3.0 *IN VITRO* ESTROGEN RECEPTOR BINDING ASSAYS

3.1 Minimum Procedural Standards

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

3.1.1 Animal Studies

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

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

3.1.2 Reference Estrogen

The displacement of a radiolabeled reference estrogen from the ER in a competitive binding study is used to identify substances that bind to the ER. 17β-Estradiol (CASRN 50-28-2) should be used as the reference estrogen in all ER binding assays; the hexa-tritium-labeled form (i.e., [2,3,6,7,16,17-^{3}H] 17β-estradiol) is recommended. The relative binding affinity (RBA), a measure of relative activity, of a test substance is equal to the IC_{50} of the unlabeled reference estrogen divided by the IC_{50} of the test substance, multiplied by 100. The IC_{50} is the (calculated) concentration that inhibits the binding of the radiolabeled reference estrogen to the ER by 50%, and is determined by simultaneously incubating the ER with a saturating amount of the radiolabeled estrogen and a range of concentrations of the test substance or the unlabeled reference estrogen. The concentration range used for the unlabeled estrogen should be 1 nM to 1 µM. IC_{50} and RBA values should be calculated and presented for all *in vitro* ER binding assays.

Section 3

Rationale: 17β-Estradiol is recommended because it is the most potent naturally occurring estrogen in the human body. The commercially available hexa-tritium-labeled form offers the highest specific radioactivity, which increases the sensitivity of competitive binding assays.

3.1.3 Dissociation Constant of the Reference Estrogen

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

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

[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 estrogen 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.

3.1.4 Preparation of Test Substances and Volume of Administered Solvent

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

Rationale: Selection of water, ethanol (95 to 100%), or DMSO as solvents is based on historical usage. Members of the Expert Panel stated that water or ethanol (95 to 100%) is

preferred to DMSO because some substances, when dissolved in DMSO, appear to bind with lower affinity to the receptor. For this reason, most investigators have not used DMSO at a final concentration greater than 0.1%. Because of possible differences in receptor protein sensitivity, the maximal concentration of a solvent that does not interfere with the performance should be determined for each test method.

3.1.5 Concentration Range of Test Substances

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

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

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

3.1.6 Negative, Solvent, and Positive Controls

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

For a competitive binding assay, a set of solvent control tubes containing the ER, the radiolabeled reference estrogen, and the solvent used to dissolve the test substance is required to determine total (maximum) binding of the radiolabeled reference estrogen to the ER. The solvent control should be added at the highest volume used to administer the test substance to the reaction mixture. A set of tubes to measure nonspecific binding and those containing a negative control substance,

Section 3

as described above, are also included in each study. In addition to the unlabeled reference estrogen, another positive control substance (e.g., norethynodrel, 4-*tert*-octylphenol) with a binding affinity that is between two and three orders of magnitude lower than the reference estrogen should be included in each study, and its IC_{50} and RBA values reported.

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

3.1.7 Within-Test Replicates

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

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

3.1.8 Data Analysis

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

The statistical methods used to calculate the B_{max}, K_d, and IC_{50} values should be justified. This includes a formal assessment of the nature of the statistical characteristics of the data (distribution, variance patterns, specific

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

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

3.1.9 Good Laboratory Practice Compliance

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

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

3.1.10 Study Acceptance Criteria

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

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

3.1.11 Interpretation of Results

A substance is classified as positive for binding to the ER if an IC_{50} value can be calculated. In general, the test substance should induce a sigmoid-shaped dose response curve over at least a few log concentrations. If a precipitous decrease in binding of the radiolabeled reference estrogen to the ER occurs over a narrow concentration range (i.e., over a one log increment), the response might reflect precipitation of the ER rather than competitive binding by the test substance. If a substance does not bind to the ER after

testing to the limit dose or to the maximum concentration possible based on its solubility (while not exceeding the limit dose), the test substance is classified as "negative" for binding to the ER under the conditions of the test. Test substances that induce a statistically significant reduction, but less than 50%, in binding of the radiolabeled reference estrogen to the ER, are classified as "equivocal".

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

3.1.12 Repeat Studies

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

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

3.1.13 Study Report

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

Reference Estrogen

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

Additional Positive Control (if used)

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

Negative Binding Control Substance

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

Test Substance

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

Solvent

- name, CASRN, purity, and supplier or source
- justification for choice of solvent
- information on the solubility of the test substance in all solvents in which it was tested

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

Estrogen Receptor

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

Study Conditions

- K_d of the reference estrogen and B_{max} of the ER
- rationale for the concentration of the radiolabeled reference estrogen in the binding assay
- protein concentration of ER used in the binding assay
- name(s) and concentration(s) of protease inhibitor(s) included in the animal tissue isolation buffer, if used
- composition of buffers used
- concentration range of the test substance, with justification
- volume of the solvent used to dissolve the test substance and the volume added to the reaction mixture
- incubation volume, duration, and temperature
- description of the solvent control
- type and composition of metabolic activation system, if used
- description of the method used to separate ER-bound and -unbound radiolabeled reference estrogen
- method used to analyze concentration of receptor-ligand complexes
- statistical method used to determine K_d, B_{max}, and IC_{50} values
- any other statistical method(s) used to assess the ability of the test substance to inhibit the binding of the radiolabeled reference estrogen

Results

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

Discussion of Results

- reproducibility of the K_d of the reference estrogen and B_{max} of the ER, compared to historical data
- historical IC_{50} values for the unlabeled reference estrogen, including ranges, means, standard deviations, and confidence intervals
- reproducibility of the IC_{50} values of the unlabeled reference estrogen, compared to historical data
- historical IC_{50} and RBA values for the additional positive control substance, if used, with ranges, means, standard deviations, and confidence intervals

- reproducibility of the IC_{50} and RBA values for the additional positive control substance compared to historical data
- the test substance dose-response relationship for inhibition of binding of the radiolabeled reference estrogen to the ER

Conclusion

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

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

Section 3

3.2 Recommended Substances for Validation of *In Vitro* Estrogen Receptor Binding Assays[2]

To facilitate validation of *in vitro* ER binding assays, ICCVAM has compiled a list of 78 recommended substances for use in future validation studies. The 78 substances are presented in **Table 3-1**, with a summary of available quantitative *in vitro* ER binding data for each substance. **Section 2.0** provides a detailed account of how these substances were selected. RBA data are available for 38 (49%) of these 78 recommended substances. Although 17β-estradiol is included in the list of recommended substances, it was not included in the count of substances for validation as it is the reference standard against which all test substances are compared. Quantitative *in vitro* ER binding data are provided for substances that induced a positive response in at least one study. This includes the median RBA value and the range of RBA values where more than one positive study had been conducted, and the number of studies and assays in which each substance was tested. In situations where only one positive study was reported, the RBA value obtained in that study is reported. The substances with RBA data are listed first, sorted by potency from strongest to weakest, based on the median or single RBA value of each substance across all positive studies. The median or single RBA values range from 234 to 0.0002, extending over seven orders of magnitude. Positive and "presumed positive" substances have been grouped into six RBA categories in log decrements: ≥10, <10 to 1, <1 to 0.1, <0.1 to 0.01, <0.01 to 0.001, and <0.001. Presumed positive substances induced a positive response in 50% or fewer of the ER binding studies in which they were tested. Substances were classified as negative if they did not induce at least a 50% reduction in the binding of the radiolabeled reference estrogen to the ER in multiple studies when tested up to the limit dose as defined in this document (i.e., 1 mM). Substances reported as negative for ER binding were classified as "presumed negative" if they had not been tested to the limit dose in multiple studies (i.e., reproducibility for a negative response had not been demonstrated at a test substance concentration up to 1 mM). Diethylhexylphthalate is the only substance that had been reported as negative when tested to the limit dose in multiple studies. The negative and presumed negative substances are listed below the sixth RBA category (<0.001) and include the highest dose tested (HDT) used among studies, if available, in addition to the number of studies and assays in which the substance was tested. No effort was made to assess the validity and quality of

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

each negative or positive response reported for each substance in each study. Following the presumed negative substances are those that have not been tested for ER binding activity. These substances have been assigned a presumed positive or negative response in *in vitro* ER binding assays based on the substances' anticipated or known mechanism of action and their response in *in vitro* ER TA assays. Presumed positive substances are listed first, followed by presumed negative substances that have been selected for the minimal list of substances (see below and **Section 2.4.4**). Both categories are sorted alphabetically by substance name. The other substances that are presumed negative are sorted alphabetically at the end of the list.

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

The quantitative and qualitative data provided with this substance list summarize information obtained primarily from peer-reviewed scientific reports. Because the positive data were obtained from studies using different *in vitro* ER 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 3-1** is provided to inform interested investigators of the historical quantitative values obtained for these substances in *in vitro* ER binding studies.

As described in **Section 2.4.4**, a subset of 53 substances has been identified that, at a minimum, should be used in any validation of *in vitro* ER binding assays. Of these substances, 75% (40) are classified as positive (22) or presumed positive (18) for ER binding, and 25% (13) are classified as negative (1) or presumed negative (12).

Table 3-1: ICCVAM Recommended Substances for Validation of *In Vitro* ER Binding Assays [a]

RBA Range [b] (Classification)	Substance	CASRN	Median RBA Value and RBA Range [c]	No. Positive Responses/ No. Times Tested	No. ER Binding Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing [d]	Comments	Chemical Class
>10 (Positive)	***meso*-Hexestrol** [e]	84-16-2	234 (58 - 302)	7/7	6		Strong ER agonist	Diphenylalkane; Bisphenol; Phenol
	4-Hydroxytamoxifen [e]	68047-06-3	172 (2.9 - 400)	18/18	13		ER antagonist	Triphenylethylene; Benzylidene; Stilbene; Phenol
	17α-Ethinyl estradiol [e]	57-63-6	148 (100 - 867)	10/10	7	U; 407; F-PA	Strong ER agonist	Steroid, phenolic
	Diethylstilbestrol [e]	56-53-1	124 (0.003 - 5000)	38/38	14	IUL	Strong ER agonist	Stilbene; Benzylidene; Diphenylalkene
	17β-Estradiol [e,f]	50-28-2	***100 (Reference estrogen)***	82/82	14	IM; IUL; FRS	Strong ER agonist; AR agonist and antagonist	Steroid, phenolic; Estrene
	Estrone [e]	53-16-7	41 (0.22 - 100)	18/18	13		Strong ER agonist; AR agonist	Steroid, phenolic; Estrene
	ICI 182,780 [e]	129453-61-8	37.5 (25 - 500)	5/5	4	IM	ER antagonist	Steroid, phenolic
	Zearalenone [e]	17924-92-4	15 (5 - 82)	12/12	10		ER agonist	Resorcylic acid lactone; Phenol
	Coumestrol [e]	479-13-0	12 (0.24 - 185)	15/15	11	IM	ER agonist	Coumestan; Benzopyranone; Coumarin; Ketone
	17α-Estradiol [e]	57-91-0	11 (0.22 - 1000)	9/9	7		ER agonist	Steroid, phenolic; Estrene
<10 to 1 (Positive)	**Tamoxifen** [e]	10540-29-1	4 (0.017 - 25)	21/21	14		ER antagonist	Triphenylethylene; Benzylidene; Stilbene
	Genistein [e]	446-72-0	1.45 (0.33 - 87)	18/18	11	U; 407	Weak ER agonist and antagonist	Flavanoid; Isoflavone; Phenol
	Apigenin [e]	520-36-5	1.15 (0.028 - 6)	4/4	3	IUL	ER agonist	Flavanoid; Flavone; Phenol
	Kaempferol [e]	520-18-3	1.05 (0.025 - 3)	4/4	3		ER agonist	Flavanoid; Flavone; Phenol

Table 3-1: ICCVAM Recommended Substances for Validation of *In Vitro* ER Binding Assays [a] *(continued)*

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

Section 3

Table 3-1: ICCVAM Recommended Substances for Validation of *In Vitro* ER Binding Assays [a] *(continued)*

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

Table 3-1: ICCVAM Recommended Substances for Validation of *In Vitro* ER Binding Assays[a] *(continued)*

RBA Range[b] (Classification)	Substance	CASRN	Median RBA Value and RBA Range[c]	No. Positive Responses/ No. Times Tested	No. of Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing[d]	Comments	Chemical Class
No RBA Value (Presumed Negative)	**Dibenzo[*a,h*]-anthracene[e]**	53-70-3	HDT - 5 µM	0/1	1			Polycyclic aromatic hydrocarbon; Anthracene
	Fluoranthene[e]	206-44-0	HDT - 5 µM	0/1	1		AR antagonist	Polycyclic aromatic hydrocarbon; Fluorene
	Corticosterone[e]	50-22-6	HDT - 100 µM	0/3	2		Binds weakly to AR	Steroid, nonphenolic
	Dexamethasone[e]	50-02-2	HDT - 100 µM	0/1	1		AR agonist	Steroid, nonphenolic
	Flavone[e,l]	525-82-6	HDT - 100 µM	0/3	3	M-PA; IM	Weak ER antagonist	Flavanoid; Flavone
	2,4,5-Trichlorophenoxyacetic acid[e]	93-76-5	HDT - 1000 µM	0/1	1		Weak ER agonist	Organochlorine; Chlorinated aromatic hydrocarbon
ANTICIPATED RESPONSES								
RBA Data Not Available (Presumed Positive)	**Fenarimol**	60168-88-9	Pos.			F-PA	Aromatase inhibitor; Weak ER agonist	Heterocycle; Pyrimidine
	Methyl testosterone	58-18-4	Pos.			H; 407; M-PA; IUL; FRS	ER and AR agonist	Steroid, nonphenolic; Androstene
RBA Data Not Available (Presumed Negative)	**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
	Phenobarbital	57-30-7	Neg.			F&M-PA; IM	Enhances thyroid hormone excretion	Heterocycle; Pyrimidine
	Propylthiouracil	51-52-5	Neg.			407; F&M-PA; IM; IUL; 2G	Inhibits T3/T4 synthesis	Pyrimidine; Uracil
	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
	Ammonium perchlorate	7790-98-9	Neg.			IUL	Thyroid disruptor	Organic acid; Organic salt

Section 3

Table 3-1: ICCVAM Recommended Substances for Validation of *In Vitro* ER Binding Assays[a] *(continued)*

RBA Range[b] (Classification)	Substance	CASRN	Median RBA Value and RBA Range[c]	No. Positive Responses/ No. Times Tested	No. ER Binding Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing[d]	Comments	Chemical Class
RBA Data Not Available (Presumed Negative)	Anastrazole	120511-73-1	Neg.			IM	Aromatase inhibitor	Nitrile; Triazole
	Apomorphine	58-00-4	Neg.			IM	Dopamine D1/D2 receptor agonist	Heterocycle; Quinoline
	Bicalutamide	90357-06-5	Neg.				AR antagonist	Anilide; Nitrile; Sulfone
	CGS 18320B	112808-99-8	Neg.			407	Aromatase inhibitor	Nitrile; Imidazole
	Cycloheximide	66-81-9	Neg.				Protein synthesis inhibitor	Piperidine; Glutaramide
	Cyproterone acetate	427-51-0	Neg.			IM	AR agonist and antagonist	Nitrile; Diphenyl ether; Organochlorine
	Finasteride	98319-26-7	Neg.			H; M-PA; IM	5α-reductase inhibitor	Steroid, nonphenolic; Androstene
	Fluoxymestrone	76-43-7	Neg.				Weak AR agonist	Steroid, nonphenolic
	Flutamide	13311-84-7	Neg.			H; 407; M-PA; IM; FRS	AR antagonist	Amide; Anilide; Nitrobenzene
	Haloperidol	52-86-8	Neg.			IM	Dopamine D2 receptor antagonist	Butyrophenone; Ketone; Piperazine
	Ketoconazole	65277-42-1	Neg.			F&M-PA; IM	Weak AR agonist	Imidazole; Piperazine
	Linuron	330-55-2	Neg.			M-PA	Weak AR agonist and antagonist	Urea
	Medroxyprogesterone acetate	71-58-9	Neg.				Weak AR agonist	Steroid, nonphenolic; Polycyclic hydrocarbon
	Methyltrienolone	965-93-5	Neg.				AR agonist	Steroid, nonphenolic; Estrene
	Mifepristone	84371-65-3	Neg.			IM	AR agonist and antagonist	Steroid, nonphenolic; Estrene
	Nilutamide	63612-50-0	Neg.				AR antagonist	Heterocycle; Imidazole

Table 3-1: ICCVAM Recommended Substances for Validation of *In Vitro* ER Binding Assays[a] *(continued)*

RBA Range [b] (Classification)	Substance	CASRN	Median RBA Value and RBA Range [c]	No. Positive Responses/ No. Times Tested	No. ER Binding Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing [d]	Comments	Chemical Class
RBA Data Not Available (Presumed Negative)	Oxazepam	604-75-1	Neg.			IM	Enhances thyroid hormone excretion	Benzodiazepine
	Pimozide	2062-78-4	Neg.			F&M-PA; IM	Dopamine receptor antagonist	Piperidine; Benzimidazole
	Procymidone	32809-16-8	Neg.				AR antagonist	Organochlorine; Cyclic imide
	Reserpine	50-55-5	Neg.			IM	Depletes dopamine	Heterocycle; Yohimban
	Spironolactone	52-01-7	Neg.				AR agonist and antagonist	Steroid, nonphenolic; Pregnene lactone
	L-Thyroxine	51-48-9	Neg.			407	Thyroid hormone	Aromatic amino acid
	17β -Trenbolone	10161-33-8	Neg.			H	Binds strongly to AR	Steroid, nonphenolic; Estrene

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

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

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

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

Table 3-1: ICCVAM Recommended Substances for Validation of *In Vitro* ER Binding Assays [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 indicated in this list. The *in utero* through lactation assay (IUL) has been recommended, but EPA has not made a decision on its further development or validation.

[e] Information regarding the median RBA value, the corresponding RBA range, the number of ER binding test methods used, and the number of positive responses per number of studies conducted was derived from data presented in **Appendix D** of the NICEATM ER Binding BRD cited in footnote c. This document contains *in vitro* ER binding data from the published literature through September 30, 2001.

[f] 17β-Estradiol is not considered a positive test substance for validation purposes, since it is the recommended reference estrogen for *in vitro* ER binding and TA assays (refer to **Section 3.2** for more information).

[g] Clomiphene citrate is classified presumed positive because only a single positive study was reported for this substance.

[h] 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.

[i] Testosterone and *p,p*'-DDE are classified presumed positive because these substances tested positive in less than 50% of the reported *in vitro* ER binding studies.

[j] Atrazine has been associated with mammary tumors in rats (O'Connor et al. 2000), but it is thought to act through a mechanism other than binding to the ER (Connor et al. 1996; O'Connor et al. 2000; Sanderson et al. 2001). To be consistent with the classification scheme used for other substances where the proportion of positive studies is 50% or less, atrazine is classified presumed positive.

[k] The HDT for diethylhexyl phthalate was 5000 µM in one study and 1000 µM in two studies.

[l] The HDT for flavone was 100 µM in one study and 10 µM in two studies.