6.0 *IN VITRO* ANDROGEN RECEPTOR TRANSCRIPTIONAL ACTIVATION ASSAYS

6.1 Minimum Procedural Standards

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

6.1.1 Reference Androgen and TA Response

6.1.1.1 Agonism Assays

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

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

6.1.1.2 Antagonism assays

In AR TA antagonism assays, test substances are evaluated for their ability to reduce the level of TA induced by a reference androgen.

Section 6

The concentration of the reference androgen selected for antagonism assays should be within the upper linear region of the dose-response curve; 70 to 80% of maximal induction is recommended. The recommended reference androgen for these assays is R1881.

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

6.1.2 Preparation of Test Substances and Volume of Administered Solvent

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

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

6.1.3 Concentration Range of the Test Substances

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

Rationale: Most test method guidelines include a limit dose to ensure that all substances are tested over the same dose range while avoiding excessive amounts of a test substance that can perturb the test system through physicochemical mechanisms. An established limit dose also minimizes the effort and cost of screening and testing. Based on the range of published EC_{50} values for AR agonists and IC_{50} values for AR antagonists (NIEHS

2002d), a limit dose of 1 mM was deemed suitable by the Expert Panel, the EDWG, and ICCVAM for assessing the ability of a test substance to act as either an AR agonist or an antagonist.

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

6.1.4 Solvent and Positive Controls

6.1.4.1 Solvent controls

Agonism Assays

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

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

Antagonism Assays

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

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

6.1.4.3 Positive control

Agonism Assays

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

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

Antagonism Assays

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

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

6.1.5 Within-Test Replicates

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

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

6.1.6 Data Analysis

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

positive controls in agonism and antagonism studies, respectively, should be calculated and presented.

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

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

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

6.1.8 Study Acceptance Criteria

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

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

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

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

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

Rationale: Repeat studies are used in a validation study to demonstrate the intralaboratory

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

6.1.11 Study Report

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

Reference Androgen

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

Additional Positive Control (if used)

- name, CASRN, purity, and supplier or source
- concentrations and volumes used

Test Substance

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

Solvent

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

Androgen Receptor

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

Reporter Plasmid

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

Cell Line

- source and nomenclature of the cell line and protocol for its maintenance before and after transfection
- source of plasticware used to culture cells and source of other materials used in the study
- passage number of cell line used for transfection and passage number of cell line used in the study
- growth parameters of the cell line before initiation of the study
- method used to transiently transfect the reporter construct into the cells
- method used to monitor transient transfection efficiency between cell preparations
- methods for establishment and propagation of a stably transfected cell line and what is

required for growth of the cell line (e.g., charcoal-stripped serum)
- method used to monitor the stability of a stably transfected cell line used for testing
- rationale, based on data, for deciding on the number of passages a cell line can undergo without a decrease in activity
- details regarding the selection requirements needed for maintaining stable cell lines

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

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

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

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

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

independent replication of the study, if deemed necessary.

6.2 Recommended Substances for Validation of *In Vitro* Androgen Receptor Transcriptional Activation Assays[1]

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

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

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

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

As described in **Section 2.4.4**, and mentioned above, a subset of 44 substances has been identified that, at a minimum, should be used in any validation of *in vitro* AR TA assays. These 44 substances are in bold type in **Table 6-1** for agonism. Of these substances, 45% (20) are classified as positive (15) or presumed positive (5) for AR agonism, and 55% (24) are classified as presumed negative. The same 44 substances are in bold type in **Table 6-2** for antagonism. Of these substances, 45% (20) are classified as positive (16) or presumed positive (4) for AR antagonism, and 55% (24) are classified as presumed negative.

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

Substance	CASRN	Median EC_{50} Value and Range Across All MCRG Studies [b] (µM)	Qualitative Response for AR Agonism Across All MCRG Studies [c]	No. of MCRG Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing [d]	Comments	Chemical Class
5α-Dihydrotestosterone [e]	521-18-6	0.00015 (3) 0.00004 - 0.000153	Pos. (21/21)	13	H	Strong AR agonist; Weak ER agonist	Steroid, nonphenolic
Testosterone [e]	58-22-0	0.0002 (3) 0.000107 - 0.000527	Pos. (11/11)	10	IM	Strong AR agonist	Steroid, nonphenolic
Methyl testosterone [e]	58-18-4	0.00081 (2) 0.0000274 - 0.000135	Pos. (2/2)	2	H; 407; M-PA; IUL; FRS	AR and ER agonist	Steroid, nonphenolic; Androstene
4-Androstenedione [e]	63-05-8	0.0015 (2) 0.000645 - 0.00242	Pos. (3/3)	3		Strong AR agonist	Steroid, nonphenolic
Mifepristone [e]	84371-65-3	0.014 (1)	Pos. (3/4)	4	IM	AR agonist and antagonist	Steroid, nonphenolic; Estrene
Estrone [e]	53-16-7	0.055 (1)	Pos. (2/2)	2		AR agonist; Strong ER agonist	Steroid, phenolic; Estrene
QUALITATIVE DATA ONLY							
17β-Estradiol [e,g]	50-28-2		Pos. (10/11)	9	IM; IUL; FRS	AR agonist and antagonist; Strong ER agonist	Steroid, phenolic; Estrene
Cyproterone acetate [e,g]	427-51-0		Pos. (8/8)	7	IM	AR agonist and antagonist	Nitrile; Diphenyl ether; Organochlorine
***Methyltrienolone (R1881)* [e,f,g]**	965-93-5		Pos. (8/8)	5		AR agonist; *Recommended reference androgen*	Steroid, nonphenolic; Estrene
Progesterone [e,g]	57-83-0		Pos. (7/9)	7	IM		Steroid, nonphenolic; Pregnenedione
Hydroxyflutamide [e,g]	52806-53-8		Pos. (5/6)	4		AR agonist and antagonist	Amide; Anilide; Nitrobenzene

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

Substance	CASRN	Median EC_{50} Value and Range Across All MCRG Studies [b] (µM)	Qualitative Response for AR Agonism Across All MCRG Studies [c]	No. of MCRG Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing [d]	Comments	Chemical Class
Medroxyprogesterone acetate [e,g]	71-58-9		Pos. (4/4)	3		Weak AR agonist	Steroid, nonphenolic; Polycyclic hydrocarbon
Dexamethasone [e,g]	50-02-2		Pos. (3/4)	3		AR agonist	Steroid, nonphenolic
Bicalutamide [e,g]	90357-06-5		Pos. (2/2)	2		AR antagonist	Anilide; Nitrile; Sulfone
Spironolactone [e,g]	52-01-7		Pos. (2/2)	2		AR agonist and antagonist	Steroid, nonphenolic; Pregnene lactone
***p,p'*-DDE [e,g]**	72-55-9		Pos. (2/3)	3	H; 407; M-PA; IM; J(1G,F,A)	Weak AR agonist and antagonist	Organochlorine; Diphenylalkene
Fluoxymestrone [e,g]	76-43-7		Pos. (1/1)[j]	1		Weak AR agonist	Steroid, nonphenolic
Linuron [e,g]	330-55-2		Pos. (1/1)[j]	1	H; M-PA	Weak AR agonist and antagonist	Urea
Nilutamide [e,g]	63612-50-0		Pos. (1/1)[j]	1		AR antagonist	Heterocycle; Imidazole
Dibenzo[*a,h*]-anthracene [e,g]	53-70-3		Pos. (1/1)[j]	1			Polycyclic aromatic hydrocarbon; Anthracene
Flutamide [e,g]	13311-84-7		Neg. (5/5)	5	H; 407; M-PA; IM; FRS	AR antagonist	Amide; Anilide; Nitrobenzene
Diethylstilbestrol [e,g]	56-53-1		Neg. (2/2)	2	IUL	Strong ER agonist	Stilbene; Benzylidene; Diphenylalkene
Kepone [e,g] (Chlordecone)	143-50-0		Neg. (2/2)	2		Binds to AR and ER	Organochlorine; Chlorinated bridged cycloalkane

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

Substance	CASRN	Median EC_{50} Value and Range Across All MCRG Studies[b] (µM)	Qualitative Response for AR Agonism Across All MCRG Studies[c]	No. of MCRG Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing[d]	Comments	Chemical Class
Atrazine[e,g]	1912-24-9		Neg. (1/1)	1	M-PA; IUL		Aromatic amine; Triazine; Arylamine
Bisphenol A[e,g]	80-05-7		Neg. (1/1)	1	U; F-PA; J(1G,F,A)	Weak ER agonist	Diphenylalkane; Bisphenol; Phenol
Corticosterone[e,g]	50-22-6		Neg. (1/1)	1		Binds weakly to AR	Steroid, nonphenolic
***o,p'*-DDT[e,g]**	789-02-6		Neg. (1/1)	1	U; J(1G,F,A)	Weak AR and ER antagonist; Weak ER agonist	Organochlorine; Diphenylalkene
Di-*n*-butyl phthalate[e,g]	84-74-2		Neg. (1/1)	1	U; M-PA; 1G; J(U,H,1G,F,A)	ER agonist	Phthalate
Diethylhexyl phthalate[e,g]	117-81-7		Neg. (1/1)	1	J(U,H,1G,F,A)		Phthalate
17α-Ethinyl estradiol[e,g]	57-63-6		Neg. (1/1)	1	U; 407; F-PA	Strong ER agonist	Steroid, phenolic
Fluoranthene[g,h]	206-44-0		Neg. (1/1)	1		AR antagonist	Polycyclic aromatic hydrocarbon; Fluorene
4-Hydroxytamoxifen[e,g]	68047-06-3		Neg. (1/1)	1		ER antagonist	Triphenylethylene; Benzylidene; Stilbene; Phenol
***p,p'*-Methoxychlor[e,g]**	72-43-5		Neg. (1/1)	1	U; F&M-PA; IUL; IM; 2G(avian); FRS	AR antagonist; Weak ER agonist	Organochlorine; Chlorinated hydrocarbon
***p*-n-Nonylphenol[e,g,i]**	104-40-5		Neg. (1/1)	1	U; 407; J(U,H,1G,F,A)	AR and ER antagonist; ER agonist	Alkylphenol; Phenol
4-*tert*-Octylphenol[e,g]	140-66-9		Neg. (1/1)	1	J(U,H,1G,F,B)	ER agonist	Alkylphenol; Phenol

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

Substance	CASRN	Median EC_{50} Value and Range Across All MCRG Studies [b] (µM)	Qualitative Response for AR Agonism Across All MCRG Studies [c]	No. of MCRG Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing [d]	Comments	Chemical Class
Phenobarbital [e,g]	57-30-7		Neg. (1/1)	1	F&M-PA; IM	Enhances thyroid hormone excretion	Heterocycle; Pyrimidine
Procymidone [e,g]	32809-16-8		Neg. (1/1)	1	H	AR antagonist	Organochlorine; Cyclic imide
Vinclozolin [e,g]	50471-44-8		Neg. (1/1)	1	H; M-PA; IM; IUL; 1G; FRS	AR antagonist	Organochlorine; Cyclic imide; Carbamate
Bisphenol B [e,g]	77-40-7		Neg. (1/1)	1		ER agonist	Diphenylalkane; Bisphenol; Phenol
Butylbenzyl phthalate [e,g]	85-68-7		Neg. (1/1)	1	IUL	ER agonist	Phthalate
Coumestrol [e,g]	479-13-0		Neg. (1/1)	1	IM	ER agonist	Coumestan; Benzopyranone; Coumarin; Ketone
4-Cumylphenol [e,g]	599-64-4		Neg. (1/1)	1		Weak ER agonist	Phenol
17α-Estradiol [e,g]	57-91-0		Neg. (1/1)	1		ER agonist	Steroid, phenolic; Estrene
Tamoxifen [e,g]	10540-29-1		Neg. (1/1)	1		ER antagonist	Triphenylethylene; Benzylidene; Stilbene
Zearalenone [e,g]	17924-92-4		Neg. (1/1)	1		ER agonist	Resorcylic acid lactone; Phenol
ANTICIPATED RESPONSES (No EC_{50} or Qualitative Agonism Data Available)							
Ketoconazole	65277-42-1		Pos.		F&M-PA; IM	Pos. for AR agonism in yeast assay	Imidazole; Piperazine

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

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

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

Substance	CASRN	Median EC_{50} Value and Range Across All MCRG Studies[b] (µM)	Qualitative Response for AR Agonism Across All MCRG Studies[c]	No. of MCRG Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing[d]	Comments	Chemical Class
2-*sec*-Butylphenol	89-72-5		Neg.				Phenol
CGS 18320B	112808-99-8		Neg.		407	Aromatase inhibitor	Nitrile; Imidazole
Clomiphene citrate	50-41-9		Neg.			Binds to the ER	Chlorinated triphenylethylene; Benzylidene; Stilbene
Cycloheximide	66-81-9		Neg.			Protein synthesis inhibitor	Piperidine; Glutaramide
Daidzein	486-66-8		Neg.			Weak ER agonist	Flavanoid; Isoflavone; Phenol
Ethyl paraben	120-47-8		Neg.			Binds weakly to ER	Paraben; Organic acid
Fenarimol	60168-88-9		Neg.		F-PA	Aromatase inhibitor	Heterocycle; Pyrimidine
Flavone	525-82-6		Neg.		M-PA; IM	Weak ER antagonist	Flavanoid; Flavone
Genistein	446-72-0		Neg.		U; 407	Weak ER agonist and antagonist	Flavanoid; Isoflavone; Phenol
Haloperidol	52-86-8		Neg.		IM	Dopamine D2 receptor antagonist	Butyrophenone; Ketone; Piperazine
meso-Hexestrol	84-16-2		Neg.			Strong ER agonist	Diphenylalkane; Bisphenol; Phenol
ICI 182,780	129453-61-8		Neg.		IM	ER antagonist; Neg. for AR agonism in yeast assay	Steroid, phenolic

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

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

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

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

[b] An EC_{50} is the effective concentration of the test substance that elicits 50% of the maximum response in a particular test system. Median EC_{50} values are derived from *in vitro* mammalian cell reporter gene studies that were either published in the peer-reviewed scientific literature or submitted to NICEATM,

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

and then reviewed and summarized in the NICEATM Background Review Document (BRD) titled "Current Status of Test Methods for Detecting Endocrine Disruptors: *In Vitro* Androgen Receptor Transcriptional Activation Assays-July 2002" (available on the ICCVAM website at *http://iccvam.niehs.nih.gov/methods/endocrine.htm*). Substances for which quantitative AR agonism data are available are ranked according to their relative potency in AR mammalian cell reporter gene agonism assays from most potent to least potent. Numbers in parentheses to the right of the EC_{50} value refer to the number of studies for which an EC_{50} value was reported in the BRD. An italicized EC_{50} value indicates the value was estimated from a graphical presentation of data. The range of values reported for a substance is listed below the median value.

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

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

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

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

[g] No EC_{50} data available for this substance.

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

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

[j] The classification for this substance is "presumed positive" for AR agonism since the substance was positive in 50% or less of reported studies, or was reported positive in the single study conducted.

Table 6-2: ICCVAM Recommended Substances for Validation of *In Vitro* AR TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems [a]

Substance	CASRN	Median EC_{50} Value and Range Across All MCRG Studies [b] (µM)	Qualitative Response for AR Antagonism Across All MCRG Studies [c]	No. of MCRG Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing [d]	Comments	Chemical Class
Mifepristone [e]	84371-65-3	0.05 (1)	Pos. (2/2)	2	IM	AR agonist and antagonist	Steroid, nonphenolic; Estrene
Cyproterone acetate [e]	427-51-0	0.1 (5) 0.01 - 45	Pos. (6/6)	5	IM	AR agonist and antagonist	Nitrile; Diphenyl ether; Organochlorine
Hydroxyflutamide [e]	52806-53-8	0.1 (6) 0.01 - 45	Pos. (11/11)	7		AR agonist and antagonist	Amide; Anilide; Nitrobenzene
Vinclozolin [e]	50471-44-8	0.28 (2) 0.05 - 0.5	Pos. (3/3)	3	H; M-PA; IM; IUL; 1G; FRS	AR antagonist	Organochlorine; Cyclic imide; Carbamate
Spironolactone [e]	52-01-7	0.3 (2) 0.09 - 0.5	Pos. (2/3)	3		AR agonist and antagonist	Steroid, nonphenolic; Pregnene lactone
Nilutamide [e]	63612-50-0	0.3 (3) 0.15 - 10	Pos. (3/3)	2		AR antagonist	Heterocycle; Imidazole
Progesterone [e]	57-83-0	0.3 (2) 0.1 - 0.5	Pos. (3/3)	3	IM		Steroid, nonphenolic; Pregnenedione
Diethylstilbestrol [e]	56-53-1	0.36 (1)	Pos. (2/2)	2	IUL	Strong ER agonist	Stilbene; Benzylidene; Diphenylalkene
17β-Estradiol [e]	50-28-2	0.5 (3) 0.05 - 1	Pos. (5/5)	4	IM; IUL; FRS	AR agonist and antagonist; Strong ER agonist	Steroid, phenolic; Estrene
Bicalutamide [e]	90357-06-5	0.63 (4) 0.5 - 18	Pos. (5/5)	4		AR antagonist	Anilide; Nitrile; Sulfone
Bisphenol A [e]	80-05-7	1 (1)	Pos. (1/2) [j]	2	U; F-PA; J(1G,F,B)	Weak ER agonist	Diphenylalkane; Bisphenol; Phenol
***p,p'*-DDE [e]**	72-55-9	3 (4) 0.75 - 15.2	Pos. (7/7)	5	H; 407; M-PA; IM; J(1G,F,B)	Weak AR agonist and antagonist	Organochlorine; Diphenylalkene

Table 6-2: ICCVAM Recommended Substances for Validation of *In Vitro* AR TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems [a] *(continued)*

Substance	CASRN	Median EC_{50} Value and Range Across All MCRG Studies [b] (µM)	Qualitative Response for AR Antagonism Across All MCRG Studies [c]	No. of MCRG Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing [d]	Comments	Chemical Class
4-*tert*-Octylphenol [f]	140-66-9	3 (1)	Pos. (1/1)[j]	1	J(U,H,1G,F,B)	ER agonist	Alkylphenol; Phenol
Fluoranthene [e]	206-44-0	4.6 (1)	Pos. (1/1)[j]	1		AR antagonist	Polycyclic aromatic hydrocarbon; Fluorene
Linuron [e]	330-55-2	5 (3) 5 - 10	Pos. (3/3)	2	H; M-PA	Weak AR agonist and antagonist	Urea
Kepone [e]	143-50-0	6.9 (1)	Pos. (1/2)[j]	2		Binds to AR and ER	Organochlorine; Chlorinated bridged cycloalkane
Procymidone [e]	32809-16-8	7.5 (2) 5 - 10	Pos. (2/2)	2	H	AR antagonist	Organochlorine; Cyclic imide
Fenarimol [f]	60168-88-9	*15*	Pos. (1/1)[j]	1	F-PA	Aromatase inhibitor	Heterocycle; Pyrimidine
Flutamide [e,g]	13311-84-7		Pos. (3/3)	3	H; 407; M-PA; IM; FRS	AR antagonist	Amide; Anilide; Nitrobenzene
***o,p'*-DDT [e,g]**	789-02-6		Pos. (2/2)	2	U; J(1G,F,B)	Weak AR and ER antagonist; Weak ER agonist	Organochlorine; Diphenylalkene
***p,p'*-Methoxychlor [e,g]**	72-43-5		Pos. (2/2)	1	U; F&M-PA; IUL; IM; FRS; 2G(avian)	AR antagonist; Weak ER agonist	Organochlorine; Chlorinated hydrocarbon
Atrazine [e,g]	1912-24-9		Neg. (1/1)	1	M-PA; IUL		Aromatic amine; Triazine; Arylamine
Fluoxymestrone [e,g]	76-43-7		Neg. (1/1)	1		Weak AR agonist	Steroid, nonphenolic
Medroxyprogesterone acetate [e,g]	71-58-9		Neg. (1/1)	1		Weak AR agonist	Steroid, nonphenolic; Polycyclic hydrocarbon

Table 6-2: ICCVAM Recommended Substances for Validation of *In Vitro* AR TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems [a] *(continued)*

Substance	CASRN	Median EC_{50} Value and Range Across All MCRG Studies [b] (µM)	Qualitative Response for AR Antagonism Across All MCRG Studies [c]	No. of MCRG Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing [d]	Comments	Chemical Class
Methyltrienolone (R1881) [e,g,h]	965-93-5		Neg. (1/1)	1		AR agonist; *Recommended reference androgen*	Steroid, nonphenolic; Estrene
Testosterone [e,g]	58-22-0		Neg. (1/1)	1	IM	Strong AR agonist	Steroid, nonphenolic
Butylbenzyl phthalate [e,g]	85-68-7		Neg. (1/1)	1	IUL	ER agonist	Phthalate
ANTICIPATED RESPONSES (No IC_{50} or Qualitative Antagonism Data Available)							
Actinomycin D	50-76-0		Neg.			RNA synthesis inhibitor	Phenoxazone; Lactone; Peptide
4-Androstenedione	63-05-8		Neg.			Strong AR agonist	Steroid, nonphenolic
Corticosterone	50-22-6		Neg.			Binds weakly to AR	Steroid, nonphenolic
Dexamethasone	50-02-2		Neg.			AR agonist	Steroid, nonphenolic
Di-*n*-butyl phthalate	84-74-2		Neg.		U; M-PA; 1G; J(U,H,1G,F,B)	ER agonist	Phthalate
Diethylhexyl phthalate	117-81-7		Neg.		J(U,H,1G,F,B)		Phthalate
5α-Dihydro-testosterone	521-18-6		Neg.		H	Weak ER agonist; Strong AR agonist	Steroid, nonphenolic
Estrone	53-16-7		Neg.			Strong ER agonist; R agonist	Steroid, phenolic; Estrene
17α-Ethinyl estradiol	57-63-6		Neg.		U; 407; F-PA	Strong ER agonist	Steroid, phenolic

Table 6-2: ICCVAM Recommended Substances for Validation of *In Vitro* AR TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems [a] *(continued)*

Substance	CASRN	Median EC_{50} Value and Range Across All MCRG Studies [b] (µM)	Qualitative Response for AR Antagonism Across All MCRG Studies [c]	No. of MCRG Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing [d]	Comments	Chemical Class
Fadrozole	102676-47-1		Neg.		F-PA; IM; FRS	Aromatase inhibitor	Imidazole; Nitrile
Finasteride	98319-26-7		Neg.		H; M-PA; IM	5α-reductase inhibitor	Steroid, nonphenolic; Androstene
4-Hydroxytamoxifen	68047-06-3		Neg.			ER antagonist	Triphenylethylene; Benzylidene; Stilbene; Phenol
Ketoconazole	65277-42-1		Neg.		F&M-PA; IM	Weak AR agonist; Neg. for AR antagonism in yeast assay	Imidazole; Piperazine
Methyl testosterone	58-18-4		Neg.		H; 407; M-PA; IUL; FRS	AR and ER agonist	Steroid, nonphenolic; Androstene
***p*-n-Nonylphenol [i]**	104-40-5		Neg.		U; 407; J(U,H,1G,F,B)	ER agonist and antagonist; Neg. for AR antagonism in yeast assay	Alkylphenol; Phenol
Phenobarbital	57-30-7		Neg.		F&M-PA; IM	Enhances thyroid hormone excretion	Heterocycle; Pyrimidine
Sodium azide	26628-22-8		Neg.			Cytotoxic	Organic salt; Azide
12-*O*-Tetradecanoyl-phorbol-13-acetate	16561-29-8		Neg.			Activates ligand independent cell division	Phorbol ester; Terpene
17β-Trenbolone	10161-33-8		Neg.		H	Binds strongly to the AR	Steroid, nonphenolic; Estrene
2,4,5-Trichlorophenoxy-acetic acid	93-76-5		Neg.			Weak ER agonist	Organochlorine; Chlorinated aromatic hydrocarbon

Table 6-2: ICCVAM Recommended Substances for Validation of *In Vitro* AR TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems [a] *(continued)*

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

Table 6-2: ICCVAM Recommended Substances for Validation of *In Vitro* AR TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems[a] *(continued)*

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

Table 6-2: ICCVAM Recommended Substances for Validation of *In Vitro* AR TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems [a] *(continued)*

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

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

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

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

Table 6-2: ICCVAM Recommended Substances for Validation of *In Vitro* AR TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems[a] *(continued)*

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

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

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

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

[g] No IC_{50} data available for this substance.

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

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

[j] The classification for this substance is "presumed positive" for AR antagonism since the substance was positive in 50% or less of reported studies, or was reported positive in the single study conducted.

[This Page Intentionally Left Blank]