PMSF to 100 ml ethanol = 100 mM. Store at 4ºC. Use 1.00 ml/100ml TEDG buffer = 1.0 mM.

5.1.3. Add 2.419 g sodium molybdate to 8.0 ml ddH_2O in a 10 ml volumetric flask; bring the total volume to 10 mls = 1.0 M. Store at 4ºC. Use 100µl/100ml TEDG buffer = 1.0 mM.

5.1.4. Add 15.4 mg DTT directly to 100 ml TEDG buffer the morning of the receptor isolation = 1.0 mM.

5.1.5. Add 147.24 g Tris-HCL + 8.0 g Tris base to 800mls ddH_2O in a volumetric flask; bring the final volume to 1.0 liter. Refrigerate to 4ºC and pH (using 4ºC pH standardizing solutions) the cooled solution to 7.4. Store at 4ºC. Use 1.0 ml/100 ml TEDG buffer = 10mM. (50 mM Tris = 50 ml 1 M Tris/1 L H2O)

5.1.6. Add 298.2 g KCL to 600 ml ddH_2O in a 1000 ml volumetric flask; bring the total volume to 1000 ml = 4.0 M. Store at room temperature. Use 10.0 ml per 100 ml high-salt TEDG buffer = 0.4M.

5.2. Preparation of Low-Salt TEDG Buffer (pH 7.4)

To make 100 mls of low-salt TEDG buffer add the following together in this order:

-87.15 ml ddH_2O

-1.0 ml 1M TRIS

-10.0 ml glycerol

-100 µl 1M sodium molybdate

-750 µl 200mM EDTA

-1.0 ml 100mM PMSF

-15.4 mg DTT

5.2.1. Check pH of the final solution to make sure it is 7.4 at 4ºC.

(Preparation of high salt buffer has been omitted by EPA)

5.3. Preparation of 50 mM TRIS Buffer

5.3.1. Add 50.0 ml 1.0 M TRIS to 950 ml ddH_2O. Store at 4ºC. Check pH of the final solution to make sure it is 7.4 at 4ºC.

5.4. Preparation of 60% Hydroxylapatite (HAP) Slurry

5.4.1. Shake BIO-RAD HT-GEL until all the HAP is in suspension (i.e., looks like milk).

5.4.2. The evening before the receptor extraction, pour 100 mls (or an appropriate volume) into a 100 ml graduated cylinder, parafilm seal the top and place in the refrigerator for at least 2h.

5.4.3. Pour off the phosphate buffer supernatant, and bring the volume to 100mls with 50 mM TRIS. Suspend the HAP by parafilm sealing the top of the graduated cylinder and inverting the cylinder several times. Place in the refrigerator overnight.

5.4.4. The next morning, repeat the washing steps x 2 with fresh 50 mM TRIS buffer.

5.4.5. After the last wash, add enough 50 mM TRIS to make the final solution a 60% slurry (i.e., if the volume of the settled HAP is 60 ml bring the final volume of the slurry to 100 mls with 50 mM TRIS).

5.4.6. Store at 4ºC until ready for use in the extraction.

5.5. Preparation of [^{3}H-17á-Methyl]-R1881 Stock Solutions

5.5.1. Steps 5.5.2 through 5.5.4 describe the **general** preparation, section 5.5 describes the preparation at Battelle..

5.5.2. Dilute the original 1.0 mCi/ml stock of [^{3}H-17á-methyl]-R1881 to 0.1 µM (i.e., 1 x 10^{-7} M). This is most easily accomplished by pipetting 1 µl of the stock solution for every specific activity unit (Ci/mmol) and diluting this to 10.0 mls with ethanol. Thus, if the specific activity of the stock vial is 86 Ci/mmol, then pipette 86.0 µl into an amber colored vial (i.e., R1881 is photosensitive) and add 10.0 mls ethanol to the vial; this solution is 1 x 10^{-7}M.

5.5.3. Calculation Check

5.5.3.1. 86 µl x 1.0 mCi/1000µl = 86 x 10^{-3} mCi R1881 = 86 x 10^{-6} Ci R1881

5.5.3.2. 86 x 10^{-6} Ci; 86.0 Ci/mmol = 1 x 10^{-6} mmol R1881 = 1 x 10^{-9} moles R1881

5.5.3.3. 1 x 10^{-9} moles R1881; 0.010 liters = 1 x 10^{-7} moles/liter = 0.1 µM

5.5.4. To prepare the 1 x 10^{-8}M stock simply make a 10-fold dilution of the 1 x 10^{-7} M stock (i.e., pipette 1.0 ml of the 1 x 10^{-7} M stock into a clean amber colored vial and add 9 mls ethanol = 0.01 µM).

5.5.5. **Specific:** The R1881 acquired by Battelle in January, 2001 had a specific activity of 75.2 Ci/mmol (rather than the 86 used in the example in section 5.5.1) at 1 mCi/ml. The stock solution was prepared by adding 75.2 µl of R1881 to 10 ml EtOH. This solution is 1 x 10^{-7}M. To prepare the 10^{-8} M stock, a ten-fold dilution of the 1 x 10^{-7}M stock was made by adding 1 ml of the 1 x 10^{-7}M stock to 9 ml of EtOH.

5.6. Preparation of 100X Radioinert R1881 Solutions

5.6.1. The R1881 comes as a 5.00 mg quantity. Dilute the original stock to 5.0 ml with ethanol = 3.52 mM. Take 56.82 µl and dilute to 20 ml in an amber vial with ethanol = 1 x 10^{-5} M R1881. This is the 10 µM Radioinert (cold) R1881 stock.

5.6.2. To make the 1.0 µM cold R1881 stock, pipette 2 ml of the 10 µM stock into an amber vial and dilute to 20 ml with ethanol = 1 x 10^{-6}M = 1.0 µM cold R1881 stock.

5.7. Compound Stock Preparations

5.7.1. Battelle-Sequim will supply test chemicals diluted in ethanol (200 proof) at a concentration of 3.0 x 10^{-2} M (i.e., 30 mM).

5.7.2. Note: Battelle-Sequim may determine that some chemicals are not soluble at this concentration, so adjustments will need to be made in the protocol depending upon the specific chemical. Likewise, some chemicals (e.g., CdCl) may not be soluble in ethanol at all, so appropriate modifications in this assay should be made to accommodate any change in solvent. Such changes must be documented.

5.7.3. Prepare serial dilutions of R1881 for standard curve and test chemical in ethanol to yield the Initial Concentrations as indicated in Table 1.

Table 1 Standard Curve

Standards	*Initial R1881 Concentration (Molar)*	**Final R1881 Concentration (Molar) in AR assay tube*
Negative Control	0 (Corticosterone)	1 × 10^{-4}
0	0 (EtOH)	0
NSB	3 × 10^{-5}	1 × 10^{-6}

S1	3×10^{-6}	1×10^{-7}
S2	3×10^{-7}	1×10^{-8}
S3	3×10^{-8}	1×10^{-9}
S4	3×10^{-9}	1×10^{-10}
S5	3×10^{-10}	1×10^{-11}

**When 10 µl of each standard is added to the AR assay tube, the final concentration will be as indicated when the total volume in the AR assay tube is 310 µl.*

5.7.4. Prepare serial dilutions of the test chemicals as indicated in Table 2.

Table 2 – Test Chemical Concentrations

Serial Dilutions of Test Chemical	*Initial Concentration (30 X) (Molar)*	**Final Concentration (Molar) in AR assay tube*
Concentration 1	3×10^{-4}	1×10^{-5}
Concentration 2	3×10^{-5}	1×10^{-6}
Concentration 3	3×10^{-6}	1×10^{-7}
Concentration 4	3×10^{-7}	1×10^{-8}
Concentration 5	3×10^{-8}	1×10^{-9}
Concentration 6	3×10^{-9}	1×10^{-10}
Tube 7	0 (vehicle only)	0

**Final Concentration of test chemical in assay tube when 10 µl of Initial Concentration is used in a total volume of 310 µl.*

Example for use at Battelle-Sequim:

Make stocks 30X above desired final (this accounts for the use of 10µl stock in 300µl cytosol)

4 (t) octyl phenol FW 206.33
1M = 206.33g/L
1mM= .20633mg/ml
final conc *x30mM*
1) 1mM = 6.1899mg x 2 = 12.37 mg/2 ml ethanol (100%)
2) 316µM =316µl of 1 +684µl ethanol (100%)
3) 100µM =100µl of 1 + 900µl ethanol (100%)
4) 31.6µM =100µl of 2 + 900µl ethanol (100%)
5) 10µM =100µl of 3 + 900µl ethanol (100%)
6) 3.16µM =100µl of 4 + 900µl ethanol (100%)
7) 1µM =100µl of 5 + 900µl ethanol (100%)

6. Tissue Homogenate Collection

6.1. Castrate 60-90 day old rats as per laboratory animal protocols.

6.2. 24 hours after castration, make low-salt TEDG buffers and place in an ice-water bucket.

6.3. Kill rat and excise ventral prostate. Tissue should be trimmed of fat and pooled. The weight of the pooled prostate tissue will be recorded.

6.4. Add low-salt TEDG buffer at 10ml/g tissue

6.5. Mince tissues with Metzenbaum scissors until all pieces are small 1-2mmcubes. Then homogenize the tissues at 4ºC with a Polytron homogenizer using 5-sec bursts of the Polytron. [Note: place probe of the Polytron in an ice-water bath with TEDG buffer to cool it down prior to its use for homogenization]

6.6. Transfer homogenates to pre-cooled centrifuge tubes, balance, and centrifuge at 30,000x g for 30 minutes (i.e., 15,262 rpm using JA-17/JA-21 Beckman rotors).

6.7. The supernatant is the low-salt cytosolic receptor. Pool the supernatant from all rats. Aliquot into 5ml and store -80ºC until needed for assay.

6.8. Determine the protein content for each batch of cytosol using the BioRad Protein Assay Kit (BioRad Chemical Division, Richmond, CA).

7. Assay Procedure, Day 1

7.1. Set up tubes:

7.1.1. Label 12 x 75mm glass tubes 1-90 (or if using pre-labeled tubes, note starting number). Place tubes in centrifuge tube holders following numbering scheme. See worksheet for assignment of tube numbers. 12x75 mm glass tubes

7.1.2. Add 30µl of 0.01µM [^{3}H] R1881 + 50µl Triamcinolone Acetonide (60µM stock) to ALL tubes

7.1.3. For 2 tubes, also add 100x inert R1881 (30µl of 10µM)

7.1.4. Place tubes in speed-vac and dry the tubes according to instructions. Remove when dry.

7.2. Add 10µl of compound stocks (see Table 2 for concentrations 1-7 in duplicate)

7.3. Remove aliquot of prostate cytosol and thaw on ice.

7.4. Add 300µl of cytosol to every tube ON ICE. Gently vortex and place tubes in refrigerator overnight in rotor (20hr).

7.5. Before leaving for the day, prepare the first wash of the HAP slurry as described in section 4.5 above.

7.6. Also, if necessary, label the HAP tubes and the scintillation vials to be used the following day.

8. Assay Procedure, Day 2

8.1. The following morning, wash the HAP as described in section 4.5 above, dilute with 50 mM TRIS to yield a 60% slurry, and transfer contents to a 100 ml Erlenmeyer flask. Place a stir bar in the flask and place the flask into a beaker containing ice-water; stir the HAP slurry by placing the beaker on a magnetic stir plate.

8.2. While the HAP slurry is constantly being stirred, pipette 500 µl of the HAP slurry into the assay tubes. Place these tubes in a rack in an ice-water bath prior to pipetting the HAP slurry and keep them in the ice-water bath for the remainder of the assay.

8.3. One tube should be prepared for each incubation tube (duplicate omitted by EPA).

8.4. Take the incubation tubes from the refrigerator and place them in an ice-water bath with the HAP tubes. Pipette 100 µl (duplicate omitted by EPA) from each of the incubation tubes into the appropriate pre-labeled tubes containing HAP. Repeat for all tubes. Quickly take each rack from the ice-water bath and vortex each rack of tubes using the whole-rack vortex unit. Place racks back into the ice-water bath and vortex as above every 5 minutes for 20 minutes.

8.5. Centrifuge the HAP tubes for 2-3 minutes at 4°C and 600 x *g* (i.e., 1780 rpm in a Beckman GLC refrigerated centrifuge). Place the tubes back into the rack and into the ice-water bath.

8.6. While the tubes remain in the ice-water bath, aspirate the supernatant from each tube using a 9-inch pipette connected to an aspiration apparatus as per the radiation safety protocol.

8.7. Add 2 ml of 50 mM TRIS to each tube, vortex and centrifuge at 600 x *g* as above. Place the tubes into decanting racks in an ice-water bath and decant the supernatant TRIS wash into the radiation safety container. Gently tap the tube openings on a clean adsorbent diaper, place the rack back in the ice-water bath and add 2 mls of 50 mM TRIS.

8.8. Repeat the TRIS washing procedure 3 or 4 times (to be determined empirically) keeping the tubes at 4°C at all times.

8.9. Following the last wash and decanting, add 1.5 ml of ethanol to each tube, vortex 3 times at 5 minute intervals and centrifuge the tubes at 600 x *g* for 10 minutes. Decant the supernatants into pre-labeled 20 ml scintillation vials. Add 14 ml of Optifluor scintillation cocktail and count samples using the single label DPM program with quench correction.

9. Data Processing

9.1. Concentration of Free [^{3}H]-R1881

9.1.1. Multiply the DPM in the total counts tubes by 1.8047×10^{-5}. This value will yield the free concentration (i.e., nM) of [^{3}H]-R1881 initially present in each incubation tube.

9.1.1.0.1. Calculation Check

X DPM = 4.5045×10^{-13} Ci = 5.4141×10^{-15} mmole = 5.4141×10^{-18} moles

2.22×10^{12} dpm/Ci 83.2 Ci/mmole 1000 mmole/mole0.0003 liters

= 1.8047×10^{-14} moles/liter = X (1.8047×10^{-5}) nM 1×10^{-9} moles/nmole

9.2. Calculation of Total, Nonspecific and Specific [^{3}H]-R1881 Binding

Total binding is calculated by multiplying the DPM from the tubes that contained only radiolabelled R1881 x (1.6242×10^{-2}). This value will be total binding in fmoles.

Nonspecific binding is calculated by multiplying the DPM from the tubes containing radiolabelled R1881 + 100-fold molar excess of cold R1881 x (1.6242×10^{-2}). This value will be nonspecific binding in fmoles.

Specific binding is calculated by subtracting nonspecific binding from total binding i.e., fmoles total binding - fmoles nonspecific binding = specific binding in fmoles.

9.3. Calculation Check

To get fmoles multiply the DPM values by 1.6242×10^{-2}. This is simply nM x 300, i.e.,

1.8047×10^{-5} nM x $\frac{0.0003 \text{ liter}}{1 \times 10^{-6} \text{ nmoles/fmole}}$ = 1.6242×10^{-2} fmoles

9.4. Graphical Presentation of the Data

9.4.1. Standard Curve and Test Chemical Competitive Binding Curves: Data for the standard curve and each test chemical will be plotted as the percent ^{3}H-R1881 bound versus the molar concentration. Estimates of the IC_{50}s will be determined using appropriate non linear curve fitting software such as GraphPad Prism (GraphPad Software, Inc., San Diego, CA). A Scatchard analysis may also be preformed for the standard curve using R1881 to demonstrate that the assay meets acceptable QA standards.

9.4.2. Relative Binding Affinity: The RBA for each competitor should be calculated by dividing the IC_{50} for R1881 by the IC_{50} of the competitor and expressing as a percent (e.g., RBA for R1881 =100 %).

10. References

file: `chemreceptor.sop (8/24/99)` supplied by EPA with Statement of Work for Contract Number: 68-W-99-033 Work Assignment 2-19, "Development of Estrogen Receptor and Androgen Receptor Binding Data".

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Segel, I.H. (1975) *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*. 1st Ed, John Wiley and Sons, Inc., New York, NY

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11. Example Worksheet

The first sixteen positions of the assay run are used to establish background and standards for the run. Positions 1 and 2 are the replicate "zero" vials, designated "0". Positions 3 and 4 are non specific binding vials containing cold receptor, designated "NSB". Positions 5 through 14 are the standard curve, designated "S1" through "S6". Positions 15 and 16 are the negative control, designated "Neg.".

There are twelve positions for each unknown, designated "U1", "U2", etc. After the last unknown, there are four positions for additional NSB and Neg., and four calibration positions for vials containing only the tracer and scintillation cocktail, these are designated as "Hot"

Androgen Receptor Competitive Binding Protocol-Rat Prostate Cytosol

Example Assay Worksheet Rat Androgen Receptor 5/2/2001

Person's Name here ***Num_Pts_Std_Curve:*** 6 ***Num_Test_Chem:*** 1 ***Num_Dilutions_Per_Chem:*** 6

Receptor: Rat Prostate, Lot 021401 ***Tracer:*** H-3 R1881, Lot 3363714

Position			***Competitor***	***Initial Concentration (M)***	*Tracer* ***(ul)***	*Inert Tracer* ***(ul)***	***Tri.*** *Acetate* ***(ul)***	***Speed Vac***	*Competitor* ***(ul)***	***Receptor (ul)***	*HAP* ***(ul)***	***Final Concentration (M)***
1	1	0	EtOH		30	-	50	<>	10	300	500	
2	2	0	EtOH		30	-	50	<>	10	300	500	
3	1	NSB	Inert R1881	1E-05	30	30	50	<>	-	300	500	1E-06
4	2	NSB	Inert R1881	1E-05	30	30	50	<>	-	300	500	1E-06
5	1	S1	Inert R1881	3E-06	30	-	50	<>	10	300	500	1E-07
6	2	S1	Inert R1881	3E-06	30	-	50	<>	10	300	500	1E-07
7	1	S2	Inert R1881	3E-07	30	-	50	<>	10	300	500	1E-08
8	2	S2	Inert R1881	3E-07	30	-	50	<>	10	300	500	1E-08
9	1	S3	Inert R1881	3E-08	30	-	50	<>	10	300	500	1E-09
10	2	S3	Inert R1881	3E-08	30	-	50	<>	10	300	500	1E-09
11	1	S4	Inert R1881	3E-09	30	-	50	<>	10	300	500	1E-10
12	2	S4	Inert R1881	3E-09	30	-	50	<>	10	300	500	1E-10
13	1	S5	Inert R1881	3E-10	30	-	50	<>	10	300	500	1E-11
14	2	S5	Inert R1881	3E-10	30	-	50	<>	10	300	500	1E-11
15	1	Neg.	Corticosterone	3E-03	30	-	50	<>	10	300	500	1E-04
16	2	Neg.	Corticosterone	3E-03	30	-	50	<>	10	300	500	1E-04
17	1	U1	Sample ID	3E-04	30	-	50	<>	10	300	500	1E-05
18	2	U1	Sample ID	3E-04	30	-	50	<>	10	300	500	1E-05
19	1	U1	Sample ID	3E-05	30	-	50	<>	10	300	500	1E-06
20	2	U1	Sample ID	3E-05	30	-	50	<>	10	300	500	1E-06
21	1	U1	Sample ID	3E-06	30	-	50	<>	10	300	500	1E-07
22	2	U1	Sample ID	3E-06	30	-	50	<>	10	300	500	1E-07
23	1	U1	Sample ID	3E-07	30	-	50	<>	10	300	500	1E-08
24	2	U1	Sample ID	3E-07	30	-	50	<>	10	300	500	1E-08
25	1	U1	Sample ID	3E-08	30	-	50	<>	10	300	500	1E-09
26	2	U1	Sample ID	3E-08	30	-	50	<>	10	300	500	1E-09
27	1	U1	Sample ID	3E-09	30	-	50	<>	10	300	500	1E-10
28	2	U1	Sample ID	3E-09	30	-	50	<>	10	300	500	1E-10
29	1	0	EtOH		30	-	50	<>	10	300	500	
30	2	0	EtOH		30	-	50	<>	10	300	500	
31	1	NSB	Inert R1881	1E-05	30	30	50	<>	-	300	500	1E-06
32	2	NSB	Inert R1881	1E-05	30	30	50	<>	-	300	500	1E-06
33	1	Neg.	Corticosterone	3E-03	30	-	50	<>	10	300	500	1E-04
34	2	Neg.	Corticosterone	3E-03	30	-	50	<>	10	300	500	1E-04
35	1	Hot	Scint. Cocktail		30	-	-	<>	-	-	-	
36	2	Hot	Scint.Cocktail		30	-	-	<>	-	-	-	
37	1	Hot	Scint.Cocktail		30	-	-	<>	-	-	-	
38	2	Hot	Scint. Cokctail		30	-	-	<>	-	-	-	

Appendix B2

Protocol for the COS Cell Binding 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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COS CELL BINDING ASSAY

Revised 2-06-02

1. Day 1- Monday

Plate 400,000 COS-1 cells/well of 6 well plate in 3 ml 10% bovine calf serum, DMEM-H/20 mM Hepes, glutamine, pen/strep (use stock of 2 M Hepes, pH 7.2, sterile filter)

(200,000 cells/12 well plate with 2 ml media for Scatchard analysis)

2. Day 2, prepare DNA

0.95 ml 1.08x TBS/well
2 µg AR DNA for 6 well comp binding (0.1-3 µg AR DNA for 12 well, 3 µg for GAL/VP vectors)
0.11 ml DEAE-dextran
(250 mg/50 ml, autoclaved water, sterile filter made fresh)

Aspirate media, add 1 ml DNA solution, incubate 30 min at 37˚C, aspirate media
Add 3 ml/6 well of chloroquine-media (2 ml/12 well)
Prepare 5 mg/ml chloroquine in dH_2O fresh, sterile filter, add 1 ml of 5 mg/ml chloroquine to 100 ml 10% BCS/DMEM-H, 20 mM Hepes media
Incubate 3 h at 37˚C, aspirate media
Glycerol shock 4 min at RT with 1 ml/6 well (or 12 well) of 15% glycerol in 10% BCS/DMEM-H
Aspirate, wash carefully 1X with 3 ml 1xTBS/6 well (2 ml/12 well)
Add 3 ml 10% BCS DMEM-H, incubate overnight in incubator

3. Day 3, leave in 10% serum containing media until next day

4. Day 4, aspirate (don't wash), set up tubes for binding assay:

Use 600 µl/6 well of 5 nM [^{3}H]R1881 labeling solution in serum free/phenol red free with or without 100 fold excess unlabeled R1881 for nonspecific binding control (400 µl/12 well)

For calculations, prepare 0.625 ml/well for all h and h+c wells in serum-free, phenol red-free media

To make h + c, # h+c wells x 0.625 ml, take this volume from 5 nM hot solution, add cold R1881 so final is 100 fold higher (500 nM) unlabeled R1881 with 5 nM [^{3}H]R1881

Incubate 2 hr at 37˚C (for Scatchard in 12 well, after 2 h labeling, take 100 µl for free counts)

For ligand dissociation experiment:

Add 10,000 fold excess of cold R1881 (50 µM final) in 0.1 ml serum free media (350 µM, 7X stock)

Amount to prepare: 100 µl x total # wells + 0.5 ml extra

Spread plates out in incubator, start timer, incubate at 37˚C for times indicated
Remove at indicated time, aspirate using radioactive flask; wash carefully 1X with 3 ml PBS
Aspirate to dry, harvest in 500 µl 1X sample buffer (2% SDS, 10% glycerol, 10 mM Tris, pH 6.8) for 6 or 12 well, add 4 ml scintillation fluid and count

	500 ml	final conc	4 liters
2X TBS:	8.18 g NaCl	280 mM NaCl	65.44 gr NaCl
	0.23 g KCl	6 mM KCl	1.84 g KCl
	0.147 g $CaCl_2$-$2H_2O$	2 mM $CaCl_2$	1.18 g $CaCl_2$-$2H_2O$
	0.1 g $MgCl_2$-$6H_2O$	1 mM $MgCl_2$	0.8 g $MgCl_2$-$6H_2O$
	0.128 g NaH_2PO_4-H_2O	1.8 mM NaH_2PO_4	1.02 NaH_2PO_4-H_2O
	3.03 g Tris	50 mM Tris pH 7.4	24.24 g Tris

pH to 7.4

	500 ml	4 liters	final	MW
1.08X TBS:	4.42 g NaCl	35.34 g NaCl	51.2 mM NaCl	58.44
	0.121 g KCl	1.0 g KCl	3.24 mM KCl	74.56
	0.08 g $CaCl_2$-$2H_20$	0.64 g $CaCl_2$-$2H_20$	1.08 mM $CaCl_2$	147.02
	0.055 g $MgCl_2$-$6H_20$	0.439 g $MgCl_2$-$6H_20$	0.54 mM $MgCl_2$	203.3
	0.067 g NaH_2PO_4-H_20	0.54 g NaH_2PO_4-H_20	0.972 mM NaH_2PO_4	137.99
	1.636 g Tris	13.09 g Tris	27 mM Tris pH 7.4	121.14

pH to 7.4
or 270 ml 2XTBS + 230 ml H_20 = 1.08xTBS

Appendix B3

Protocol for Measuring Androgen-Binding Sites on Androgen Receptors or Binding Proteins

(Provided by Dr. Benjamin Danzo, Department of Obstetrics and Gynecology, Vanderbilt University School of Medicine, Nashville, TN, USA)

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Protocol for Measuring Androgen-Binding Sites on Androgen Receptors or Binding Proteins

1. Source of androgen receptors:

Androgen receptors are present in tissues of the male reproductive tract, for example, the prostate, epididymis, and seminal vesicle. In intact adult males, the binding site on the androgen receptors is occupied by endogenous androgen, therefore, it is necessary that they be castrated to eliminate endogenous androgens, freeing the androgen binding-site for occupation by exogenous radiolabeled ligand used in the assay. While the epididymis contains androgen receptor, it also contains androgen-binding protein (ABP), which is present at a concentration at least 10-times greater than that of the receptor. Special precautions are necessary when assaying androgen receptor in the presence of ABP, therefore, it is suggested that the epididymis not be used as a routine source of androgen receptor. A good source of androgen receptor is the prostate of adult rats that have been castrated for 24-48 hr. In sexually immature male animals, the binding site on the androgen receptors is not occupied by endogenous hormone, therefore, reproductive tract tissues of immature males provide a good source of androgen receptor. We successfully have used frozen prostates from 21 day-old rats, purchased from Harlan Bioproducts for Science, Indianapolis, IN, as a source of androgen receptor. Androgen receptors are also present in female reproductive tract tissues, for example, the chicken oviduct, and the uterus and vagina of most mammals. Thus, these tissues are also potential sources of androgen receptor for assay purposes.

2. Preparation of Cytosol:

a. Weigh the androgen receptor-containing tissues, mince thoroughly, on ice, using razor blades or scissors [1]. If using frozen tissues, pulverize in a mortar and pestle. Transfer the minced (or pulverized) tissue to an appropriately sized cold test tube and add ice cold TE buffer(10mM Tris-HCl, pH 7.5, 1.0 mM EDTA) at a ratio of 1:10 or 1:4 weigh/volume to the minced tissue. Keep samples on ice. It is recommended that a ratio of 1:4, or even 1:2, be tried initially. You will be diluting the cytosol later in the assay and you want to make sure that you have enough receptor present to detect. Once you become familiar with the amount of receptor in the cytosol from a given source, you can standardize your tissue to buffer ratio. As a precaution against proteolysis of the androgen receptor, you may wish to add a cocktail of protease inhibitors to the buffer, for example, 2 mM PMSF, 10 ug/ml antipain, 10 mM molybdate, 5 mM leupeptin [2, 3]. If you are planning to freeze the cytosol for later use, homogenize in TE buffer containing 10% v/v glycerol.

b. Homogenize the tissues in the above buffer using a Polytron (Brinkman Instruments, Westbury , NY) in a cold room (cool Polytron before use) or use cold glass-glass or glass-teflon homoginizers.

c. Centrifuge the homogenate at about 200,000 x g for 1 hr in a refrigerated centrifuge—the supernatant is the cytosol. Pour off and save cytosol for the assay—keep on ice.

3. The "binding check":

The purpose of the binding check is to determine how much cytosol you will need to use in the assay to obtain an androgen receptor measurement that is clearly above background.

a. Decide on the volumes of cytosol that you wish to test, for example, 10 ul, 50 ul, 200 ul.

b. We want the final volume of the assay to be 500 ul, so set up as follows in an ice bath, add components to the tubes in the order shown:

Tube No.*	Vol. of TE	Vol oflabel**	Vol. ofcold***	Vol. of cytosol
1-2	440 ul	50 ul	-------	10 ul
3-4	340 ul	50 ul	100 ul	10 ul
5-6	400 ul	50 ul	--------	50 ul
7-8	300 ul	50 ul	100 ul	50 ul
9-10	250 ul	50 ul	------	200 ul
11-12	150 ul	50 ul	100 ul	200 ul
13-14	450 ul	50 ul	------	------

*We typically use 12 x 75 mm borosilicate tubes.

**To standardize the assay, we dilute the label ([^{3}H]5α-DHT, 130 Ci/mmole, Dupont/NEN, Boston, MA) with TE so that when 50 ul of it are added to the assay tubes, the appropriate concentration of radiolabeled steroid (approx. 7 nM) will be present (working solution).

***This is the volume of unlabeled 5α-DHT at 1 ng/ in TE, will completely inhibit binding of label to the receptor.

c. Incubate the samples on ice for 2 hr; binding equilibrium may not have occurred during this time, but you will probably have enough binding to see what volume of cytosol to use. If not, incubate longer, for example 4 hr or overnight.

d. At the conclusion of the incubation add 0.5 of ice-cold dextran-coated charcoal to each tube, vortex each tube for 10 sec, then incubate all tubes on ice for 10 min [1]. When the incubation time is up, centrifuge the tubes for 10 min at 1500 x g in a swinging- bucket- type rotor at 4 C. When centrifugation is completed, carefully, but swiftly decant the supernatant from each tube into a separate scintillation vial, add scintillation fluid that is designed for aqueous samples, and count. The decanting process soon becomes almost innate—shortly, you will have less than a

1% variance among replicates. Alternatively, you may use a pipette to aspirate a specific volume of each supernatant.

e. Determining your results:
1) Average the duplicates 2) Subtract the duplicates containing unlabeled hormone from those that contained labeled hormone only. This will give you the amount of specific binding in cpm; appropriate calculations will yield fmol or pmol of bound label. This specifically bound 5α-DHT is presumed to be bound by the androgen receptor. One or more of the volumes of cytosol used should give you the amount to be used for measuring the androgen receptor. 3) The tubes containing only label and buffer give you the true background, that is, the amount of label not adsorbed by the charcoal. This number should be low--500-1500 cpm. If the background is higher than this, reduce the amount of label added per tube. This background number also gives an indication of the quality of the labeled preparation; as the solution of labeled hormone ages, the background increases. To reduce the occurrence of degradation of the labeled compound, store the stock solution (in ethanol as it comes from Dupont or diluted with additional ethanol) at –20 C and only make up enough working solution (stock solution diluted in TE) to last for a few days. Store the working solution at 4 C and keep on ice while in use. The stock solution stored under these condition should last at least a year without significant signs of degradation.

f. The "binding check" seems to be a tedious procedure, and it is, but you should do it to get an estimate of how much receptor is in a given tissue. Once you know this, you can use cytosol from that tissue source in the future without having to do a binding check.

4. Competitive Binding Assay:

a. Set up the assay essentially as shown for the "binding check", however, for the assay we always use triplicate determinations and incubate on ice or at 4 C for 4 hr or overnight. For the assay a standard curve is set up in which a fixed concentration of labeled hormone and a fixed amount of cytosol (receptor) is used together with varying concentrations of unlabeled hormone—add cytosol last! For example:

Tube No.	Vol. of TE	Vol of label*	Vol. of cold**	Vol. of cytosol
1-3	250 ul	50 ul	-----	200 ul
4-6	240 ul	50 ul	0.1 ng (10ul, 0.1ng.ul)	200 ul
7-9	200 ul	"	0.5 ng(50 ul ")	"
10-12	240 ul	"	1.0 ng(10ul, 0.1 ng/ul)	"
13-15	230 ul	"	2.0 ng(20ul ")	"
16-18	200 ul	"	5.0 ng(50ul ")	"
19-21	150 ul	"	10ng(100ul ")	"
22-24	200 ul	"	50 ng (50ul, 1 ng/ul)	"
25-27	150 ul	"	100 ng (100ul, ")	"
28-30	450 ul	"	-------	-----

*Using labeled 5α-DHT at 7 nM
** Using unlabeled 5α-DHT in TE buffer.

b. As with the binding check: average triplicates, subtract the value of the average of tubes 25-27 from the average of the other samples to yield specific binding.

c. Plotting: Calculate the percentage of control of each concentration of inhibitor, using the value of the samples having no competitor added as 100%, and plot against the log of the unlabeled hormone concentration—this will yield a sigmoid curve.

d. When using competitors other than unlabeled 5α-DHT, for example environmental toxicants, set up assays as for the standard curve and plot the resulting data in the same manner. Remember that steroid hormone receptors have affinities for toxicants that are orders of magnitude lower than for the physiological hormones and plan to use concentrations of competitors to reflect this. We make up stock solutions of toxicants DMSO. The toxicant stocks are diluted with TE to make the working solutions. Higher concentrations of toxicants may come out of solution at 4 C. To minimize this, add cytosol to assay tubes that are at room temperature, vortex, and then add to the ice bath.

e. Calculating the RBA (relative binding affinity): Use the standard curve, determine the concentration of unlabeled 5α-DHT that causes a 50% inhibition of binding of radiolabeled 5α-DHT (IC_{50}). Use the competition curve, calculate the concentration of unlabeled toxicant, etc. that causes a 50% inhibition of radiolabeled 5α-DHT. Divide the IC_{50} of the physiological ligand by the IC_{50} of the toxicant and multiply by 100 to obtain the RBA [4].

5. Costs

a. The [^{3}H]5α-DHT is the most expensive item with 250 uCi costing approximately $500.00. This is enough radioactivity to set up about 4000 tubes.
b. Frozen prostates from 21 day-old rats are about $4.00 each. To do a standard curve and a competition curve could take 25-30 prostates. A less expensive source of androgen receptor is the 24-hr castrated adult rats, approximate cost $30.00.
c. The costs of unlabeled 5α-DHT, charcoal, dextran, and reagents for buffers is negligible.
d. Technical costs: It would take about 1 hr to prepare the solutions and reagents necessary for the assay. A technician can set up the 57 tubes required for the two curves in less than 30 min. Another 30 min is necessary for adding of charcoal to the assay tubes, centrifuging them, decanting the supernatants, adding the scintillation fluid to the assay vials, and placing the vials in the scintillation counter. Calculations and plotting takes about 1 hr. Total technician time—approximately 3 hr.
e. The total cost for the two curves as shown above would be about $175 (high end) or about $85 (low end).

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Appendix B4

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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Technical Perspective on the U.S. EPA Endocrine Disruptor Screening Program:

In Vitro EDSTAC Guideline Protocols[1]

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

[1] 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.

- 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 steroidogenisis 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 Receptor Binding Assays

III. A. Purpose & General Design

The purpose of this procedure is to screen chemicals for the capacity to compete for binding to mammalian nuclear hormone receptors. This technique has been used in the mechanistic evaluation of chemical-receptor interactions. It is assumed that if a test material binds to a receptor with some degree of affinity, then some biological activity on the part of the chemical is usually inferred.

The binding of ligand to the receptor (i.e., specific binding) is a saturable process. Unsaturable binding of ligand is called nonspecific binding and is due to ligand binding to non-receptor elements in a preparation. Total binding is defined as the sum of specific (saturable) and non-specific (unsaturable binding):

Total binding = Saturable binding + Unsaturable binding.

Total and nonspecific binding are determined empirically, while specific binding is calculated as their difference. Total ligand binding is determined by incubating the receptor preparations with increasing concentrations of radiolabelled ligand (^{3}H, ^{125}I etc) for sufficient time to reach equilibrium. The total bound ligand (i.e., saturable + unsaturable binding) is separated from free ligand and quantified using liquid scintillation spectrometry. Nonspecific binding is determined exactly as above except that a 100-fold molar excess of radioinert ligand is included in all

incubations, together with the increasing concentrations of radiolabelled ligand (i.e., binding of radiolabelled ligand in the presence of a 100-fold molar excess of radioinert ligand represents nonsaturable binding). Specific binding is defined and calculated as the difference of total binding and non-specific binding:

Specific binding = Total binding - Nonspecific binding

Specific binding is analyzed graphically via Scatchard analysis to determine the K_d and B_{max}. Radiolabelled 17β-estradiol and methyltrienolone (R1881) are generally recommended for use as ligands for the estrogen and androgen receptors, respectively.

The general protocol followed herein is based on the use of isolated mammalian receptor preparations as currently being pursued by the National Center for Toxicology Research (NCTR) as part of their Quantitative Structure Activity Relationship (QSAR) modeling effort (Blair *et al.,* 2000). This methodology is recommended as the standard that would be validated under an ICCVAM process. The use of recombinant or purified receptors is not precluded once proper validation exercises are performed against the standard procedure so as to ensure equivalency of the technique in terms of precision, reproducibility and sensitivity.

III. B. General Methodology

The receptor protein to be used in the receptor binding assays should be initially characterized by determining the apparent K_d for endogenous ligand binding (i.e., androgen or estrogen) and the maximum number of binding sites/tube (B_{max}) in the receptor preparation. These objectives are normally accomplished by completing an initial Scatchard analysis on each receptor preparation. Once the receptor preparation has been characterized it can be used to assess the ability of test chemicals to displace endogenous ligand from the receptor in binding assays. Appropriate performance criteria will need to be established, for example receptor Kd's in the 0.1-1.0 nM range.

For the purposes of screening test chemicals, an initial three point assay at zero (vehicle) and two concentrations, at the upper solubility limit and 2 log concentrations below is recommended. Chemicals that inhibit receptor binding by 50% (IC_{50}) or more at either of these concentrations in at least two of three replicate assays should be considered positive (i.e., able to bind the respective nuclear hormone receptor and displace endogenous ligand). In these three point binding assays the concentration of radiolabelled ligand is held constant at a value equal to its K_d (determined above) and competing test chemical is added with and without a 100-fold molar excess of radioinert ligand (nonspecific binding).

Alterations in nonspecific binding by test chemical reflect possible direct interference of the test chemical with the assay (i.e., protein denaturation, precipitation, etc) and should preclude an assessment of the test chemical on nuclear hormone receptor binding. Triplicate analyses using a positive control test chemical (concentrations of a chemical known to inhibit receptor binding by 90% or more) should be included in every screening assay for quality control.

Positives should be further assessed using relative binding affinity experiments to more precisely define the dose-response relationship between test chemical concentration and inhibition of receptor binding. Relative binding affinity (RBA) assays determine in a quantitative manner the relative ability of test chemicals to compete with radiolabelled ligand. The ligand is held constant at concentrations equal to its K_d for binding to the nuclear hormone receptor and competing test chemical is added with and without a 100-fold molar excess of radioinert ligand (nonspecific binding). Concentrations of test chemicals used in RBA assays should be deliberately broad ranging from 10 pM up to 25 uM (or the upper limit of solubility of the chemical in the receptor preparation) in 10-fold concentration increments.

Specific binding is then calculated by subtracting nonspecific from total binding at each concentration and the data are plotted in a line graph. Specific binding (% total binding that occurs in the absence of added chemical) is plotted on the ordinate vs log dose of test chemical on the abscissa. The IC_{50} value is calculated as the concentration of test chemical that displaces 50% of the radiolabelled ligand from the receptor.

$$RBA = IC_{50} \text{ Test chemical} / IC_{50} \text{ Radioinert ligand} \times 100.$$

RBA values of test chemicals can be compared to determine relative potency. RBA values should be compared only when the slopes of the RBA data curves between 20 and 80 percent of the maximal response are parallel. Non-parallel slopes suggest atypical interactions of ligand and receptor; binding by these compounds should be evaluated separately for the presence of different interfering mechanisms, which may preclude the use of receptor binding assays.

III. C. Data Evaluation & Assay Pass-Fail Criteria

- IC_{50} and RBA values for each test chemical and the positive controls 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.

- Chemicals that inhibit receptor binding by 50% (IC_{50}) or more in at least two of three replicate assays will be considered positives.

- The percent coefficient of variation (%CV) of replicate samples at each concentration of test or control chemical cannot exceed 20% in any assay as per GLP. 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 positive control test chemical must reduce radiolabelled ligand binding by at least 90% within a 20% CV or the assay will be considered unacceptable.

- Scatchard analyses for each receptor preparation should be completed and the calculated K_d and B_{max} clearly indicated and within prescribed limits.

III. D. Limitations

Several limitations of receptor binding assays should be recognized:

- Agonist and antagonist activity cannot be discriminated using receptor binding assays.
- Positive results may occur *in vitro* at concentrations that far exceed those that are caable of existing *in vivo*.
- Only receptor-ligand interactions are assessed.

Furthermore, as part of a Tier I testing scheme if data from a validated *in vitro* gene transcriptional activation assay (discussed below) is already available, (based on that assay's requirement for receptor binding), there should generally be no need for the performance of the hormone receptor binding assays.

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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 Binding Assays:

Information Sorted by Substance Name

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Information Sorted by Substance Name

Substance Name	Synonyms	CASRN	Chemical Class	Product class
4-Acetoxy-4-androstene-3,17-dione	4-(Acetyloxy)androst-4-ene-3,17-dione	61630-32-8	Steroid, nonphenolic	
Allyltrienolone			Steroid, nonphenolic	Pharmaceutical
Androstanediol	5α-Androstane-3α, 17β-diol	1852-53-5	Steroid, nonphenolic	Pharmaceutical
5α-Androstane-3β,17β-diol	Androstan-3-ol, 17-((2-aminoethyl)methylamino)-, (3β, 5α,17β)-	126061-67-4	Steroid, nonphenolic	Pharmaceutical
4-Androstenedione	4-Androstene-3,17-dione; delta-4-Androstenedione	63-05-8	Steroid, nonphenolic	Natural product
Atrazine	1,3,5-Triazine-2,4-diamine, 6-chloro-N-ethyl-N'-(1-methylethyl)-	1912-24-9	Triazine	Pesticide
Bicalutamide	Casodex, ICI 176,334	90357-06-5	Anilide; Nitrile	Pharmaceutical
2,2-Bis-(*p* -hydroxyphenyl)-1,1,1-trichloroethane	1,1,1-Trichloro-2,2-bis(4-hydroxyphenyl)ethane	2971-36-0	Organochlorine	Pesticide
Boldenone	1,2-Dehydrotestosterone	846-48-0	Steroid, nonphenolic	Pharmaceutical
Canrenone	17-Hydroxy-3-oxo-17α-pregna-4,6-diene-21-carboxylic acid gamma-lactone	976-71-6	Steroid, nonphenolic	Pharmaceutical
Chlormadinone acetate	CMA	302-22-7	Steroid, nonphenolic	Pharmaceutical
11β-Chloromethyl estradiol	Org 4333	71794-60-0	Steroid, phenolic	
Cimetidine	1-Cyano-2-methyl-3-(2-(((5-methyl-4-imidazolyl)methyl)thio)ethyl)guanidine	51481-61-9	Amidine; Imidazole	Pharmaceutical
Corticosterone	17-Deoxycortisol; 11β, 21-Dihydroxyprogesterone	50-22-6	Steroid, nonphenolic	Pharmaceutical
Cortisol	Hydrocortisone	50-23-7	Steroid, phenolic	Pharmaceutical
Cyanoketone	2α-Cyano-4,4,17α-trimethyl-17β-hydroxy-5-androsten-3-one	4248-66-2	Steroid, nonphenolic; Nitrile	
Cyproterone acetate	1,2α-Methylene-6-chloro-(sup 4,6)-pregnadiene-17α-ol-3,20-dione 17α-acetate	427-51-0	Steroid, nonphenolic	Pharmaceutical
Danazol	17α-2,4-Pregnadien-20-yno(2,3-d)isoxazol-17-ol	17230-88-5	Steroid, nonphenolic	Pharmaceutical
***p,p′* -DDD**	1,1-Dichloro-2,2-bis(*p* -chlorophenyl) ethane	72-54-8	Organochlorine	Pesticide
***p,p′* -DDE**	4,4'-DDE	72-55-9	Organochlorine	Pesticide
***o,p′* -DDT**	2-(*o* -Chlorophenyl)-2-(*p* -chlorophenyl)-1,1,1-trichloroethane	789-02-6	Organochlorine	Pesticide
***p,p′* -DDT**	1,1,1-Trichloro-2,2-bis(*p* -chlorophenyl)ethane	50-29-3	Organochlorine	Pesticide
Dehydroepiandrosterone	Prasterone	53-43-0	Steroid, nonphenolic	Pharmaceutical
15-Dehydroetonogestrel			Steroid, nonphenolic	
15-Dehydronorethisterone			Steroid, nonphenolic	
Desogestrel	13-Ethyl-11-methylene-18,19-dinor-17α-pregn-4-en-20-yn-17-ol	54024-22-5	Steroid, nonphenolic	Pharmaceutical

Information Sorted by Substance Name

Substance Name	Synonyms	CASRN	Chemical Class	Product class
Dexamethasone	(11β,16α)-9-Fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione	50-02-2	Steroid, nonphenolic	Pharmaceutical
Diazoxide	7-Chloro-3-methyl-2H-1,2,4-benzothiadiazine 1,1 dioxide	364-98-7	Benzothiadiazine	Pharmaceutical
3,4-Dichloroacetanilide	Acetanilide, dichloro-	31620-87-8	Anilide	Pesticide metabolite
3,4-Dichloroaniline	1-Amino-3,4-dichlorobenzene	95-76-1	Aniline	Chemical intermediate
3',5'-Dichloro-2-hydroxy-2-methylbut-3-enanilide	Vinclozolin metabolite M2	16776-82-1	Organochlorine	Pesticide metabolite
2-[[3,5-(Dichlorophenyl)carbamoyl]oxy]-2-methyl-3-butenoic acid	Vinclozolin metabolite M1	119209-27-7	Organochlorine	Pesticide metabolite
1-(3,4-Dichlorophenyl)urea	Urea, (3,4-dichlorophenyl)-	2327-02-8	Urea	Pesticide metabolite
Dieldrin		60-57-1	Organochlorine	Pesticide
Diethylstilbestrol	DES	56-53-1	Stilbene	Pharmaceutical
Dihydrospirorenone	Drospirenone	67392-87-4	Steroid, nonphenolic	Pharmaceutical
5α-Dihydrotestosterone	Dihydrotestosterone; Androstanolone; Stanolone	521-18-6	Steroid, nonphenolic	Pharmaceutical
5β-Dihydrotestosterone	17β-Hydroxy-5β-androstan-3-one	571-22-2	Steroid, nonphenolic	Pharmaceutical
Diphenylhydantoin	Phenytoin	57-41-0	Imidazole	Pharmaceutical
Diuron		330-54-1	Urea	Pesticide
DTIB	4-[4,4-Dimethyl-3-(4-hydroxybutyl)-5-oxo-2-thioxo-1-imidazolidinyl]-2-iodobenzonitrile		Imidazole	
Epitestosterone	(17α)-17-Hydroxyandrost-4-en-3-one	481-30-1	Steroid, nonphenolic	Natural product
17β-Estradiol	Estradiol	50-28-2	Steroid, phenolic	Pharmaceutical
Estriol	Estratriene-3,16α,17β-triol	50-27-1	Steroid, phenolic	Pharmaceutical
Estrone	Estra-1,3,5(10)-trien-17-one, 3-hydroxy-	53-16-7	Steroid, phenolic	Pharmaceutical
17α-Ethinyl estradiol	17α-Ethinylestradiol	57-63-6	Steroid, phenolic	Pharmaceutical
Etonogestrel	3-Keto-desogestrel	54048-10-1	Steroid, phenolic	Pharmaceutical
Fluoxymesterone		76-43-7	Steroid, nonphenolic	Pharmaceutical
Flutamide	4'-Nitro-3'-trifluoromethylisobutyranilide	13311-84-7	Anilide	Pharmaceutical
Gestodene	Gestoden	60282-87-3	Steroid, nonphenolic	Pharmaceutical
2,2',4,4',5,5'-Hexachlorobiphenyl	PCB 153; 2,2', 4,4', 5,5'-Hexachloro-1,1'-biphenyl	35065-27-1	Polychlorinated biphenyl	Dielectric fluid
δ-Hexachlorocyclohexane		319-86-8	Organochlorine	Pesticide
γ-Hexachlorocyclohexane	Lindane	58-89-9	Organochlorine	Pesticide

Information Sorted by Substance Name

Substance Name	Synonyms	CASRN	Chemical Class	Product class
4-Hydroxyandrostenedione	Formestane; 4-Hydroxyandrost-4-ene-3,17-dione	566-48-3	Steroid, nonphenolic	Pharmaceutical
Hydroxyflutamide	Sch 16423; 2-Hydroxy-2-methyl-N-(4-nitro-3-(trifluoromethyl)phenyl)propanamide	52806-53-8	Anilide	Pharmaceutical metabolite
Hydroxylinuron			Urea	Pesticide metabolite
17α-Hydroxyprogesterone	17-Hydroxypregn-4-en-3,20-dione	68-96-2	Steroid, nonphenolic	Pharmaceutical
Kepone	Chlordecone	143-50-0	Organochlorine	Pesticide
11-Ketotestosterone		564-35-2	Steroid, nonphenolic	Natural product
Levonorgestrel	(-)-13-Ethyl-17-hydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one	797-63-7	Steroid, nonphenolic	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
Megestrol acetate	17-Acetoxy-6-methylprenga-4,6-diene-3,20-dione	595-33-5	Steroid, nonphenolic	Pharmaceutical
Melengestrol acetate	MGA; 17α-Acetoxy-6-methyl-16-methylenpregna-4,6-dien-3,20-dione	2919-66-6	Steroid, nonphenolic	Pharmaceutical
Melengestrol acetate-metabolite 10			Steroid, nonphenolic	Pharmaceutical metabolite
Melengestrol acetate-metabolite 6			Steroid, nonphenolic	Pharmaceutical metabolite
Melengestrol acetate-metabolite 7			Steroid, nonphenolic	Pharmaceutical metabolite
Methoxychlor	Benzene, 1,1'-(2,2,2-trichloroethylidene)bis(4-methoxy-	72-43-5	Organochlorine	Pesticide
1-Methylandrosta-1,4-diene-3,17-dione	Atamestane	96301-34-7	Steroid, nonphenolic	Aromatase inhibitor
11-Methylene-15-dehydronorethisterone			Steroid, nonphenolic	
11-Methylenenorethisterone			Steroid, nonphenolic	
Methyltestosterone		58-18-4	Steroid, nonphenolic	Pharmaceutical
Methyltrienolone	R1881; 17α-Methyl-17β-hydroxy-estra-4,9,11-trien-3-one	965-93-5	Steroid, nonphenolic	Pharmaceutical
Mibolerone	7α,17α-Dimethyl-19-nortestosterone	3704-09-4	Steroid, nonphenolic	Pharmaceutical
Mifepristone	RU-486	84371-65-3	Steroid, nonphenolic	Pharmaceutical
Moxestrol	11β-Methoxyethinylestradiol; R2858	34816-55-2	Steroid, phenolic	Pharmaceutical
MSD L-642,022			Steroid, nonphenolic	
MSD L-642,317			Steroid, nonphenolic	

Information Sorted by Substance Name

Substance Name	Synonyms	CASRN	Chemical Class	Product class
Nilutamide	RU 23908; Anandron; Nilandron	63612-50-0	Imidazole	Pharmaceutical
Nonylphenol	2,6-Dimethyl-4-heptylphenol, (*o* and *p*)	25154-52-3	Phenol	Chemical intermediate
Norethisterone	Norethindrone	68-22-4	Steroid, nonphenolic	Pharmaceutical
Norethisterone acetate	Norethindrone acetate	51-98-9	Steroid, nonphenolic	Pharmaceutical
Norgestrel	13-Ethyl-17β-hydroxy-18,19-dinor-pregn-4-en-20 yn-3-one	6533-00-2	Steroid, nonphenolic	Pharmaceutical
19-Nortestosterone	19-NT; Nandrolone	434-22-0	Steroid, nonphenolic	Pharmaceutical
ORG 2058	16α-Ethyl-21-hydroxy-19-norpregn-4-ene-3,20-dione	24320-06-7	Steroid, nonphenolic	
ORG 30659			Steroid, nonphenolic	
Oxandrolone		53-39-4	Steroid, nonphenolic	Pharmaceutical
P1	Vinclozolin metabolite P1		Organochlorine	Pesticide metabolite
2,2',4',5,5'-Pentachloro-4-biphenylol			Polychlorinated biphenyl	
Pentachlorophenol	1-Hydroxy-2,3,4,5,6-pentachlorobenzene	87-86-5	Organochlorine	Pesticide
Potassium canrenoate	Aldadiene-potassium	2181-04-6	Steroid, nonphenolic	Pharmaceutical
Pregnenolone	3β-Hydroxypregn-5-en-20-one	145-13-1	Steroid, nonphenolic	Pharmaceutical
Procymidone	N-(3',5'-Dichlorophenyl)-1,2-dimethylcyclopropane-1,2-dicarboximide	32809-16-8	Imide	Pesticide
Progesterone	Pregn-4-ene-3,20-dione	57-83-0	Steroid, nonphenolic	Pharmaceutical
Promegestone	R 5020; 17,21-Dimethyl-19-nor-4,9-pregnadiene-3,20-dione	34184-77-5	Steroid, nonphenolic	Pharmaceutical
17α-Propylmesterolone		79243-67-7	Steroid, nonphenolic	
R 2956	17β-Hydroxy-2α,2β,17α-trimethyl-8α-estra-4,9,11-trien-3-one	42438-88-0	Steroid, phenolic	Pharmaceutical
RU 56187	4-(3,4,4-Trimethyl-5-oxo-2-thioxo-1-imidazolidinyl)-2-(trifluoromethyl)benzonitrile	143782-25-6	Imidazole; Nitrile	Pharmaceutical
RU 57073	4-[4,4-Dimethyl-3-(2-hydroxyethyl)-5-oxo-2-thioxo-1-imidazolidinyl]-2-trifluoromethyl-benzonitrile		Imidazole; Nitrile	
RU 59063	4-[4,4-Dimethyl-3-(4-hydroxybutyl)-5-oxo-2-thioxo-1-imidazolidinyl]-2-trifluoromethylbenzonitrile	155180-53-3	Imidazole; Nitrile	Pharmaceutical
Spironolactone	17-Hydroxy-7α-mercapto-3-oxo-17α-pregn-4-ene-21-carboxylic acid, gamma-lactone acetate	52-01-7	Steroid, nonphenolic	Pharmaceutical
Testolactone	1,2-Didehydrotestololactone	968-94-3	Steroid, nonphenolic	Pharmaceutical

Information Sorted by Substance Name

Substance Name	Synonyms	CASRN	Chemical Class	Product class
Testosterone		58-22-0	Steroid, nonphenolic	Pharmaceutical
17α-Trenbolone	17α-TbOH; 17α-Hydroxy-estra-4,9,11-trien-3-one	80657-17-6	Steroid, nonphenolic	Pharmaceutical metabolite
17β-Trenbolone	17β-TbOH; 17β-Hydroxy-estra-4,9,11-trien-3-one	10161-33-8	Steroid, nonphenolic	Pharmaceutical
Trendione	TbO; Estra-4,9,11-trien-3,17-dione	4642-95-9	Steroid, nonphenolic	Pharmaceutical metabolite
Triamcinolone acetonide	9α-Fluoro-11β,21-dihydroxy-16α,17α-isopropylidenedioxypregna-1,4-diene-3,20-dione	76-25-5	Steroid, nonphenolic	Pharmaceutical
Vinclozolin	3-(3,5-Dichlorophenyl)-5-ethenyl-5-methyl-2,4-oxazolidinedione	50471-44-8	Organochlorine	Pesticide

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Appendix D

Substances Tested in the *In Vitro* AR Binding Assays

D1 Information Sorted by Substance Name, Assay, and Reference Androgen

D2 References

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Appendix D1

Substances Tested in the *In Vitro* AR Binding Assays

Information Sorted by Substance Name, Assay and Reference Androgen

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Information Sorted by Substance Name, Assay, and Reference Androgen

Assay*	Reference Androgen **	Substance	CASRN†	Purity (%)††	IC_{50} (µM)††	K_i (µM)††	Ki (µM) SEM††	HDT (µM)†††	RBA	log RBA	Reference
HGF	DHT	4-Acetoxy-4-androstene-3,17-dione	61630-32-8		12				0.02	-1.69897	Breiner et al. (1986)
rhAR	DHT	Allyltrienolone							75.42	1.87749	Bauer et al. (2000)
RPC	DHT	Androstanediol	1852-53-5		0.8				2	0.30103	Wilson and French (1976)
LnCaP cytosol	T	Androstanediol	1852-53-5						1.2	0.07918	Sonnenschein et al. (1989)
REC	R1881	Androstanediol	1852-53-5		3.7	1.85			0.054	-1.26761	Waller et al. (1996)
RECNR	R1881	Androstanediol	1852-53-5						1.4	0.14613	Kelce et al. (1994)
RPC	M	Androstanediol	1852-53-5		0.2337				0.57	-0.24413	Schilling and Liao (1984)
LnCaP cytosol	T	5α-Androstane-3β, 17β-diol	126061-67-4						14	1.14613	Sonnenschein et al. (1989)
HGF	R1881	4-Androstenedione	63-05-8			0.058			2	0.30103	Eil and Edelson (1984)
REC	R1881	4-Androstenedione	63-05-8		3.54	1.77			0.056	-1.25181	Waller et al. (1996)
RPC	DHT	Atrazine	1912-24-9		62				0.00177	-2.75203	Danzo (1997)
COS-1 cells +hAR	R1881	Bicalutamide	90357-06-5		0.055				6.36	0.80346	Kemppainen and Wilson (1996)
RPC	T	Bicalutamide	90357-06-5						1.8	0.25527	Teutsch et al. (1994)
REC	R1881	2,2-Bis-(*p* -hydroxyphenyl)-1,1,1-trichloroethane	2971-36-0		34.4	17.2			0.0058	-2.23657	Waller et al. (1996)
rhAR	DHT	Boldenone	846-48-0						48.76	1.68806	Bauer et al. (2000)
HGF	R1881	Canrenone	976-71-6			0.14			0.84	-0.07572	Eil and Edelson (1984)
rhAR	DHT	Chlormadinone acetate	302-22-7						14.61	1.16465	Bauer et al. (2000)
LnCaP cytosol	T	11β-Chloromethyl estradiol	71794-60-0						1.2	0.07918	Sonnenschein et al. (1989)
HGF	R1881	Cimetidine	51481-61-9			140			0.00084	-3.07572	Eil and Edelson (1984)
REC	R1881	Corticosterone	50-22-6		2940	1470			0.000068	-4.16749	Waller et al. (1996)
HGF	DHT	Cortisol	50-23-7		neg.			10	n.a.	n.a.	Breiner et al. (1986)
RPC	DHT	Cortisol	50-23-7		neg.			2	n.a.	n.a.	Wilson and French (1976)
RPC	M	Cortisol	50-23-7		neg.			0.3	n.a.	n.a.	Schilling and Liao (1984)
HGF	DHT	Cyanoketone	4248-66-2		neg.			10	n.a.	n.a.	Breiner et al. (1986)
COS-1 cells +hAR	DHT	Cyproterone acetate	427-51-0		0.17				0.588	-0.23062	Kemppainen et al. (1999)
COS-1 cells +hAR	R1881	Cyproterone acetate	427-51-0		0.18				2.2	0.34242	Kemppainen et al. (1992)
COS-1 cells +hAR	R1881	Cyproterone acetate	427-51-0		0.125				2.8	0.44716	Wong et al. (1995)
HGF	DHT	Cyproterone acetate	427-51-0		0.115				2.09	0.32015	Breiner et al. (1986)
HGF	DHT	Cyproterone acetate	427-51-0						2	0.30102	Brown et al. (1981)
HGF	R1881	Cyproterone acetate	427-51-0		0.05				10	1.00000	Brown et al. (1981)
HGF	R1881	Cyproterone acetate	427-51-0			0.0095			12.4	1.09342	Eil and Edelson (1984)
HGF	T	Cyproterone acetate	427-51-0						9.4	0.97312	Brown et al. (1981)
REC	R1881	Cyproterone acetate	427-51-0		0.08				2.5	0.39794	Kelce et al. (1994)
RECNR	R1881	Cyproterone acetate	427-51-0		2				3	0.47712	Kelce et al. (1994)
RPC	DHT	Cyproterone acetate	427-51-0		neg.			2	n.a.	n.a.	Wilson and French (1976)
RPC	T	Cyproterone acetate	427-51-0						10	1.00000	Teutsch et al. (1994)
HGF	R1881	Danazol	17230-88-5			0.00285			41.4	1.61700	Eil and Edelson (1984)
RPC	R1881	*p,p′* -DDD	72-54-8		90				0.0011	-2.95861	Kelce et al. (1995)
REC	R1881	*p,p′* -DDE	72-55-9		34.4	17.2			0.0058	-2.23657	Waller et al. (1996)
RPC	DHT	*p,p′* -DDE	72-55-9		6.8				0.016	-1.79588	Danzo (1997)
RPC	R1881	*p,p′* -DDE	72-55-9		5				0.02	-1.69897	Kelce et al. (1995)
REC	R1881	*o,p′* -DDT	789-02-6		344	172			0.00058	-3.23657	Waller et al. (1996)
RPC	DHT	*o,p′* -DDT	789-02-6		14				0.00786	-2.10458	Danzo (1997)
RPC	R1881	*o,p′* -DDT	789-02-6		95				0.00105	-2.97881	Kelce et al. (1995)
REC	R1881	*p,p′* -DDT	50-29-3		372	186			0.00054	-3.26761	Waller et al. (1996)
RPC	DHT	*p,p′* -DDT	50-29-3		22				0.005	-2.30103	Danzo (1997)
RPC	R1881	*p,p′* -DDT	50-29-3		75				0.0013	-2.88606	Kelce et al. (1995)
HGF	DHT	Dehydroepiandrosterone	53-43-0		13				0.0185	-1.73283	Breiner et al. (1986)
MCF-7 cytosol	DHT	15-Dehydroetonogestrel							2.2	0.34242	Deckers et al. (2000)
MCF-7 cytosol	DHT	15-Dehydronorethisterone							2.5	0.39794	Deckers et al. (2000)

Information Sorted by Substance Name, Assay, and Reference Androgen

Assay*	Reference Androgen **	Substance	CASRN†	Purity (%)††	IC_{50} (µM)††	K_i (µM)††	Ki (µM) SEM††	HDT (µM)†††	RBA	log RBA	Reference
HGF	DHT	Desogestrel	54024-22-5		3.9				0.062	-1.20761	Breiner et al. (1986)
HGF	DHT	Dexamethasone	50-02-2		neg.			10	n.a.	n.a.	Breiner et al. (1986)
RPC	M	Dexamethasone	50-02-2		neg.			0.3	n.a.	n.a.	Schilling and Liao (1984)
HGF	R1881	Diazoxide	364-98-7		neg.			100	n.a.	n.a.	Eil and Edelson (1984)
CUC	DHT	3,4-Dichloroacetanilide	31620-87-8						0.0131	-1.88273	Bauer et al. (1998)
CUC	DHT	3,4-Dichloroaniline	95-76-1	98					0.0062	-2.20761	Bauer et al. (1998)
COS-1 cells +hAR	R1881	3',5'-Dichloro-2-hydroxy-2-methylbut-3-enanilide	16776-82-1		0.4				0.875	-0.05799	Wong et al. (1995)
REC	R1881	3',5'-Dichloro-2-hydroxy-2-methylbut-3-enanilide	16776-82-1	>99	5				0.04	-1.39794	Kelce et al. (1994)
REC	R1881	3',5'-Dichloro-2-hydroxy-2-methylbut-3-enanilide	16776-82-1	>99	38	19			0.0053	-2.27572	Waller et al. (1996)
RECNR	R1881	3',5'-Dichloro-2-hydroxy-2-methylbut-3-enanilide	16776-82-1	>99	50				0.12	-0.92082	Kelce et al. (1994)
COS-1 cells +hAR	R1881	2-[[3,5-(Dichlorophenyl)carbamoyl]oxy]-2-methyl-3-butenoic acid	119209-27-7		20				0.0175	-1.75696	Wong et al. (1995)
REC	R1881	2-[[3,5-(Dichlorophenyl)carbamoyl]oxy]-2-methyl-3-butenoic acid	119209-27-7	>98	50				0.004	-2.39794	Kelce et al. (1994)
REC	R1881	2-[[3,5-(Dichlorophenyl)carbamoyl]oxy]-2-methyl-3-butenoic acid	119209-27-7	>98	312	156			0.00064	-3.19382	Waller et al. (1996)
RECNR	R1881	2-[[3,5-(Dichlorophenyl)carbamoyl]oxy]-2-methyl-3-butenoic acid	119209-27-7	>98	300				0.02	-1.69897	Kelce et al. (1994)
CUC	DHT	1-(3,4-Dichlorophenyl)urea	2327-02-8	98					0.0075	-2.12494	Bauer et al. (1998)
RPC	DHT	Dieldrin	60-57-1		74				0.00148	-2.82974	Danzo (1997)
HGF	DHT	Diethylstilbestrol	56-53-1		6.75				0.036	-1.44370	Breiner et al. (1986)
LnCaP cytosol	T	Diethylstilbestrol	56-53-1		neg.			5	n.a.	n.a.	Sonnenschein et al. (1989)
REC	R1881	Diethylstilbestrol	56-53-1		30.6	15.3			0.0065	-2.18709	Waller et al. (1996)
RPC	M	Diethylstilbestrol	56-53-1		neg.			0.3	n.a.	n.a.	Schilling and Liao (1984)
RPC	R1881	Diethylstilbestrol	56-53-1		10				0.01	-2.00000	Kelce et al. (1995)
HGF	DHT	Dihydrospirorenone	67392-87-4		0.695				0.35	-0.45593	Breiner et al. (1986)
COS-1 cells +hAR	DHT	5α-Dihydrotestosterone	521-18-6		0.001				100	2.00000	Kemppainen et al. (1999)
COS-1 cells +hAR	R1881	5α-Dihydrotestosterone	521-18-6		0.0035				100	2.00000	Kemppainen and Wilson (1996)
COS-1 cells +hAR	R1881	5α-Dihydrotestosterone	521-18-6		0.035				11.4	1.05690	Kemppainen et al. (1992)
COS-1 cells +hAR	R1881	5α-Dihydrotestosterone	521-18-6		0.05				10	1.00000	Lambright et al. (2000)
COS-1 cells +hAR	R1881	5α-Dihydrotestosterone	521-18-6		0.02				17.5	1.24304	Wong et al. (1995)
COS-1 cells +rtARα	M	5α-Dihydrotestosterone	521-18-6		0.0006				133	2.12385	Takeo and Yamashita (2000)
COS-1 cytosol +hAR	DHT	5α-Dihydrotestosterone	521-18-6		0.003				100	2.00000	Tilley et al. (1989)
CUC	DHT	5α-Dihydrotestosterone	521-18-6						100	2.00000	Bauer et al. (1998)
HGF	DHT	5α-Dihydrotestosterone	521-18-6		0.0024				100	2.00000	Breiner et al. (1986)

Information Sorted by Substance Name, Assay, and Reference Androgen

Assay*	Reference Androgen **	Substance	CASRN†	Purity (%)††	IC_{50} (µM)††	K_i (µM)††	Ki (µM) SEM††	HDT (µM)†††	RBA	log RBA	Reference
HGF	DHT	5α-Dihydrotestosterone	521-18-6		0.005				100	2.00000	Brown et al. (1981)
HGF	R1881	5α-Dihydrotestosterone	521-18-6						49	1.69019	Brown et al. (1981)
HGF	R1881	5α-Dihydrotestosterone	521-18-6			0.00087			136	2.12353	Eil and Edelson (1984)
HGF	T	5α-Dihydrotestosterone	521-18-6						93	1.96800	Brown et al. (1981)
LnCaP cytosol	T	5α-Dihydrotestosterone	521-18-6						100	2.00000	Sonnenschein et al. (1989)
MCF-7 cytosol	DHT	5α-Dihydrotestosterone	521-18-6						100	2.00000	Deckers et al. (2000)
MCF-7 cytosol	DHT	5α-Dihydrotestosterone	521-18-6						100	2.00000	Schoonen et al. (1995)
REC	R1881	5α-Dihydrotestosterone	521-18-6		0.0294	0.0147			6.8	0.83251	Waller et al. (1996)
RECNR	R1881	5α-Dihydrotestosterone	521-18-6						80	1.90309	Kelce et al. (1994)
rhAR	DHT	5α-Dihydrotestosterone	521-18-6						100	2.00000	Bauer et al. (2000)
RPC	DHT	5α-Dihydrotestosterone	521-18-6		0.0011				100	2.00000	Danzo (1997)
RPC	DHT	5α-Dihydrotestosterone	521-18-6		0.016				100	2.00000	Wilson and French (1976)
RPC	M	5α-Dihydrotestosterone	521-18-6		0.000572				233	2.36736	Schilling and Liao (1984)
RPC	M	5α-Dihydrotestosterone	521-18-6		0.00329	0.00069	0.0002		108.8	2.03663	Van Dort et al. (2000)
RPC	T	5α-Dihydrotestosterone	521-18-6						180	2.25527	Teutsch et al. (1994)
RPC	M	5β-Dihydrotestosterone	571-22-2		0.02909				4.6	0.66276	Schilling and Liao (1984)
HGF	R1881	Diphenylhydantoin	57-41-0		neg.			100	n.a.	n.a.	Eil and Edelson (1984)
CUC	DHT	Diuron	330-54-1						0.0024	-2.61979	Bauer et al. (1998)
RPC	M	DTIB			0.0033	0.00071	0.00022		108.5	2.03543	Van Dort et al. (2000)
rhAR	DHT	Epitestosterone	481-30-1						1.63	0.21219	Bauer et al. (2000)
COS-1 cells +hAR	DHT	17β-Estradiol	50-28-2		0.12				0.833	-0.07935	Kemppainen et al. (1999)
COS-1 cells +hAR	R1881	17β-Estradiol	50-28-2		0.1				3.5	0.54407	Kemppainen and Wilson (1996)
COS-1 cells +hAR	R1881	17β-Estradiol	50-28-2		0.25				1.6	0.20412	Kemppainen et al. (1992)
COS-1 cytosol +hAR	DHT	17β-Estradiol	50-28-2		0.06				5	0.69897	Tilley et al. (1989)
HGF	DHT	17β-Estradiol	50-28-2		0.52				0.46	-0.33724	Breiner et al. (1986)
HGF	R1881	17β-Estradiol	50-28-2			0.024			4.9	0.69020	Eil and Edelson (1984)
LnCaP cytosol	T	17β-Estradiol	50-28-2						0.9	-0.04576	Sonnenschein et al. (1989)
REC	R1881	17β-Estradiol	50-28-2		3.96	1.98			0.05	-1.30103	Waller et al. (1996)
RECNR	R1881	17β-Estradiol	50-28-2						2.2	0.34242	Kelce et al. (1994)
rhAR	DHT	17β−Estradiol	50-28-2						4.88	0.68842	Bauer et al. (2000)
RPC	DHT	17β-Estradiol	50-28-2		98				0.00112	-2.95078	Danzo (1997)
RPC	DHT	17β-Estradiol	50-28-2		0.95				1.7	0.23045	Wilson and French (1976)
RPC	M	17β-Estradiol	50-28-2		0.01574				8.5	0.92942	Schilling and Liao (1984)
RPC	R1881	17β-Estradiol	50-28-2		0.5				0.2	-0.69897	Kelce et al. (1995)
LnCaP cytosol	T	Estriol	50-27-1		neg.			5	n.a.	n.a.	Sonnenschein et al. (1989)
LnCaP cytosol	T	Estrone	53-16-7						0.1	-1.00000	Sonnenschein et al. (1989)
HGF	DHT	17α-Ethinyl estradiol	57-63-6		0.82				0.29	-0.53760	Breiner et al. (1986)
LnCaP cytosol	T	17α-Ethinyl estradiol	57-63-6						1.4	0.14613	Sonnenschein et al. (1989)
MCF-7 cytosol	DHT	Etonogestrel	54048-10-1						6.2	0.79239	Deckers et al. (2000)
MCF-7 cytosol	DHT	Etonogestrel	54048-10-1						4.5	0.65321	Schoonen et al. (1995)
COS-1 cells +hAR	DHT	Fluoxymesterone	76-43-7		0.3				0.3	-0.52288	Kemppainen et al. (1999)
COS-1 cells +hAR	R1881	Flutamide	13311-84-7		neg.			0.5	n.a.	n.a.	Kemppainen et al. (1992)
CUC	DHT	Flutamide	13311-84-7						0.0065	-2.18708	Bauer et al. (1998)
HGF	R1881	Flutamide	13311-84-7			1.2			0.079	-1.10237	Eil and Edelson (1984)
REC	R1881	Flutamide	13311-84-7		10				0.02	-1.69897	Kelce et al. (1994)
RECNR	R1881	Flutamide	13311-84-7		neg.			100	n.a.	n.a.	Kelce et al. (1994)
HGF	DHT	Gestodene	60282-87-3		0.019				12.6	1.10037	Breiner et al. (1986)
MCF-7 cytosol	DHT	Gestodene	60282-87-3						5.2	0.71600	Deckers et al. (2000)
MCF-7 cytosol	DHT	Gestodene	60282-87-3						6.1	0.78533	Schoonen et al. (1995)

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REC	R1881	2,2',4,4',5,5'-Hexachlorobiphenyl	35065-27-1		2940	1470			0.000068	-4.16749	Waller et al. (1996)
RPC	DHT	δ-Hexachlorocyclohexane	319-86-8		36				0.0031	-2.50864	Danzo (1997)
RPC	DHT	γ-Hexachlorocyclohexane	58-89-9		44				0.0025	-2.60206	Danzo (1997)
HGF	R1881	4-Hydroxyandrostenedione	566-48-3			0.15			0.79	-0.10237	Eil and Edelson (1984)
COS-1 cells +hAR	DHT	Hydroxyflutamide	52806-53-8		0.46				0.217	-0.66354	Kemppainen et al. (1999)
COS-1 cells +hAR	R1881	Hydroxyflutamide	52806-53-8		0.35				1	0.00000	Kemppainen and Wilson (1996)
COS-1 cells +hAR	R1881	Hydroxyflutamide	52806-53-8		0.52				0.769	-0.11407	Kemppainen et al. (1992)
COS-1 cells +hAR	R1881	Hydroxyflutamide	52806-53-8		0.2				1.75	0.24304	Wong et al. (1995)
REC	R1881	Hydroxyflutamide	52806-53-8		0.5				0.4	-0.39794	Kelce et al. (1994)
REC	R1881	Hydroxyflutamide	52806-53-8		160	80			0.00143	-2.88606	Waller et al. (1996)
RECNR	R1881	Hydroxyflutamide	52806-53-8		7				0.857	-0.06702	Kelce et al. (1994)
RPC	R1881	Hydroxyflutamide	52806-53-8		0.5				0.2	-0.69897	Kelce et al. (1995)
RPC	R1881	Hydroxyflutamide	52806-53-8		0.75				0.133	-0.87506	Lambright et al. (2000)
RPC	T	Hydroxyflutamide	52806-53-8						0.8	-0.09691	Teutsch et al. (1994)
REC	R1881	Hydroxylinuron			3320	1660			0.00006	-4.22185	Waller et al. (1996)
HGF	DHT	17α-Hydroxyprogesterone	68-96-2		2.75				0.087	-1.06048	Breiner et al. (1986)
HGF	R1881	17α-Hydroxyprogesterone	68-96-2			0.28			0.42	-0.37675	Eil and Edelson (1984)
REC	R1881	17α-Hydroxyprogesterone	68-96-2		3540	1770			0.000056	-4.25181	Waller et al. (1996)
REC	R1881	Kepone	143-50-0		318	159			0.00063	-3.20066	Waller et al. (1996)
RPC	R1881	Kepone	143-50-0		125				0.0008	-3.09691	Kelce et al. (1995)
COS-1 cells +rtARα	M	11-Ketotestosterone	564-35-2		0.008				10	1.00000	Takeo and Yamashita (2000)
MCF-7 cytosol	DHT	Levonorgestrel	797-63-7						10.3	1.01284	Deckers et al. (2000)
MCF-7 cytosol	DHT	Levonorgestrel	797-63-7						8.2	0.91381	Schoonen et al. (1995)
COS-1 cells +hAR	R1881	Linuron	330-55-2	99.8	20				0.025	-0.16021	Lambright et al. (2000)
CUC	DHT	Linuron	330-55-2						0.01	-2.00000	Bauer et al. (1998)
REC	R1881	Linuron	330-55-2		200	100			0.001	-3.00000	Waller et al. (1996)
RPC	R1881	Linuron	330-55-2	99.8	200				0.0005	-0.33010	Lambright et al. (2000)
COS-1 cells +hAR	DHT	Medroxyprogesterone acetate	71-58-9		0.075				1.33	0.12385	Kemppainen et al. (1999)
HGF	DHT	Medroxyprogesterone acetate	71-58-9		0.0235				10.2	1.00860	Breiner et al. (1986)
HGF	R1881	Medroxyprogesterone acetate	71-58-9			0.01			11.6	1.06446	Eil and Edelson (1984)
MCF-7 cytosol	DHT	Medroxyprogesterone acetate	71-58-9						30	1.47712	Schoonen et al. (1995)
rhAR	DHT	Medroxyprogesterone acetate	71-58-9						48.61	1.68673	Bauer et al. (2000)
HGF	R1881	Megestrol acetate	595-33-5			0.0087			13.6	1.13354	Eil and Edelson (1984)
rhAR	DHT	Melengestrol acetate	2919-66-6						0.31	-0.50864	Bauer et al. (2000)
rhAR	DHT	Melengestrol acetate-metabolite 10			neg.			10	n.a.	n.a.	Bauer et al. (2000)
rhAR	DHT	Melengestrol acetate-metabolite 6			neg.			10	n.a.	n.a.	Bauer et al. (2000)
rhAR	DHT	Melengestrol acetate-metabolite 7			neg.			10	n.a.	n.a.	Bauer et al. (2000)
REC	R1881	Methoxychlor	72-43-5		2940	1470			0.000068	-4.16749	Waller et al. (1996)
RPC	DHT	Methoxychlor	72-43-5		110				0.001	-3.00000	Danzo (1997)
HGF	DHT	1-Methylandrosta-1,4-diene-3,17-dione	96301-34-7		neg.			10	n.a.	n.a.	Breiner et al. (1986)
MCF-7 cytosol	DHT	11-Methylene-15-dehydronorethisterone							2	0.30103	Deckers et al. (2000)
MCF-7 cytosol	DHT	11-Methylenenorethisterone							5	0.69897	Deckers et al. (2000)
COS-1 cells +rtARα	M	Methyltestosterone	58-18-4		0.002				40	1.60206	Takeo and Yamashita (2000)
rhAR	DHT	Methyltestosterone	58-18-4						35.93	1.55546	Bauer et al. (2000)

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COS-1 cells +hAR	DHT	Methyltrienolone	965-93-5		0.015				6.7	0.82607	Kemppainen et al. (1999)
COS-1 cells +hAR	R1881	Methyltrienolone	965-93-5		0.0035				100	2.00000	Kemppainen and Wilson (1996)
COS-1 cells +hAR	R1881	Methyltrienolone	965-93-5		0.004				100	2.00000	Kemppainen et al. (1992)
COS-1 cells +hAR	R1881	Methyltrienolone	965-93-5		0.0035				100	2.00000	Wong et al. (1995)
COS-1 cytosol +hAR	DHT	Methyltrienolone	965-93-5		0.0028				107	2.02938	Tilley et al. (1989)
HGF	DHT	Methyltrienolone	965-93-5		0.0022				109	2.03743	Breiner et al. (1986)
HGF	DHT	Methyltrienolone	965-93-5		0.0025				200	2.30103	Brown et al. (1981)
HGF	R1881	Methyltrienolone	965-93-5			0.00118			100	2.00000	Eil and Edelson (1984)
HGF	T	Methyltrienolone	965-93-5						126	2.10030	Brown et al. (1981)
LnCaP cytosol	T	Methyltrienolone	965-93-5						200	2.30103	Sonnenschein et al. (1989)
REC	R1881	Methyltrienolone	965-93-5		0.002				100	2.00000	Kelce et al. (1994)
REC	R1881	Methyltrienolone	965-93-5		0.002	0.001			100	2.00000	Waller et al. (1996)
RECNR	R1881	Methyltrienolone	965-93-5		0.06				100	2.00000	Kelce et al. (1994)
RPC	R1881	Methyltrienolone	965-93-5		0.001				100	2.00000	Kelce et al. (1995)
RPC	T	Methyltrienolone	965-93-5						290	2.46240	Teutsch et al. (1994)
COS-1 cells +hAR	R1881	Methyltrienolone	965-93-5		0.005				100	2.00000	Lambright et al. (2000)
HGF	R1881	Methyltrienolone	965-93-5						100	2.00000	Brown et al. (1981)
RPC	M	Methyltrienolone	965-93-5		0.000906				147	2.16732	Schilling and Liao (1984)
RPC	R1881	Methyltrienolone	965-93-5		0.001				100	2.00000	Lambright et al. (2000)
COS-1 cells +hAR	DHT	Mibolerone	3704-09-4		0.012				8.3	0.91908	Kemppainen et al. (1999)
COS-1 cells +rtARα	M	Mibolerone	3704-09-4		0.0008				100	2.00000	Takeo and Yamashita (2000)
LnCaP cytosol	T	Mibolerone	3704-09-4						15	1.17609	Sonnenschein et al. (1989)
REC	R1881	Mibolerone	3704-09-4		0.002	0.001			100	2.00000	Waller et al. (1996)
RPC	DHT	Mibolerone	3704-09-4		0.08				20	1.30103	Wilson and French (1976)
RPC	M	Mibolerone	3704-09-4		0.00358	0.00075	0.0008		100	2.00000	Van Dort et al. (2000)
RPC	M	Mibolerone	3704-09-4		0.001335				100	2.00000	Schilling and Liao (1984)
COS-1 cells +hAR	R1881	Mifepristone	84371-65-3		0.18				2.2	0.34242	Kemppainen et al. (1992)
LnCaP cytosol	T	Moxestrol	34816-55-2		neg.			5	n.a.	n.a.	Sonnenschein et al. (1989)
HGF	R1881	MSD L-642,022			neg.			100	n.a.	n.a.	Eil and Edelson (1984)
HGF	R1881	MSD L-642,317				3.1			0.038	-1.42022	Eil and Edelson (1984)
HGF	DHT	Nilutamide	63612-50-0		0.3				1.67	0.22272	Brown et al. (1981)
HGF	R1881	Nilutamide	63612-50-0						0.2	-0.69890	Brown et al. (1981)
HGF	T	Nilutamide	63612-50-0						1.7	0.23040	Brown et al. (1981)
RPC	T	Nilutamide	63612-50-0						0.8	-0.09691	Teutsch et al. (1994)
RPC	DHT	Nonylphenol	25154-52-3		104				0.0011	-2.95861	Danzo (1997)
MCF-7 cytosol	DHT	Norethisterone	68-22-4						3.2	0.50515	Deckers et al. (2000)
MCF-7 cytosol	DHT	Norethisterone	68-22-4						6	0.77815	Schoonen et al. (1995)
HGF	DHT	Norethisterone acetate	51-98-9		0.09				2.6	0.41497	Breiner et al. (1986)
HGF	DHT	Norgestrel	6533-00-2		0.0145				16.6	1.22011	Breiner et al. (1986)
rhAR	DHT	19-Nortestosterone	434-22-0						75.22	1.87633	Bauer et al. (2000)
MCF-7 cytosol	DHT	ORG 2058	24320-06-7						0.14	-0.85387	Schoonen et al. (1995)
MCF-7 cytosol	DHT	ORG 30659							2.8	0.44716	Schoonen et al. (1995)
COS-1 cells +hAR	DHT	Oxandrolone	53-39-4		0.26				0.38	-0.42022	Kemppainen et al. (1999)
REC	R1881	P1			296	148			0.00068	-3.16749	Waller et al. (1996)
REC	R1881	2,2',4',5,5'-Pentachloro-4-biphenylol			264	132			0.00076	-3.11919	Waller et al. (1996)
RPC	DHT	Pentachlorophenol	87-86-5		68				0.0016	-2.79588	Danzo (1997)
HGF	DHT	Potassium canrenoate	2181-04-6		neg.			10	n.a.	n.a.	Breiner et al. (1986)

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LnCaP cytosol	T	Pregnenolone	145-13-1		neg.			5	n.a.	n.a.	Sonnenschein et al. (1989)
REC	R1881	Pregnenolone	145-13-1		2940	1470			0.000068	-4.16749	Waller et al. (1996)
REC	R1881	Procymidone	32809-16-8		2940	1470			0.000068	-4.16749	Waller et al. (1996)
COS-1 cells +hAR	DHT	Progesterone	57-83-0		0.062				1.613	0.20763	Kemppainen et al. (1999)
COS-1 cells +hAR	R1881	Progesterone	57-83-0		0.09				3.89	0.58995	Kemppainen and Wilson (1996)
COS-1 cytosol +hAR	DHT	Progesterone	57-83-0		0.15				2	0.30103	Tilley et al. (1989)
HGF	DHT	Progesterone	57-83-0		0.5				0.48	-0.31876	Breiner et al. (1986)
HGF	R1881	Progesterone	57-83-0			0.018			6.6	0.81954	Eil and Edelson (1984)
LnCaP cytosol	T	Progesterone	57-83-0						19	1.27875	Sonnenschein et al. (1989)
MCF-7 cytosol	DHT	Progesterone	57-83-0						1.5	0.17609	Schoonen et al. (1995)
REC	R1881	Progesterone	57-83-0		3540	1770			0.000056	-4.25181	Waller et al. (1996)
rhAR	DHT	Progesterone	57-83-0						3.83	0.58320	Bauer et al. (2000)
RPC	DHT	Progesterone	57-83-0		neg.			2	n.a.	n.a.	Wilson and French (1976)
RPC	M	Progesterone	57-83-0		0.03816				3.5	0.54283	Schilling and Liao (1984)
LnCaP cytosol	T	Promegestone	34184-77-5						11	1.04139	Sonnenschein et al. (1989)
MCF-7 cytosol	DHT	Promegestone	34184-77-5		neg.			10	n.a.	n.a.	Schoonen et al. (1995)
HGF	DHT	17α-Propylmesterolone	79243-67-7		0.0105				22.85	1.35902	Breiner et al. (1986)
HGF	DHT	R 2956	42438-88-0		0.0375				13	1.11394	Brown et al. (1981)
HGF	R1881	R 2956	42438-88-0						5.6	0.74818	Brown et al. (1981)
HGF	R1881	R 2956	42438-88-0			0.008			14.8	1.17026	Eil and Edelson (1984)
HGF	T	R 2956	42438-88-0						20	1.30100	Brown et al. (1981)
COS-1 cells +hAR	DHT	RU 56187	143782-25-6		0.035				2.86	0.45637	Kemppainen et al. (1999)
RPC	T	RU 56187	143782-25-6						92	1.96379	Teutsch et al. (1994)
RPC	T	RU 57073							163	2.21219	Teutsch et al. (1994)
RPC	M	RU 59063	155180-53-3		0.0106	0.00223	0.0005		33.8	1.52892	Van Dort et al. (2000)
RPC	T	RU 59063	155180-53-3						300	2.47712	Teutsch et al. (1994)
HGF	DHT	Spironolactone	52-01-7		0.315				0.76	-0.11919	Breiner et al. (1986)
HGF	R1881	Spironolactone	52-01-7			0.00176			67	1.82607	Eil and Edelson (1984)
HGF	DHT	Testolactone	968-94-3		neg.			10	n.a.	n.a.	Breiner et al. (1986)
HGF	R1881	Testolactone	968-94-3			41			0.0029	-2.53760	Eil and Edelson (1984)
COS-1 cells +hAR	DHT	Testosterone	58-22-0		0.22				0.45	-0.34679	Kemppainen et al. (1999)
COS-1 cells +rtARα	M	Testosterone	58-22-0		0.003				26.7	1.42651	Takeo and Yamashita (2000)
COS-1 cytosol +hAR	DHT	Testosterone	58-22-0		0.015				21	1.32222	Tilley et al. (1989)
HGF	DHT	Testosterone	58-22-0		0.0125				19	1.27875	Breiner et al. (1986)
HGF	DHT	Testosterone	58-22-0		0.004				125	2.09691	Brown et al. (1981)
HGF	R1881	Testosterone	58-22-0						47	1.67210	Brown et al. (1981)
HGF	R1881	Testosterone	58-22-0			0.00101			117	2.06814	Eil and Edelson (1984)
HGF	T	Testosterone	58-22-0						100	2.00000	Brown et al. (1981)
LnCaP cytosol	T	Testosterone	58-22-0						100	2.00000	Sonnenschein et al. (1989)
REC	R1881	Testosterone	58-22-0		0.0382	0.191			5.23	-0.71850	Waller et al. (1996)
RECNR	R1881	Testosterone	58-22-0						40	1.60206	Kelce et al. (1994)
rhAR	DHT	Testosterone	58-22-0						31.31	1.49568	Bauer et al. (2000)
RPC	DHT	Testosterone	58-22-0		0.06				27	1.43136	Wilson and French (1976)
RPC	M	Testosterone	58-22-0		0.001478				90	1.95424	Schilling and Liao (1984)
RPC	M	Testosterone	58-22-0		0.00667	0.0014	0.0004		53.7	1.72997	Van Dort et al. (2000)
RPC	T	Testosterone	58-22-0						100	2.00000	Teutsch et al. (1994)
rhAR	DHT	17α-Trenbolone	80657-17-6						4.49	0.65225	Bauer et al. (2000)
rhAR	DHT	17β-Trenbolone	10161-33-8						108.86	2.03687	Bauer et al. (2000)
rhAR	DHT	Trendione	4642-95-9						0.36	-0.44370	Bauer et al. (2000)

Information Sorted by Substance Name, Assay, and Reference Androgen

Assay*	Reference Androgen **	Substance	CASRN†	Purity (%)††	IC_{50} (µM)††	K_i (µM)††	Ki (µM) SEM††	HDT (µM)†††	RBA	log RBA	Reference
COS-1 cytosol +hAR	DHT	Triamcinolone acetonide	76-25-5		neg.			0.3	n.a.	n.a.	Tilley et al. (1989)
RPC	M	Triamcinolone acetonide	76-25-5		neg.			0.3	n.a.	n.a.	Schilling and Liao (1984)
COS-1 cells +hAR	R1881	Vinclozolin	50471-44-8		0.035				0.035	-1.45593	Wong et al. (1995)
REC	R1881	Vinclozolin	50471-44-8	>99	neg.			200	n.a.	n.a.	Kelce et al. (1994)
REC	R1881	Vinclozolin	50471-44-8	>99	2940	1470			0.000068	-4.16749	Waller et al. (1996)
RECNR	R1881	Vinclozolin	50471-44-8	>99	neg.			200	n.a.	n.a.	Kelce et al. (1994)

* Assays used for testing listed alphabetically

**R1881 = Methyltrienolone, DHT = 5α−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

††† Empty cells in the HDT column indicate that this information was unnecessary since RBA's could be calculated or were provided

COS-1 cells + hAR = COS-1 cells containing human AR

COS-1 cytosol + hAR = Cytosol from COS-1 cells containing hAR

COS-1 cells + rtARα = COS1 cells containing the ARα from rainbow trout

CUC = calf uterine cytosol

HGF = human genital fibroblasts

LnCaP cytosol = cytosol from human supraclavicular lymph node from patient with prostatic adenocarcinoma

REC = rat epididymal cytosol

RECNR = rat epididymal cells, nuclear receptor

RPC = rat prostate cytosol

neg. = No IC_{50} could be determined since displacement of radioligand was less than 50%, or no binding was observed; therefore, a RBA could not be calculated

n.a.= not available

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Appendix D2

Substances Tested in the *In Vitro* AR Binding Assays

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