5.0   *IN VITRO* ANDROGEN RECEPTOR BINDING ASSAYS

5.1 Minimum Procedural Standards

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

protocols, and scientific justification should be provided for any deviations.

5.1.1 Animal Studies

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

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

5.1.2 Reference Androgen

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

Rationale: 5α-Dihydrotestosterone (DHT) has been frequently used as the reference androgen in AR binding studies, especially when recombinant proteins are used as the

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source of the AR. However, since DHT is metabolized by animal tissue cytosolic preparations, R1881 is the reference androgen of choice for such binding assays. Since DHT is metabolized by many cell lines, R1881 is the reference androgen of choice for *in vitro* AR TA assays (see **Section 6.0**). Thus, to allow for a more direct comparison of the relative performance of *in vitro* AR binding and TA assays, R1881 is recommended as the most suitable reference androgen for AR binding assays.

5.1.3 Dissociation Constant of the Reference Androgen

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

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[1] The ligand binding array differs from the conventional binding assay in that the competitive binding assay is conducted using a range of concentrations of both the radiolabeled reference androgen and the test substance that generates an array of isotherms that permits the simultaneous calculation of K_d and B_{max} for the radiolabeled reference estrogen and the IC_{50} values of the unlabeled reference estrogen and the test substance.

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

5.1.4 Preparation of Test Substances and Volume of Administered Solvent

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

information, the stock solution should be prepared fresh prior to use.

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

5.1.5 Concentration Range of Test Substances

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

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

The seven recommended test substance concentrations, spaced at log intervals, should

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

5.1.6 Negative, Solvent and Positive Controls

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

For a competitive binding assay, a set of solvent control tubes containing the AR, the radiolabeled reference androgen, and the solvent used to dissolve the test substance is required to determine total (maximum)

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

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

5.1.7 Within-Test Replicates

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

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

5.1.8 Data Analysis

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

and the test substance are used to calculate the RBA value of the test substance.

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

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

5.1.9 Good Laboratory Practice Compliance

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

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

5.1.10 Study Acceptance Criteria

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

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

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5.1.11 Interpretation of Results

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

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

5.1.12 Repeat Studies

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

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

5.1.13 Study Report

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

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

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

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

Test Substance
- name, chemical structure (if known), and CASRN (if known), and supplier or source
- physical nature (solid or liquid) and purity, if known (every attempt should be made to determine the purity)

- physicochemical properties relevant to the study (e.g., solubility, pH, stability, volatility)
- concentrations and volumes used

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

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

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

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

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

Discussion of Results
- reproducibility of the K_d of the reference androgen and B_{max} of the AR, compared to historical data

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- historical IC_{50} values for the unlabeled reference androgen, including ranges, means, standard deviations, confidence intervals
- reproducibility of the IC_{50} values of the unlabeled reference androgen, compared to historical data
- historical IC_{50} and RBA values for the additional positive control substance (if used) with ranges, means, standard deviations, and confidence intervals
- reproducibility of the IC_{50} and RBA values for the additional positive control substance, if used, compared to historical data
- the test substance dose-response relationship for inhibition of binding of the radiolabeled reference androgen to the AR

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

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

5.2 Recommended Substances for Validation of *In Vitro* Androgen Receptor Binding Assays [2]

To facilitate validation of *in vitro* AR binding assays, ICCVAM has compiled a list of 78

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

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

negative response had not been demonstrated at test substance concentration up to 1 mM). Using these criteria, no substances could be classified as negative for AR binding. The presumed negative substances are listed below the sixth RBA category (<0.001) and include the maximum HDT used among studies, if available, in addition to the number of studies and assays in which the substance was tested. No effort was made to assess the validity and quality of each negative or positive response reported for each substance in each study. Following the presumed negative substances are those that have not been tested for AR binding activity. These substances have been assigned a presumed positive or negative response in *in vitro* AR binding assays based on the substances' anticipated or known mechanism of action and their response in *in vitro* AR TA assays. Presumed positive substances are listed first, followed by presumed negative substances that have been selected for the minimal list of substances (see below and **Section 2.4.4**). Both categories are sorted alphabetically by substance name. The other substances that are presumed negative are sorted alphabetically at the end of the list.

Substances have been classified as presumed positive even when reported as positive for AR binding in less than 50% of the studies conducted. This classification is because erroneous positive studies are probably less likely than erroneous negative studies due to the nature of AR binding assays and the protocols generally used. For example, in many negative studies, the HDT was below the IC_{50} value obtained in positive studies reported for that substance. The classification of a substance as positive (and its ranking), presumed positive, or presumed negative in this list is based sometimes on the results of a single study and, therefore, the accuracy of the classification is questionable. However, it is anticipated that testing these presumed positive and negative

substances will provide critical information on the comparative sensitivity and reproducibility of different *in vitro* AR binding assays, when such methods are standardized and conducted using the recommended minimum procedural standards.

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

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

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Table 5-1: ICCVAM Recommended Substances for Validation of *In Vitro* AR Binding Assays [a]

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

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Table 5-1: ICCVAM Recommended Substances for Validation of *In Vitro* AR Binding Assays [a]*(continued)*

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

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Table 5-1: ICCVAM Recommended Substances for Validation of *In Vitro* AR Binding Assays [a] *(continued)*

RBA Range [b] (Classification)	Substance	CASRN	Median RBA Value and RBA Range [c]	No. Positive Responses/ No. Times Tested	No. AR Binding Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing [d]	Comments	Chemical Class
<0.01 to 0.001 (Positive)	*o,p'*-DDT [e]	789-02-6	0.00105 (0.00058 - 0.00786)	3/3	2	U; J(1G,F,A)	Weak AR and ER antagonist; Weak ER agonist	Organochlorine; Diphenylalkene
	4-tert-Octylphenol [h,k]	140-66-9	0.0001	1/1	1	J(U,H,1G,F,A)	ER agonist	Alkylphenol; Phenol
<0.001 (Presumed Positive)	Kepone (Chlordecone) [e]	143-50-0	0.00072 (0.00063 - 0.008)	2/2	2		Binds to AR and ER	Organochlorine; Chlorinated bridged cycloalkane
	p,p'-Methoxychlor [e]	72-43-5	0.00053 (0.000068 - 0.001)	2/2	2	U; F&M-PA; IUL; IM; FRS; 2G(avian)	AR antagonist; Weak ER agonist	Organochlorine; Chlorinated hydrocarbon
	Corticosterone [e]	50-22-6	0.000068	1/1	1			Steroid, nonphenolic
	Procymidone [e]	32809-16-8	0.000068	1/1	1	H	AR antagonist	Organochlorine; Cyclic imide
	Bisphenol A [k]	80-05-7	0.000094	1/1	1	U; F-PA; J(1G,F,A)	Weak ER agonist	Diphenylalkane; Bisphenol; Phenol
No RBA Value (Presumed Negative)	Diethylhexyl phthalate [k]	117-81-7	HDT - 10 μM	0/1	1	J(U,H,1G,F,A)		Phthalate
	Dexamethasone [e,l]	50-02-2	HDT - 10 μM	0/2	2		AR agonist	Steroid, nonphenolic
ANTICIPATED RESPONSES								
RBA Data Not Available (Presumed Positive)	Fluoranthene	206-44-0	Pos.				AR antagonist	Polycyclic aromatic hydrocarbon; Fluorene
	Ketoconazole	65277-42-1	Pos.			F&M-PA; IM	Weak AR agonist	Imidazole; Piperazine

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Table 5-1: ICCVAM Recommended Substances for Validation of *In Vitro* AR Binding Assays [a](continued)

RBA Range [b] (Classification)	Substance	CASRN	Median RBA Value and RBA Range [c]	No. Positive Responses/ No. Times Tested	No. AR Binding Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing [d]	Comments	Chemical Class
RBA Data Not Available (Presumed Positive)	*p-n*-Nonylphenol [m]	104-40-5	Pos.			U; 407; J(U,H,1G,F,A)	AR antagonist	Alkylphenol; Phenol
RBA Data Not Available (Presumed Negative)	**Actinomycin D**	50-76-0	Neg.				RNA synthesis inhibitor	Phenoxazone; Lactone; Peptide
	Di-*n*-butyl phthalate	84-74-2	Neg.			U; M-PA; 1G; J(U,H,1G,F,A)	ER agonist	Phthalate
	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-Hydroxy-tamoxifen	68047-06-3	Neg.				ER antagonist	Triphenylethylene; Benzylidene; Stilbene; 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-13acetate	16561-29-8	Neg.				Activates ligand independent cell division	Phorbol ester; Terpene
	2,4,5-Trichloro-phenoxyacetic 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

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Table 5-1: ICCVAM Recommended Substances for Validation of *In Vitro* AR Binding Assays[a]*(continued)*

RBA Range[b] (Classification)	Substance	CASRN	Median RBA Value and RBA Range[c]	No. Positive Responses/No. Times Tested	No. AR Binding Assays in Which Tested	Completed/Anticipated *In Vivo* Testing[d]	Comments	Chemical Class
	Anastrazole	120511-73-1	Neg.			IM	Aromatase inhibitor	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
	Butylbenzyl phthalate	85-68-7	Neg.			IUL	ER agonist	Phthalate
RBA Data Not Available (Presumed Negative)	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
	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
	Dibenzo[*a,h*]-anthracene	53-70-3	Neg.					Polycyclic aromatic hydrocarbon; Anthracene

Table 5-1: ICCVAM Recommended Substances for Validation of *In Vitro* AR Binding Assays [a]*(continued)*

RBA Range [b] (Classification)	Substance	CASRN	Median RBA Value and RBA Range [c]	No. Positive Responses/ No. Times Tested	No. AR Binding Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing [d]	Comments	Chemical Class
	17α-Estradiol	57-91-0	Neg.				ER agonist	Steroid, phenolic; Estrene
	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
RBA Data Not Available (Presumed Negative)	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	Steroid, phenolic
	Kaempferol	520-18-3	Neg.				ER agonist	Flavanoid; Flavone; Phenol
	Morin	480-16-0	Neg.				Binds weakly to the ER	Flavanoid; Flavone; Phenol
	Norethynodrel	68-23-5	Neg.				Binds to the ER	Steroid, nonphenolic; Norpregnene
	Oxazepam	604-75-1	Neg.			IM	Enhances thyroid hormone excretion	Benzodiazepine
	Phenolphthalin	81-90-3	Neg.					Triphenylmethane; Diphenylalkane carboxylic acid

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Table 5-1: ICCVAM Recommended Substances for Validation of *In Vitro* AR Binding Assays[a]*(continued)*

RBA Range[b] (Classification)	Substance	CASRN	Median RBA Value and RBA Range[c]	No. Positive Responses/ No. Times Tested	No. AR Binding Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing[d]	Comments	Chemical Class
RBA Data Not Available (Presumed Negative)	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.

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

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

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

Table 5-1: ICCVAM Recommended Substances for Validation of *In Vitro* AR Binding Assays ᵃ*(continued)*

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

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

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

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

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

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

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

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

m   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 con-tains only one isomer consisting of a linear alkyl chain (CASRN 104-40-5). ICCVAM recommends the linear form, which has a uniform chemical structure, for validation studies.

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