4.0 *IN VITRO* ESTROGEN RECEPTOR TRANSCRIPTIONAL ACTIVATION ASSAYS

4.1 Minimum Procedural Standards

More than 95 different *in vitro* assays have been used to evaluate the ability of substances to act as ER TA agonists or antagonists (NIEHS 2002b). Of the 95 *in vitro* ER TA assays considered in the ER TA BRD, 63 used mammalian cell lines, 22 used yeast cells, and 10 measured cell proliferation. The Expert Panel recommended that assays using yeast and those measuring cell proliferation not be considered for future validation efforts. Yeast-based assays were not recommended due to the poor transport of many substances across the yeast cell wall, while assays based on cell proliferation were not recommended because cell proliferation can be mediated through pathways other than those involving transcriptional activation of estrogen responsive genes. No validation studies have been conducted to assess the performance and reliability of these test methods, and the few substances tested multiple times using the same or different test methods preclude an assessment of comparative assay performance. Although the Expert Panel concluded that no specific *in vitro* ER TA test method could be recommended currently as a priority for validation, assays using cells with an endogenous or stably transfected ER and a stably or transiently transfected reporter vector containing the luciferase (Luc) gene were thought to be the most effective and reliable (see **Appendix A**). To assist in the development, standardization, and validation of *in vitro* ER TA assays, NICEATM and the EDWG developed proposed minimum procedural standards for consideration by the Expert Panel (NIEHS 2002b). 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. Such standards also enhance any assessment of the comparative performance of different ER TA assays. The minimum procedural standards provided here have been revised to incorporate recommendations and comments of the Expert Panel, the EDWG, and the public. Except where noted, all *in vitro* ER TA assays should incorporate these minimal procedural standards in their protocols, and scientific justification should be provided for any deviations.

4.1.1 Reference Estrogen and TA Response

4.1.1.1 Agonism assays

The purpose of the reference estrogen in ER TA agonism assays is to demonstrate the adequacy of the test method for detecting ER agonists (i.e., the reference estrogen serves as a positive control). The recommended reference estrogen is 17β-estradiol (CASRN 50-28-2). The TA-inducing ability of the reference estrogen should be demonstrated by generating a full dose-response curve in each study. The concentration of 17β-estradiol used in most *in vitro* TA agonism assays ranges from 1 pM to 1 μM.

Rationale: 17β-Estradiol is the most potent naturally occurring estrogen in the human body, and virtually all published *in vitro* ER TA agonism studies have used this substance as the reference estrogen. Test acceptance criteria for the positive control should be established based on historical data for the maximum induction and on the calculated concentration of the reference estrogen that induces a half-maximal response (i.e., the effective concentration [EC_{50}] value).

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4.1.1.2 Antagonism assays

In ER TA antagonism assays, test substances are evaluated for their ability to reduce the level of TA induced by a reference estrogen. The concentration of the reference estrogen selected for antagonism assays should be within the upper linear region of the dose-response curve; 70 to 80% of maximal induction is recommended. The recommended reference estrogen for these assays is 17β-estradiol.

Rationale: 17β-Estradiol is the most potent naturally occurring estrogen in the human body, and virtually all published *in vitro* ER TA antagonism studies have used this substance as the reference estrogen. The ability to detect a weak antagonist depends on the magnitude of the TA response induced by the reference estrogen. Using a reference estrogen concentration that elicits a response within the upper linear portion of the dose-response curve maximizes the sensitivity of the test method.

4.1.2 Preparation of Test Substances and Volume of Administered Solvent

Test substances should be dissolved in a solvent that is miscible with the cell medium. Water, ethanol (95 to 100%), or DMSO is the preferred solvent. Preference should be given to the solvent that allows testing of the test substance at the maximal concentration possible without exceeding the limit dose (see **Section 4.1.3**). However, in testing situations where more than one solvent could be used, preference should be given to water, followed by ethanol (95 to 100%), and then DMSO. Other solvents may be used if it can be demonstrated that they are not cytotoxic and otherwise do not interact with the test system. The volume of the solvent included in the reaction mixture generally has ranged from 0.1 to 1% of the total volume. For any solvent, it should be demonstrated that the maximum volume used does not interfere with the test system. This can be accomplished by comparing the maximum fold induction and the mean EC_{50} value for the reference estrogen in the presence and absence of the solvent at the highest volume to be used in the TA studies. The stability of the dissolved test substance should be determined prior to testing. In the absence of stability information, the stock solution should be prepared fresh prior to use.

Rationale: Selection of water, ethanol (95 to 100%), or DMSO as suitable solvents is based on historical usage. Members of the Expert Panel stated that water or ethanol (95 to 100%) is preferred to DMSO because some substances, when dissolved in DMSO, might result in reduced activity (see **Section 4.1.4**). For this reason, most investigators have limited the final concentration of DMSO to less than 0.1%. Because of differences in the sensitivity of various cell lines, the maximal concentration of a solvent that does not interfere with performance should be determined for each test method.

4.1.3 Concentration Range of the Test Substances

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

Rationale: Most test method guidelines include a limit dose to ensure that all substances are tested over the same dose range while avoiding excessive amounts of a test substance

that can perturb the test system through physicochemical mechanisms. An established limit dose also minimizes the effort and cost of screening and testing. Based on the range of published EC_{50} values for ER agonists and IC_{50} values for ER antagonists (NIEHS 2002b), a limit dose of 1 mM was deemed suitable by the Expert Panel, the EDWG, and ICCVAM for assessing the ability of a test substance to act as either an ER agonist or an antagonist.

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

4.1.4 Solvent and Positive Controls

4.1.4.1 Solvent controls

Agonism Assays

In each study, a set of concurrent solvent control cultures should be included. The solvent control consists of the solvent in which the reference estrogen and the test substance are dissolved plus the cell line containing the ER, but without the reference estrogen. The solvent for the reference estrogen and test substance should be present at the highest volume that is used to add these substances to the test system. As indicated in **Section 4.1.2**, the solvent at the concentration used must not be cytotoxic or otherwise interact with the test system.

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

Antagonism Assays

A concurrent set of solvent control cultures should be included in each study. The solvent controls consist of the solvent in which the reference estrogen and the test substance are dissolved, the cell line containing the ER, and the test method specific concentration of the reference estrogen (based on achieving 70 to 80% of the maximum TA of the reference estrogen). The solvent for the reference estrogen and test substance should be present at the highest volume that is used to add these substances to the test system. As indicated in **Section 4.1.2**, the solvent at the concentration used must not be cytotoxic or otherwise interact with the test system.

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

4.1.4.3 Positive control

Agonism Assays

In addition to the standard potent reference estrogen, it might be useful to include in each study a positive control estrogen (e.g., genistein) with a maximal TA response two to three orders of magnitude lower than the reference estrogen.

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

study should be evaluated during the validation process.

Antagonism Assays

A known ER antagonist (e.g., ICI 182,780) should be included as a positive antagonist control in each antagonism study. The concentration of the reference antagonist that is used should be one that reduces the ability of the reference estrogen to induce TA in the test system by 70 to 90%. The positive antagonist control should also be tested in the absence of the reference estrogen to determine whether it alone can induce TA.

Rationale: The purpose of the positive antagonist control is to demonstrate the sensitivity and reproducibility of the *in vitro* ER TA antagonism assay. A range of doses of a positive control antagonist that inhibits the ability of the reference estrogen to induce TA will allow for historical confidence intervals to be calculated, which can be used as a QC measure to ensure the adequacy of each study. ICI 182,780 is suggested as the candidate ER antagonist as this substance historically has been shown to be negative as an agonist but positive as an antagonist. Other substances that may be used as a positive control antagonist should produce a similar response.

4.1.5 Within-Test Replicates

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

Rationale: The purpose of triplicate tubes for each concentration and volume of the various controls, the reference 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 has been collected using an optimized test method protocol.

4.1.6 Data Analysis

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

intervals for the EC_{50} or IC_{50} values for the reference estrogen/positive controls in agonism and antagonism studies, respectively, should be calculated and presented.

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

4.1.7 Good Laboratory Practice Compliance

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

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

4.1.8 Study Acceptance Criteria

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

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

4.1.9 Interpretation of Results

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

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

4.1.10 Repeat Studies

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

Rationale: Repeat studies are used in a validation study to demonstrate the intralaboratory repeatability and reproducibility of a test

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

4.1.11 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
- concentrations and volumes used

Additional Positive Control (if used)

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

Test Substance

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

Solvent

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

Estrogen Receptor

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

Reporter Plasmid

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

Cell Line

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

- method used to monitor the stability of a stably transfected cell line used for testing
- rationale, based on data, for deciding on the number of passages a cell line can undergo without a decrease in activity
- details regarding selection requirements needed for maintaining stable cell lines

Study Conditions

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

Results

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

Discussion of Results

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

Conclusion

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

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

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independent replication of the study, if deemed necessary.

4.2 Recommended Substances for Validation of *In Vitro* Estrogen Receptor Transcriptional Activation Assays[1]

To facilitate validation of *in vitro* ER TA assays, ICCVAM has compiled a list of 78 recommended substances for use in future validation studies. Separate lists are provided of the available quantitative and qualitative data and anticipated responses of each of the 78 substances in *in vitro* ER TA agonism (**Table 4-1**) and antagonism (**Table 4-2**) assays. **Section 2.0** provides a detailed account of how these substances were selected. EC_{50} and IC_{50} data are available for 18 (23%) and 8 (10%) of these 78 recommended substances for agonism and antagonism, respectively. Qualitative data are available for 27 (35%) and 10 (13%) of these 78 recommended substances for agonism and antagonism, respectively. Thus, there is incomplete information regarding how all 78 of the recommended substances will respond in *in vitro* ER TA agonism and antagonism assays utilizing mammalian cell reporter gene systems. 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 a required component of the test system to measure antagonism and is the positive control for agonism studies. Quantitative *in vitro* ER TA data are provided for the substances inducing a positive response in at least one study. This includes the median EC_{50} or IC_{50} values for agonism and antagonism studies, respectively, a range of values where more than one study had been conducted, and the number of studies and test methods in which each substance was tested. In situations where only one positive study was reported, the EC_{50} or IC_{50} value obtained in that study is reported. The substances with EC_{50} or IC_{50} data are listed first, sorted by potency from strongest to weakest, based on the median EC_{50} or IC_{50} value of each substance across all positive studies. Substances that induced a positive response in 50% or fewer of the ER TA studies in which they were tested are classified in this table as "presumed positive" for ER agonism or antagonism. No effort was made to assess the validity and quality of each negative or positive study reported for each substance. These substances are sorted by most positive responses per number of times tested. Substances were classified as negative for ER TA agonism or antagonism activity if they were reported as negative in multiple studies when tested up to the limit dose as defined in this document (i.e., 1 mM). Substances were classified as "presumed negative" for ER TA activity if they had not been tested to the limit dose in multiple studies (i.e., reproducibility for a negative response had not been demonstrated at test substance concentration up to 1 mM). Using these criteria, no substances could be classified as negative for ER TA activity. Following the presumed negative substances are those without relevant *in vitro* ER TA data. Substances lacking either quantitative or qualitative data have been assigned a presumed positive or negative response in *in vitro* ER TA assays, based on the substances' anticipated or known mechanism of action and response in *in vitro* ER binding assays. Presumed positive substances are listed first, followed by presumed negative substances that have been selected for the minimal list of substances (see below and **Section 2.4.4**). Both categories are sorted alphabetically by substance name. The remaining substances that are presumed

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

negative are sorted alphabetically at the end of the list.

Substances have been classified as presumed positive for agonism even when less than 50% of the studies were positive. Without detailed information regarding the experimental protocol used, it is not possible to assess the quality of the data. However, with the ER TA agonism tests, false positive responses are possible if the cell line used in the study contains a glucocorticoid or progesterone receptor and the mouse mammary tumor virus hormone response element is incorporated into the reporter construct. The classification of a substance as positive (and its ranking) or negative in this list is based sometimes on the results of a single study and, therefore, the accuracy of the classification is questionable. However, it is anticipated that testing these presumed positive and negative substances will provide critical information on the comparative sensitivity and reproducibility of different *in vitro* ER TA 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 TA assays, they show a great deal of variability and, thus, the reported values should not be used as definitive target values to be obtained during the validation process. The data summaries presented in **Tables 4-1** and **4-2** are provided to inform interested investigators of the historical quantitative values obtained for these substances in *in vitro* ER TA assays.

As described in **Section 2.4.4**, and mentioned above, a subset of 53 substances has been identified that, at a minimum, should be used in any validation of *in vitro* ER TA assays. These 53 substances are in bold type in **Table 4-1** for agonism. Of these substances, 64% (34) are classified as positive (21) or presumed positive (13) for ER agonism, and 36% (19) are classified as presumed negative. The same 53 substances are in bold type in **Table 4-2** for antagonism. Of these substances, 21% (11) are classified as positive (5) or presumed positive (6) for ER antagonism, and 79% (42) are classified as presumed negative.

Table 4-1: ICCVAM Recommended Substances for Validation of *In Vitro* ER TA Agonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems[a]

Substance	CASRN	Median EC_{50} Value and Range Across All MCRG Studies[b] (µM)	Qualitative Response for ER Agonism Across All MCRG Studies[c]	No. of MCRG Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing[d]	Comments	Chemical Class
17α-Ethinyl estradiol[e]	57-63-6	0.000011 (2) 0.0000073 - 0.0000144	Pos. (2/2)	2	U; 407; F-PA	Strong ER agonist	Steroid, phenolic
Diethylstilbestrol[e]	56-53-1	0.000019 (3) 0.000015 - 0.000024	Pos. (8/8)	8	IUL	Strong ER agonist	Stilbene; Benzylidene; Diphenylalkene
17α-Estradiol[e]	57-91-0	0.000046 (1)	Pos. (2/2)	2		ER agonist	Steroid, phenolic; Estrene
17β-Estradiol[e,f]	50-28-2	0.0001 (29) 0.000005 - 0.0099	Pos. (77/77)	45	IM; IUL; FRS	Strong ER and AR agonist; AR antagonist	Steroid, phenolic; Estrene
***meso*-Hexestrol**[e]	84-16-2	0.0002 (1)	Pos. (1/1)[j]	1		Strong ER agonist	Diphenylalkane; Bisphenol; Phenol
Zearalenone[e]	17924-92-4	0.002 (3) 0.001 - 0.0073	Pos. (8/8)	6		ER agonist	Resorcylic acid lactone; Phenol
Estrone[e]	53-16-7	0.0032 (2) 0.00002 - 0.0063	Pos. (3/3)	3		Strong ER agonist; AR agonist	Steroid, phenolic; Estrene
Methyl testosterone[e]	58-18-4	0.011 (2) 0.00573 - 0.0158	Pos. (2/2)	2	H; 407; M-PA; IUL; FRS	ER and AR agonist	Steroid, nonphenolic; Androstene
Coumestrol[e]	479-13-0	0.015 (3) 0.01 - 0.017	Pos. (8/8)	7	IM	ER agonist	Coumestan; Benzopyranone; Coumarin; Ketone
Genistein[e]	446-72-0	0.062 (5) 0.00423 - 0.1	Pos. (11/11)	10	U; 407	Weak ER agonist and antagonist	Flavanoid; Isoflavone; Phenol
***p*-n-Nonylphenol**[e,g]	104-40-5	0.085 (3) 0.0356 - 0.26	Pos. (4/4)	4	U; 407; J(U,H,1G,F,A)	ER and AR antagonist; ER agonist	Alkylphenol; Phenol
Bisphenol B[e]	77-40-7	0.088 (2) 0.0624 - 0.114	Pos. (2/2)	2		ER agonist	Diphenylalkane; Bisphenol; Phenol

Table 4-1: ICCVAM Recommended Substances for Validation of *In Vitro* ER TA Agonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems[a] *(continued)*

Substance	CASRN	Median EC_{50} Value and Range Across All MCRG Studies[b] (µM)	Qualitative Response for ER Agonism Across All MCRG Studies[c]	No. of MCRG Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing[d]	Comments	Chemical Class
Daidzein[e]	486-66-8	0.29 (2) 0.09 - 0.49	Pos. (5/5)	5		Weak ER agonist	Flavanoid; Isoflavone; Phenol
4-Cumylphenol[e]	599-64-4	0.322 (2) 0.248 - 0.395	Pos. (2/2)	2		Weak ER agonist	Phenol
Bisphenol A[e]	80-05-7	0.40 (10) 0.000033 - 0.89	Pos. (15/15)	13	U; F-PA; J(1G,F,A)	ER agonist	Diphenylalkane; Bisphenol; Phenol
***o,p'*-DDT[e]**	789-02-6	0.66 (1)	Pos. (7/8)	8	U; J(1G,F,A)	Weak ER and AR antagonist; Weak ER agonist	Organochlorine; Diphenylalkene
***p,p'*-Methoxychlor[e]**	72-43-5	8.85 (2) 5.7 - 12	Pos. (12/13)	13	U; F&M-PA; IUL; IM; FRS; 2G(avian)	Weak ER agonist; AR antagonist	Organochlorine; Chlorinated hydrocarbon
Fenarimol[h]	60168-88-9	*27* (1)	Pos. (1/1)[j]	1	F-PA	Aromatase inhibitor	Heterocycle; Pyrimidine
QUALITATIVE DATA ONLY							
Apigenin[e,i]	520-36-5		Pos. (6/6)	5	IUL	ER agonist	Flavanoid; Flavone; Phenol
Tamoxifen[e,i]	10540-29-1		Pos. (5/7)	6		ER antagonist	Triphenylethylene; Benzylidene; Stilbene
Kepone[e,i] (Chlordecone)	143-50-0		Pos. (4/6)	6		Binds to ER and AR	Organochlorine; Chlorinated bridged cycloalkane
Butylbenzyl phthalate[e,i]	85-68-7		Pos. (3/4)	4	IUL	ER agonist	Phthalate
4-Hydroxytamoxifen[e,i]	68047-06-3		Pos. (3/8)[j]	8		ER antagonist	Triphenylethylene; Benzylidene; Stilbene; Phenol

Table 4-1: ICCVAM Recommended Substances for Validation of *In Vitro* ER TA Agonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems[a] *(continued)*

Substance	CASRN	Median EC_{50} Value and Range Across All MCRG Studies[b] (µM)	Qualitative Response for ER Agonism Across All MCRG Studies[c]	No. of MCRG Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing[d]	Comments	Chemical Class
Kaempferol[e,i]	520-18-3		Pos. (2/2)	2		ER agonist	Flavanoid; Flavone; Phenol
***4-tert*-Octylphenol**[e,i]	140-66-9		Pos. (2/3)	3	J(U,H,1G,F,A)	ER agonist	Alkylphenol; Phenol
***p,p'*-DDE**[e,i]	72-55-9		Pos. (2/4)[j]	4	H; 407; M-PA; IM; J(1G,F,B)	Weak AR agonist and antagonist	Organochlorine; Diphenylalkene
Di-*n*-butyl phthalate[e,i]	84-74-2		Pos. (2/4)[j]	4	U; M-PA; 1G; J(U,H,1G,F,A)	ER agonist	Phthalate
Flavone[e,i]	525-82-6		Pos. (2/5)[j]	4	M-PA; IM	Weak ER antagonist	Flavanoid; Flavone
Dexamethasone[e,i]	50-02-2		Pos. (1/1)[j]	1		AR agonist	Steroid, nonphenolic
5α-Dihydro-testosterone[e,i]	521-18-6		Pos. (1/1)[j]	1	H	Weak ER agonist; Strong AR agonist	Steroid, nonphenolic
2,4,5-Trichloro-phenoxyacetic acid[e,i]	93-76-5		Pos. (1/1)[j]	1		Weak ER agonist	Organochlorine; Chlorinated aromatic hydrocarbon
Dibenzo[*a,h*]-anthracene[e,i]	53-70-3		Pos. (1/2)[j]	2			Polycyclic aromatic hydrocarbon; Anthracene
ICI 182,780[e,i]	129453-61-8		Neg. (9/10)	9	IM	ER antagonist	Steroid, phenolic
Atrazine[e,i]	1912-24-9		Neg. (3/3)	3	M-PA; IUL		Aromatic amine; Triazine; Arylamine
Progesterone[e,i]	57-83-0		Neg. (2/2)	2	IM		Steroid, nonphenolic; Pregnenedione

Table 4-1: ICCVAM Recommended Substances for Validation of *In Vitro* ER TA Agonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems [a] *(continued)*

Substance	CASRN	Median EC_{50} Value and Range Across All MCRG Studies [b] (µM)	Qualitative Response for ER Agonism Across All MCRG Studies [c]	No. of MCRG Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing [d]	Comments	Chemical Class
Testosterone [e,i]	58-22-0		Neg. (2/2)	2	IM	Strong AR agonist	Steroid, nonphenolic
Corticosterone [e,i]	50-22-6		Neg. (1/1)	1		Binds weakly to AR	Steroid, nonphenolic
Phenobarbital [e,i]	57-30-7		Neg. (1/1)	1	F&M-PA; IM	Enhances thyroid hormone excretion	Heterocycle; Pyrimidine
Vinclozolin [e,i]	50471-44-8		Neg. (1/1)	1	H; M-PA; IM; IUL; 1G; FRS	AR antagonist	Organochlorine; Cyclic imide; Carbamate
Cyproterone acetate [e,i]	427-51-0		Neg. (1/1)	1	IM	AR agonist and antagonist	Nitrile; Diphenyl ether; Organochlorine
Flutamide [e,i]	13311-84-7		Neg. (1/1)	1	H; 407; M-PA; IM; FRS	AR antagonist	Amide; Anilide; Nitrobenzene
Linuron [e,i]	330-55-2		Neg. (1/1)	1	H; M-PA	Weak AR agonist and antagonist	Urea
Methyltrienolone [e,i]	965-93-5		Neg. (1/1)	1		AR agonist	Steroid, nonphenolic; Estrene
Mifepristone [e,i]	84371-65-3		Neg. (1/1)	1	IM	AR agonist and antagonist	Steroid, nonphenolic; Estrene
Procymidone [e,i]	32809-16-8		Neg. (1/1)	1		AR antagonist	Organochlorine; Cyclic imide
ANTICIPATED RESPONSES (No EC_{50} or Qualitative Agonism Data Available)							
Clomiphene citrate	50-41-9		Pos.			Binds to the ER; Selective estrogen receptor modulator	Chlorinated triphenylethylene; Benzylidene; Stilbene

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Table 4-1: ICCVAM Recommended Substances for Validation of *In Vitro* ER TA Agonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems[a] *(continued)*

Substance	CASRN	Median EC_{50} Value and Range Across All MCRG Studies[b] (µM)	Qualitative Response for ER Agonism Across All MCRG Studies[c]	No. of MCRG Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing[d]	Comments	Chemical Class
Ethyl paraben	120-47-8		Pos.			Binds weakly to ER; Pos. in yeast ER agonism assay	Paraben; Organic acid
Norethynodrel	68-23-5		Pos.			Binds to ER	Steroid, nonphenolic; Norpregnene
Actinomycin D	50-76-0		Neg.			RNA synthesis inhibitor	Phenoxazone; Lactone; Peptide
4-Androstenedione	63-05-8		Neg.			Strong AR agonist; Neg. for ER agonism in yeast assay	Steroid, nonphenolic
2-*sec*-Butylphenol	89-72-5		Neg.				Phenol
Diethylhexyl phthalate	117-81-7		Neg.		J(U,H,1G,F,A)	Neg. ER binding	Phthalate
Fadrozole	102676-47-1		Neg.		F-PA; IM; FRS	Aromatase inhibitor	Imidazole; Nitrile
Fluoranthene	206-44-0		Neg.			AR antagonist; Neg. for ER agonism in yeast assay	Polycyclic aromatic hydrocarbon; Fluorene
Hydroxyflutamide	52806-53-8		Neg.			AR agonist and antagonist	Amide; Anilide; Nitrobenzene
Morin	480-16-0		Neg.			Binds weakly to ER	Flavanoid; Flavone; Phenol
Phenolphthalin	81-90-3		Neg.				Triphenylmethane; Diphenylalkane carboxylic acid

Table 4-1: ICCVAM Recommended Substances for Validation of *In Vitro* ER TA Agonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems [a] *(continued)*

Substance	CASRN	Median EC_{50} Value and Range Across All MCRG Studies [b] (µM)	Qualitative Response for ER Agonism Across All MCRG Studies [c]	No. of MCRG Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing [d]	Comments	Chemical Class
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
Anastrazole	120511-73-1		Neg.		IM	Aromatase inhibitor	Nitrile; Triazole
Apomorphine	58-00-4		Neg.		IM	Dopamine D1/D2 receptor agonist; Neg. for ER agonism in yeast assay	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
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

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Table 4-1: ICCVAM Recommended Substances for Validation of *In Vitro* ER TA Agonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems[a] *(continued)*

Substance	CASRN	Median EC_{50} Value and Range Across All MCRG Studies[b] (µM)	Qualitative Response for ER Agonism Across All MCRG Studies[c]	No. of MCRG Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing[d]	Comments	Chemical Class
Haloperidol	52-86-8		Neg.		IM	Dopamine D2 receptor antagonist; Neg. for ER agonism in yeast assay	Butyrophenone; Ketone; Piperazine
Ketoconazole	65277-42-1		Neg.		F&M-PA; IM	Weak AR agonist	Imidazole; Piperazine
Medroxyprogesterone acetate	71-58-9		Neg.			Weak AR agonist	Steroid, nonphenolic; Polycyclic hydrocarbon
Nilutamide	63612-50-0		Neg.			AR antagonist	Heterocycle; Imidazole
Oxazepam	604-75-1		Neg.		IM	Enhances thyroid hormone excretion	Benzodiazepine
Pimozide	2062-78-4		Neg.		F&M-PA	Dopamine receptor antagonist	Piperidine; Benzimidazole
Reserpine	50-55-5		Neg.		IM	Depletes dopamine; Neg. for ER agonism in yeast assay	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 the AR; Neg. for ER agonism in yeast assay	Steroid, nonphenolic; Estrene

Table 4-1: ICCVAM Recommended Substances for Validation of *In Vitro* ER TA Agonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems[a] *(continued)*

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

[c] Numbers in parentheses refer to the number of studies in which the substance was reported positive or negative compared to the number of studies in which it was tested. A substance is classified as "positive" for ER agonism if it was positive in more than 50% of reported studies. A substance is classified as "presumed positive" for ER agonism if it was positive in 50% or less of reported studies, or if it was reported positive in the single study conducted. Substances reported negative in their respective studies are classified as "presumed negative" instead of "negative" for ER agonism, since they had not been tested in multiple studies at or above the limit dose of 1 mM recommended in **Section 4.1.3**. Substances without data are classified "presumed positive" or "presumed negative" based on available information, including their known mechanism of action or their responses in ER binding assays, AR binding assays, or AR TA assays.

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

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

[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 4.2** for more information).

[g] 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.

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

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

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

Table 4-2: ICCVAM Recommended Substances for Validation of *In Vitro* ER TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems[a]

Substance	CASRN	Median EC_{50} Value and Range Across All MCRG Studies[b] (µM)	Qualitative Response for ER Antagonism Across All MCRG Studies[c]	No. of MCRG Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing[d]	Comments	Chemical Class
4-Hydroxtamoxifen[e,f]	68047-06-3	*0.00025* (1)	Pos. (10/10)	9		ER antagonist	Triphenylethylene; Benzylidene; Stilbene; Phenol
ICI 182,780[e,f]	129453-61-8	*0.003* (4) *0.00001 - 0.016*	Pos. (14/15)	13	IM	ER antagonist	Steroid, phenolic
Tamoxifen[e,f]	10540-29-1	*0.018* (2) *0.01 - 0.025*	Pos. (7/8)	7		ER antagonist	Triphenylethylene; Benzylidene; Stilbene
Zearalenone[e,f]	17924-92-4	*5* (1)	Pos. (2/3)	3		ER agonist	Resorcylic acid lactone; Phenol
Coumestrol[e,f]	479-13-0	*5* (1)	Pos. (1/3)[j]	3	IM	ER agonist	Coumestan; Benzopyranone; Coumarin; Ketone
Flavone[e,f]	525-82-6	*15* (1)	Pos. (1/1)[j]	1	M-PA; IM	Weak ER antagonist	Flavanoid; Flavone
Apigenin[e,f]	520-36-5	*20* (1)	Pos. (3/5)	4	IUL	ER agonist	Flavanoid; Flavone; Phenol
Genistein[e,f]	446-72-0	*25* (1)	Pos. (1/3)[j]	3	U; 407	Weak ER agonist and antagonist	Flavanoid; Isoflavone; Phenol
17β-Estradiol[e,g,h]	50-28-2		Neg. (8/8)	7	IM; IUL; FRS	Strong ER agonist; AR agonist and antagonist	Steroid, phenolic; Estrene
Kaempferol[e,g]	520-18-3		Neg. (3/3)	3		ER agonist	Flavanoid; Flavone; Phenol
Bisphenol A[e,g]	80-05-7		Neg. (2/2)	2	U; F-PA; J(1G,F,A)	ER agonist	Diphenylalkane; Bisphenol; Phenol
Daidzein[e,g]	486-66-8		Neg. (2/2)	2		Weak ER agonist	Flavanoid; Isoflavone; Phenol

Table 4-2: ICCVAM Recommended Substances for Validation of *In Vitro* ER TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems [a] *(continued)*

Substance	CASRN	Median EC_{50} Value and Range Across All MCRG Studies [b] (µM)	Qualitative Response for ER Antagonism Across All MCRG Studies [c]	No. of MCRG Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing [d]	Comments	Chemical Class
***p,p'*-DDE** [e,g]	72-55-9		Neg. (2/2)	2	H; 407; M-PA; IM; J(1G,F,A)	Weak AR agonist and antagonist	Organochlorine; Diphenylalkene
Kepone [e,g] **(Chlordecone)**	143-50-0		Neg. (2/2)	2		Binds to ER and AR	Organochlorine; Chlorinated bridged cycloalkane
***p,p'*-Methoxychlor** [e,g]	72-43-5		Neg. (2/2)	2	U; F&M-PA; IUL; IM; FRS; 2G(avian)	Weak ER agonist; AR antagonist	Organochlorine; Chlorinated hydrocarbon
Atrazine [e,g]	1912-24-9		Neg. (1/1)	1	IM		Aromatic amine; Triazine; Arylamine
Butylbenzyl phthalate [e,g]	85-68-7		Neg. (1/1)	1	IUL	ER agonist	Phthalate
Di-*n*-butyl phthalate [e,g]	84-74-2		Neg. (1/1)	1	U; M-PA; 1G; J(U,H,1G,F,A)	ER agonist	Phthalate
ANTICIPATED RESPONSES (No IC_{50} or Qualitative Antagonism Data Available)							
Clomiphene citrate	50-41-9		Pos.			Binds to the ER; Selective estrogen receptor modulator	Chlorinated triphenylethylene; Benzylidene; Stilbene
***o,p'*-DDT**	789-02-6		Pos.		U; J(1G,F,A)	Weak AR antagonist; Weak ER agonist; Pos. for ER antagonism in yeast assay	Organochlorine; Diphenylalkene
Dibenzo[*a,h*]-anthracene	53-70-3		Pos.				Polycyclic aromatic hydrocarbon; Anthracene

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Table 4-2: ICCVAM Recommended Substances for Validation of *In Vitro* ER TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems[a] *(continued)*

Substance	CASRN	Median EC_{50} Value and Range Across All MCRG Studies[b] (µM)	Qualitative Response for ER Antagonism Across All MCRG Studies[c]	No. of MCRG Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing[d]	Comments	Chemical Class
Actinomycin D	50-76-0		Neg.			RNA synthesis inhibitor	Phenoxazone; Lactone; Peptide
4-Androstenedione	63-05-8		Neg.			Strong AR agonist	Steroid, nonphenolic
Bisphenol B	77-40-7		Neg.			ER agonist	Diphenylalkane; Bisphenol; Phenol
2-*sec*-Butylphenol	89-72-5		Neg.				Phenol
Corticosterone	50-22-6		Neg.			Binds weakly to the AR	Steroid, nonphenolic
4-Cumylphenol	599-64-4		Neg.			Weak ER agonist	Phenol
Dexamethasone	50-02-2		Neg.			AR agonist	Steroid, nonphenolic
Diethylhexyl phthalate	117-81-7		Neg.		J(U,H,1G,F,A)		Phthalate
Diethylstilbestrol	56-53-1		Neg.		IUL	Strong ER agonist	Stilbene; Benzylidene; Diphenylalkene
5α-Dihydrotestosterone	521-18-6		Neg.		H	Weak ER agonist; Strong AR agonist	Steroid, nonphenolic
17α-Estradiol	57-91-0		Neg.			ER agonist	Steroid, phenolic; Estrene
Estrone	53-16-7		Neg.			Strong ER agonist; AR agonist	Steroid, phenolic; Estrene
17α-Ethinyl estradiol	57-63-6		Neg.		U; 407; F-PA	Strong ER agonist	Steroid, phenolic

Table 4-2: ICCVAM Recommended Substances for Validation of *In Vitro* ER TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems[a] *(continued)*

Substance	CASRN	Median EC_{50} Value and Range Across All MCRG Studies[b] (µM)	Qualitative Response for ER Antagonism Across All MCRG Studies[c]	No. of MCRG Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing[d]	Comments	Chemical Class
Ethyl paraben	120-47-8		Neg.			Binds weakly to ER	Paraben; Organic acid
Fadrozole	102676-47-1		Neg.		F-PA; IM; FRS	Aromatase inhibitor	Imidazole; Nitrile
Fenarimol	60168-88-9		Neg.		F-PA	Aromatase inhibitor	Heterocycle; Pyrimidine
Fluoranthene	206-44-0		Neg.			AR antagonist; Neg. for ER antagonism in yeast assay	Polycyclic aromatic hydrocarbon; Fluorene
***meso*-Hexestrol**	84-16-2		Neg.			Strong ER agonist	Diphenylalkane; Bisphenol; Phenol
Hydroxyflutamide	52806-53-8		Neg.			AR agonist and antagonist	Amide; Anilide; Nitrobenzene
Methyl testosterone	58-18-4		Neg.		H; 407; M-PA; IUL; FRS	ER and AR agonist	Steroid, nonphenolic; Androstene
Morin	480-16-0		Neg.			Binds weakly to ER	Flavanoid; Flavone; Phenol
***p*-n-Nonylphenol[i]**	104-40-5		Neg.		U; 407; J(U,H,1G,F,A)	ER agonist; AR antagonist	Alkylphenol; Phenol
Norethynodrel	68-23-5		Neg.			Binds to ER	Steroid, nonphenolic; Norpregnene
***4-tert*-Octylphenol**	140-66-9		Neg.		J(U,H,1G,F,A)	ER agonist; Neg. for ER antagonism in yeast assay	Alkylphenol; Phenol
Phenobarbital	57-30-7		Neg.		F&M-PA; IM	Enhances thyroid hormone excretion	Heterocycle; Pyrimidine

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Table 4-2: ICCVAM Recommended Substances for Validation of *In Vitro* ER TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems[a] *(continued)*

Substance	CASRN	Median EC_{50} Value and Range Across All MCRG Studies[b] (µM)	Qualitative Response for ER Antagonism Across All MCRG Studies[c]	No. of MCRG Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing[d]	Comments	Chemical Class
Phenolphthalin	81-90-3		Neg.				Triphenylmethane; Diphenylalkane carboxylic acid
Progesterone	57-83-0		Neg.		IM		Steroid, nonphenolic; Pregnenedione
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
Testosterone	58-22-0		Neg.		IM	Strong AR agonist	Steroid, nonphenolic
12-*O*-Tetradecanoyl-phorbol-13-acetate	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
Vinclozolin	50471-44-8		Neg.		H; M-PA; IM; IUL; 1G; FRS	AR antagonist	Organochlorine; Cyclic imide; Carbamate
Ammonium perchlorate	7790-98-9		Neg.		IUL	Thyroid disruptor	Organic acid; Organic salt
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

Table 4-2: ICCVAM Recommended Substances for Validation of *In Vitro* ER TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems [a] *(continued)*

Substance	CASRN	Median EC_{50} Value and Range Across All MCRG Studies [b] (µM)	Qualitative Response for ER Antagonism Across All MCRG Studies [c]	No. of MCRG Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing [d]	Comments	Chemical Class
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.		H; 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 4-2: ICCVAM Recommended Substances for Validation of *In Vitro* ER TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems[a] *(continued)*

Substance	CASRN	Median EC_{50} Value and Range Across All MCRG Studies[b] (µM)	Qualitative Response for ER Antagonism Across All MCRG Studies[c]	No. of MCRG Assays in Which Tested	Completed/ Anticipated *In Vivo* Testing[d]	Comments	Chemical Class
Oxazepam	604-75-1		Neg.		IM	Enhances thyroid hormone excretion	Benzodiazepine
Pimozide	2062-78-4		Neg.		F&M-PA	Dopamine receptor antagonist	Piperidine; Benzimidazole
Procymidone	32809-16-8		Neg.		H	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 the 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-chlorophenyl]ethylene; DDT = Dichlorodiphenyltrichloroethane; ER = Estrogen receptor; HDT = Highest dose tested; Neg. = Negative; Pos. = Positive; RBA = Relative binding affinity; T3 = Triiodothyronine; T4 = Thyroxine; TA = Transcriptional activation.

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

[b] An IC_{50} is the concentration of the test substance that inhibits 50% of the response of 17β-estradiol in a particular test system. Median IC_{50} values are derived from *in vitro* mammalian cell reporter gene studies that were either published in the peer-reviewed scientific literature or submitted to NICEATM, and then reviewed and summarized in the NICEATM Background Review Document (BRD) titled "Current Status of Test Methods for Detecting Endocrine Disruptors: *In Vitro* Estrogen Receptor Transcriptional Activation Assays-August 2002" (available on the ICCVAM website at *http://iccvam.niehs.nih.gov/methods/endocrine.htm*). Substances for which quantitative ER antagonism data are available are ranked according to their relative potency in ER mammalian cell reporter gene antagonism assays from most potent to least potent. Numbers in parentheses to the right of the IC_{50} value refer to the number of studies for which an IC_{50} value was reported in the BRD. An italicized IC_{50} value indicates the value was estimated from a graphical presentation of data. The range of values reported for a substance is listed below the median value.

Table 4-2: ICCVAM Recommended Substances for Validation of *In Vitro* ER TA Antagonism Assays That Use Mammalian Cell Reporter Gene (MCRG) Systems[a] *(continued)*

[c] Numbers in parentheses refer to the number of studies in which the substance was reported positive or negative compared to the number of studies in which it was tested. A substance is classified as "positive" for ER antagonism if it was positive in more than 50% of reported studies. A substance is classified as "presumed positive" for ER antagonism if it was positive in 50% or less of reported studies, or if it was reported positive in the single study conducted. Substances reported negative in their respective studies are classified as "presumed negative" instead of "negative" for ER antagonism, since they had not been tested in multiple studies at or above the limit dose of 1 mM recommended in **Section 4.1.3**. Substances without data are classified "presumed positive" or "presumed negative" based on available information, including their known mechanism of action or their responses in ER binding assays, AR binding assays, or AR TA assays.

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

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

[f] IC_{50} data were abstracted from publications reviewed for the NICEATM ER TA BRD; however, these data were not included in the BRD.

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

[h] 17β-Estradiol is not considered a test substance for validation purposes, since it is the recommended reference estrogen for *in vitro* ER binding and TA assays (refer to **Section 4.2** for more information).

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

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

Section 4

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