Aromatase Assay (Human Recombinant)
OCSPP Guideline 890.1200

Standard Evaluation Procedure (SEP)

ENDOCRINE DISRUPTOR SCREENING PROGRAM
U.S. Environmental Protection Agency
Washington, DC 20460

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I. INTRODUCTION

A. Use of the Standard Evaluation Procedure

This document was developed by EPA to provide guidance to EPA staff who will be reviewing the data submitted in response to Tier 1 Orders issued under the Endocrine Disruptor Screening Program (EDSP), and may also be of interest to parties submitting such data. This document provides general guidance and is not binding on either EPA or any outside parties. The use of language such as "will," "is," "may," "can" or "should" in this document does not connote any requirement for either EPA or any outside parties. As such, EPA may depart from the guidance where circumstances warrant and without prior notice.

This Standard Evaluation Procedure (SEP) provides guidance on how to review studies conducted using the OCSPP Guideline 890 890.1200 Aromatase (Human Recombinant) that are submitted to support requirements imposed under the U.S. Environmental Protection Agency's Endocrine Disruptor Screening Program (EDSP). The product of the review will be a Data Evaluation Record (DER) that reflects how well the study conforms to the Guideline, and evaluates how well the study was performed, and provides the appropriate conclusions supported by the data. The DER will include, for example, a list of any significant deviations from the protocol as well as their potential impacts, a list of significant information missing from the study report, and any other information about the performance of the study that affects interpretation of the data within the context of the EDSP.

The DER should contain adequate information to provide the EPA with the ability to determine whether the study was performed according to the guideline. The objective of EDSP Tier 1 assays is to characterize the potential of a chemical to interact with the endocrine system.

The Guideline recommends the critical materials, methods, and analyses that lead to successful performance of the assay. If a particular material, method, or analysis is named in the Guideline, it is usually because other materials, methods, or analyses are either known to be inappropriate or at least have not been validated or that there is concern for their potential influence on results. The Agency has posted Corrections and Clarifications on Technical Aspects of the EDSP Tier 1 Assays (OCSPP Test Guideline Series 890) in the docket; the link to this document may be found by way of the EDSP web page (http://www.epa.gov/endo/). It is therefore important to note deviations from specific materials, methods, or analyses in the DER, and provide the Agency's opinion on whether the deviation/deficiency has an impact on the performance and results of the study or the acceptability of the study.

II. AROMATASE ASSAY

A. Purpose of the Assay

The aromatase assay identifies chemicals that inhibit the catalytic activity of the cytochrome P450 enzyme aromatase (also known as CYP19). Aromatase is responsible for converting androgens to estrogens during steroidogenesis and is expressed in multiple tissues,

such as the ovary, placenta, uterus, testis, brain, and adipose tissue. This enzyme is essential for normal reproductive development and function in males and females. Specifically, inhibition of the catalytic activity of aromatase causes a reduction in estrogen levels in tissues and ultimately disrupts estrogen mediated physiological responses. A number of compounds, including several flavonoids and related phytoestrogens, various pesticides, and other environmental contaminants have been shown to inhibit the catalytic activity of aromatase by blocking the active site of the enzyme or by altering its conformation resulting in an inability of the substrate (androgens) to activate the enzymatic reaction.

B. Study Design

The Aromatase Test Guideline is an *in vitro* assay that measures enzyme (i.e., aromatase) activity in the presence of a range of test chemical concentrations. This assay is limited to the detection of chemicals that inhibit the catalytic activity of aromatase. The assay is conducted by incubating human recombinant microsomes with increasing concentrations of test chemical, radiolabeled [1β-^{3}H] androstenedione ([^{3}H] ASDN, an aromatase substrate), and an essential cofactor (NADPH) for a designated period of time. The rate of tritiated water (^3H_2O) released during the conversion of [^{3}H] ASDN to estrone is quantified (following a methylene chloride extraction) by liquid scintillation counting as a measure of aromatase activity. Each time the assay is conducted (i.e., a single "run" of the assay), a series of controls are included for Quality Assurance (QA) and compared to the specified performance criteria. For each test chemical, production of ^3H_2O (i.e., aromatase activity) is plotted as a percent of the solvent control (i.e., maximal aromatase activity) versus the log of the concentration of the test chemical. Response curves (sigmoidal) are fitted by weighted least squares nonlinear regression analysis. The test chemical is classified as an inhibitor, non-inhibitor, or equivocal depending upon the shape of the response curve and percent inhibition observed at the highest concentration of the test chemical.

C. Pre-test Preparation

Prior to using the assay for the evaluation of test chemicals, the Guideline recommends that the laboratory demonstrate proficiency with running the assay. The initial step is to meet the performance criteria for the full activity control, background activity controls, and the recommended positive control chemical as described in section III. D. The next step is to conduct the assay with proficiency chemicals as described in section III. E. As the performance of an assay is directly linked to the laboratory personnel conducting the assay, it is recommended that these procedures be repeated if a change in laboratory personnel occurs.

III. EVALUATION OF STUDY CONDUCT

A. Test Compound

EPA recommends that the Aromatase Assay be performed with the technical form of the chemical intended for commercial use. The following information on the test chemical will typically be provided in the DER:

- CAS Number
- Molecular Formula/Weight
- Supplier/Source
- Lot Number
- Purity
- Storage Conditions
- Solvent (Usually ethanol or dimethyl sulfoxide (DMSO))
- Solubility Limit (if insoluble at any guideline concentration)
- Highest Concentration Tested
- Description of how stock solution was prepared
- Stock solution: storage conditions
- Stability of the Stock Solution

Most labs will likely select the solvent based on the physical properties of the test chemical (e.g. partition coefficient, hydrophobicity, solubility, etc.). Fresh serial dilutions of each test chemical will be prepared in the same solvent as the stock solution on the day of use such that the target concentration can be achieved by the addition of 20 μL of the dilution to a 2 mL assay volume. It is recommended that the total volume of the test chemical formulation used in each assay be no more than 1% of the total assay volume (20 μL in a 2 mL assay) in order to minimize the potential of the solvent to inhibit the enzyme activity, and that the solvent concentration remains consistent among all assay tubes.

Other information to be included in the DER:

Unlabelled ASDN
Supplier, Lot Number, Purity (purity should be ≥ 98%)

[^{3}H]ASDN
Supplier, Lot Number, Purity (radiochemical purity should be ≥ 95%)

B. Microsomes

Based on the assay validation, any source of human recombinant microsomes containing CYP19 + reductase can be successfully used in this assay provided the performance criteria are met. EPA recommends that the aromatase activity of each new lot of recombinant microsomes be measured to demonstrate sufficient activity for use with the test chemicals. The recommended minimum acceptable aromatase activity is 0.1 nmol/mg-protein/min. EPA recommends the protein concentration of the microsome preparation be determined on the day the assay is performed. The Agency also recommends if commercial sources of microsomes are used, supplier-provided values for protein concentration, cytochrome c reductase activity and aromatase activity be reported in the DER.

C. Range of Test Chemical Concentrations

The range of concentrations used for the assay may vary slightly among the test chemicals, and will depend upon multiple factors to include the physical properties and solubility limitations of each chemical, and where applicable, the effective concentration range for those chemicals that inhibit aromatase activity. Overall, the Test Guidelines recommend using eight concentrations of the test chemical in the assay with the first run typically covering a range from 10^{-3} and 10^{-10} M such that each concentration decreases by a factor of 10 (e.g., log units). Table 1 shows the test chemical design for each test run.

If cloudiness or a precipitate is observed (indicating test chemical insolubility) at the highest chemical concentration (10^{-3} M), the Test Guidelines recommend that mid-log concentrations ($10^{-3.5}$ M) be evaluated until a soluble concentration is determined. If the highest concentration to be tested is equal to or lower than 10^{-5} M, then the eight concentrations can be spaced at mid-log intervals. Solubility issues should be noted in the DER.

Results from the first run are confirmed with subsequent runs. In some cases, data from the first run may indicate the need for modifying the concentration range in the additional (or subsequent) runs to better bracket the expected response curve. For example, the range for strong inhibitors may need to be shifted toward the lower concentrations (e.g., $\leq 10^{-11}$ M) to ensure that a full response curve can be obtained. For chemicals that display a weaker ability to inhibit aromatase activity, the inclusion of mid-log concentration intervals can provide a more defined response curve when approaching the limit of solubility or 10^{-3} M. The review should assess the adequacy of the concentrations selected for the runs.

Table 1. Test Chemical Study Design for each Test Run

Sample Type	Repetitions (Tubes)	Description	Test Chemical (M)
Test Chemical Conc 1	3	All test components[a] plus test chemical	1×10^{-3}
Test Chemical Conc 2	3	All test components plus test chemical	1×10^{-4}
Test Chemical Conc 3	3	All test components plus test chemical	1×10^{-5}
Test Chemical Conc 4	3	All test components plus test chemical	1×10^{-6}
Test Chemical Conc 5	3	All test components plus test chemical	1×10^{-7}
Test Chemical Conc 6	3	All test components plus test chemical	1×10^{-8}
Test Chemical Conc 7	3	All test components plus test chemical	1×10^{-9}
Test Chemical Conc 8	3	All test components plus test chemical	1×10^{-10}

[a] buffer, propylene glycol, microsomal protein, [^{3}H]ASDN, and NADPH

D. Control Groups

The guideline recommends prior to using the assay for the evaluation of test chemicals, that each technician demonstrate proficiency with running the assay. The initial step is to meet the performance criteria for the full activity control, background activity controls, and the recommended positive control chemical.

The <u>full activity control</u> is used to determine the maximum aromatase activity of the assay system as measured by the production of $^3H_20$ during the conversion of ^{3}H-ASDN to estrone.

The background activity control is used to quantify non-specific radioactivity that is not related to aromatase activity.

The positive control, 4-hydroxy androstenedione, ([4-OH] ASDN), is a known inhibitor of aromatase activity.

The study design for the controls when tested at concentrations ranging from 10^{-10} to 10^{-5} M and plotted as recommended by the test guideline is shown in Table 2.

Table 2. Control Study Design

Sample Type	Repetitions (Tubes)	Description	4-OH ASDN (M)
Full Activity Control	4	All test components[a] plus solvent vehicle	N/A
Bkgd Activity Control	4	Same as above without NADPH	N/A
4-OH ASDN Conc 1	3	All test components plus 4-OH ASDN[b]	1×10^{-5}
4-OH ASDN Conc 2	3	All test components plus 4-OH ASDN	1×10^{-6}
4-OH ASDN Conc 3	3	All test components plus 4-OH ASDN	$1\times10^{-6.5}$
4-OH ASDN Conc 4	3	All test components plus 4-OH ASDN	1×10^{-7}
4-OH ASDN Conc 5	3	All test components plus 4-OH ASDN	$1\times10^{-7.5}$
4-OH ASDN Conc 6	3	All test components plus 4-OH ASDN	1×10^{-8}
4-OH ASDN Conc 7	3	All test components plus 4-OH ASDN	1×10^{-9}
4-OH ASDN Conc 8	3	All test components plus 4-OH ASDN	1×10^{-10}

a The complete assay contains buffer, propylene glycol, microsomal protein, [^{3}H]-ASDN, and NADPH
b Positive control for aromatase inhibition

The positive control produces a sigmoid inhibition curve.

The performance criteria for the positive control are listed below:

- Mean full activity control (e.g., maximum aromatase activity of assay system) ≥0.1 nmol/mg-protein/min.
- Mean background control activity ≤ 15% of the full activity control.
- Coefficient of variation (CV) < 15% for replicates within each sample type and concentration of 4-OH ASDN.
- Performance criteria (Table 3) are provided and serve as guidance in identifying runs that provide parameters in the preferred ranges.

Table 3. Performance Criteria for the Positive Control

Parameter	Lower Limit	Upper Limit
Slope	-1.2	-0.8
Top (%)	90	110
Bottom (%)	-5	+6
Log IC_{50}	-7.3	-7.0

E. Proficiency Demonstration

After successfully conducting the positive control assay, it is recommended that each new technician conduct a full-scale assay using the four proficiency chemicals in listed in Table 4. A full-scale assay consists of three independent experiments (runs). Each run tests the response of aromatase activity at 8 test concentrations performed in triplicate (*i.e.*, three tubes of each test

concentration). The proficiency chemicals were selected to span the range of responses expected in the assay based on known effects on aromatase activity determined from previously conducted validation studies (EPA, 2007). Successful demonstration of proficiency with the assay is typically achieved when the positive controls meet performance criteria as described in Table 3 above and all proficiency chemicals are correctly classified as shown in Table 4. The Test Guidelines recommend that proficiency chemicals be tested at the same concentrations as the other test chemicals (see Table 5).

Table 4. Proficiency Chemicals

Compound	CAS No.	Class
Econazole	24169-02-6	Inhibitor
Fenarimol	60168-88-9	Inhibitor
Nitrofen	1836-75-5	Inhibitor
Atrazine	1912-24-9	Non-inhibitor

F. Test Chemical Study Design

In most cases, each run should follow the template below (Table 5); however, a lab may need to adjust the range of test chemical concentrations based on solubility limits and/or to better define an aromatase inhibition curve. In addition, EPA expects that the performance criteria for the positive control (Table 3) controls would be met for each run. The performance criteria for these for parameters are not rigid boundaries but should be evaluated on a case-by-case basis. The performance criteria are particularly important in cases when the test chemical presents equivocal or variable data, in order to demonstrate that the test system is responding appropriately.

Table 5. Test Chemical Study Design

Sample Type	Repetitions (Tubes)	Description	4-OH ASDN (M)
Full Activity Control	4	All test components[a] plus solvent vehicle	N/A
Bkgd Activity Control	4	Same as above without NADPH	N/A
4-OH ASDN Conc 1	2	All test components plus 4-OH ASDN	1×10^{-5}
4-OH ASDN Conc 2	2	All test components plus 4-OH ASDN	1×10^{-6}
4-OH ASDN Conc 3	2	All test components plus 4-OH ASDN	$1\times10^{-6.5}$
4-OH ASDN Conc 4	2	All test components plus 4-OH ASDN	1×10^{-7}
4-OH ASDN Conc 5	2	All test components plus 4-OH ASDN	$1\times10^{-7.5}$
4-OH ASDN Conc 6	2	All test components plus 4-OH ASDN	1×10^{-8}
4-OH ASDN Conc 7	2	All test components plus 4-OH ASDN	1×10^{-9}
4-OH ASDN Conc 8	2	All test components plus 4-OH ASDN	1×10^{-10}
Test Chemical Conc 1	3	All test components plus test chemical	1×10^{-3}
Test Chemical Conc 2	3	All test components plus test chemical	1×10^{-4}
Test Chemical Conc 3	3	All test components plus test chemical	1×10^{-5}
Test Chemical Conc 4	3	All test components plus test chemical	1×10^{-6}
Test Chemical Conc 5	3	All test components plus test chemical	1×10^{-7}
Test Chemical Conc 6	3	All test components plus test chemical	1×10^{-8}
Test Chemical Conc 7	3	All test components plus test chemical	1×10^{-9}
Test Chemical Conc 8	3	All test components plus test chemical	1×10^{-10}

[a] The complete assay contains buffer, propylene glycol, microsomal protein, [^{3}H]-ASDN, and NADPH

IV. STUDY INTERPRETATION

A. Data Interpretation and Graphical Representation

The Guideline recommends that raw data be used to calculate aromatase activity (nmol/mg protein/min) and percent control. The reported data may be available in an electronic format (spreadsheet or comma-separate values). The reviewer should verify that the derived information was calculated correctly. The Guideline recommends that the following raw data be included in the study report so the reviewer can verify calculations and adequately evaluate the study.

- Disintegrations per minute (DPM)/mL for each aliquot of extracted aqueous incubation mixture.
- Average DPM/mL for each aqueous portion (after extraction).
- Total DPM for each aqueous portion (after extraction).
- The total DPM present in the assay tube at initiation. The volume (mL) of substrate solution added to the incubation multiplied by the substrate solution's specific activity (DPM/mL) yields the total DPM present in the assay tube at initiation.
- Percent of substrate converted to product. The total DPM remaining in the aqueous portion after extraction divided by the total DPM present in the assay tube at initiation times 100 yields the percent of the substrate that was converted to product.
- Total DPM after extraction correct for background. The total DPM remaining in the aqueous portion after extraction is corrected for background by subtracting the average DPM present in the aqueous portion of the background tubes. This corrected DPM is then converted to nmol product formed by dividing by the substrate specific activity (DPM/nmol).
- Aromatase activity. The activity of the enzyme reaction is expressed in nmol/mg protein/min and is calculated by dividing the amount of $^{3}H_2O$ (nmol) produced by the product of mg microsomal protein used times the incubation time (15 minutes).
- Average activity in the full activity control tubes.
- Percent of control activity remaining in the presence of various inhibitor concentrations, including the positive control. This value is calculated by dividing the aromatase activity at a given concentration by the average full activity control and multiplying by 100.

Note: nominally, one might expect the percent of control activity values for an inhibitor to vary between 0% near the higher chemical concentrations and 100% near the low chemical concentrations. However, due to experimental variation, individual observed percent of control values will sometimes extend slightly below 0% or above 100% as noted in the performance criteria.

Data from the assay will be used to classify chemicals according to their ability to inhibit aromatase. In general, to be classified as an inhibitor, the data will fit the 4-parameter regression model to yield a sigmoidal inhibition curve and result in greater than 50% inhibition at the highest concentration. Average the value of the inhibition curve at each of three runs at the highest concentration and compare with the following criteria (Table 6):

Table 6. Interpretation of Results

Criteria		Interpretation
Data fit 4-parameter nonlinear regression model	Average curve across runs crossed 50%[a]	Inhibitor
	Average lowest portion of curves across runs is between 50% and 75% activity[b]	Equivocal
	Average lowest portion of curves across runs is greater than 75% activity[b]	Non-inhibitor
Data do not fit model	---	

[a] Ordinarily, an inhibition curve will fall from 90% to 10% over 2 log units with a slope near -1. Unusually steep curves may indicate protein denaturing or solubility issues. If the slope of the curve is steeper than -2.0, the result is interpreted as equivocal.

[b] If the test compound was not soluble at 10^{-5} M and the inhibition curve does not cross 50%, the chemical is interpreted as untestable.

B. Model Fitting

The Test Guideline recommends that model fitting be conducted as follows: The response curve is fitted by weighted least squares nonlinear regression analysis with weights equal to 1/Y; model fits are to be carried out using a non-linear regression program such as Prism software (Version 3 or higher); the software (version) used for analysis in the study should be reported in the Data Evaluation Record (DER); concentration response trend curves are fitted to the percent of control activity values within each of the repeat tubes at each test chemical concentration; and concentration is expressed on the log scale. In agreement with past convention, common logarithms (i.e., base 10) are used. The variables in the response curve are defined as follows:

Y = percent of control activity in the inhibitor tube
X = logarithm (base 10) of the concentration
T = average DPMs across the repeat tubes with the same test chemical concentration that define the top of the curve
B = average DPMs across the repeat tubes with the same test chemical concentration that define the bottom of the curve
β = slope of the concentration response curve (β will be negative)
$\mu = \log_{10}(IC_{50})$; (IC_{50} is the concentration corresponding to percent of control activity equal to 50%)

The following concentration-response curve is fitted to relate percent of control activity to logarithm of concentration within each run:

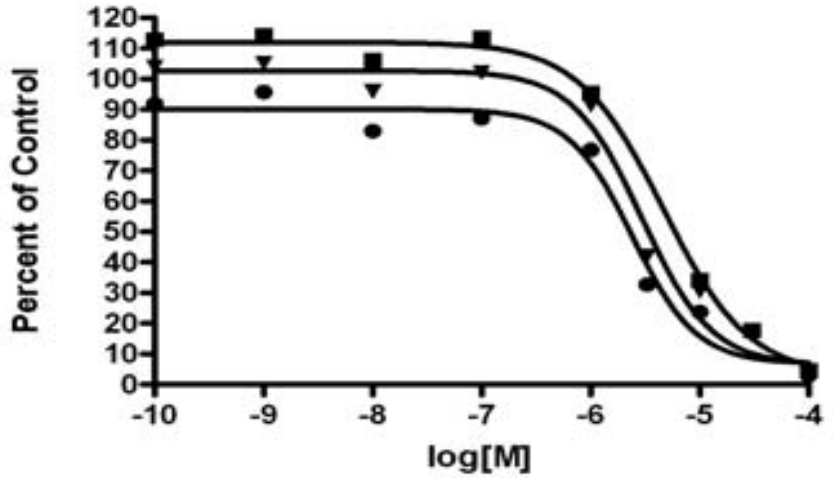
$$Y = B + \frac{(T - B)}{1 + 10^{(\log(IC_{50}) - X)\beta + \log(\frac{(T-B)}{(50-B)} - 1)}}$$

A concentration-response model is fitted for each test run for each test chemical.

C. Graphical and Analysis of Variance Comparisons Among Concentration Response Curve Fits

For each run the individual percent of control values are plotted versus logarithm of the test chemical concentration to generate a sigmoidal-shaped response curve. The fitted concentration response curve is superimposed on the plot (see Figure 1.).

Figure 1. Test Chemical Inhibition.



EPA requests that additional plots be prepared to compare the percent of control activity values across runs through the following steps:

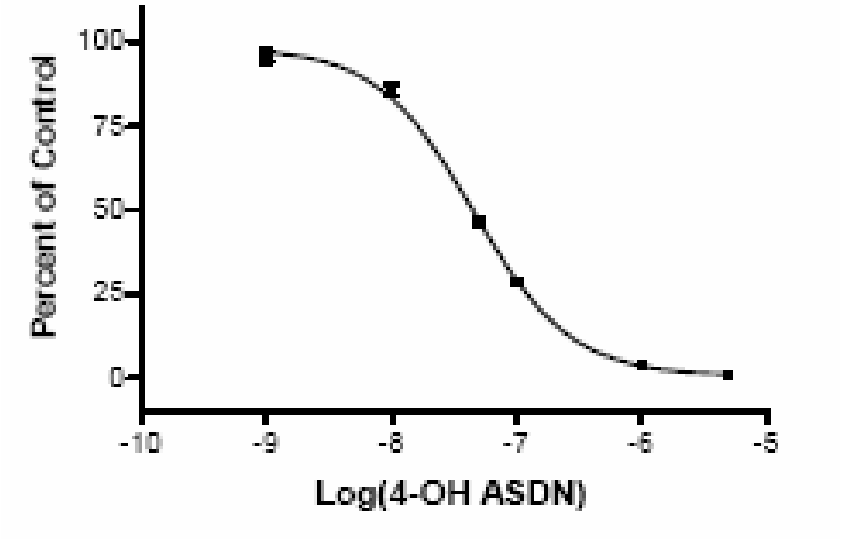
- For each run, plot the average percent of control values versus logarithm of test chemical concentration, and place all of the runs for one chemical on the same graph.
- Use different symbols to distinguish among runs.
- Superimpose the fitted concentration response curves for each run on the graph.
- On a separate graph, plot the average percent of control values for each run versus the logarithm of test chemical concentration.
- Superimpose the average concentration response curve across runs on the same plot.

For each run, treat (β, μ) as a random variables with mean (β_{avg}, μ_{avg}). Let X and Y ($0 < Y < 100$) denote logarithm of concentration and percent of control, as defined above. The average response curve is:

$$Y = B + \frac{(T - B)}{1 + 10^{(\mu_{avg} - X)\beta_{avg} + \log\left(\frac{(T-B)}{(50-B)^{-1}}\right)}} + \varepsilon$$

Compare slope (β) and $\log_{10}IC_{50}$ (μ) across runs based on one-way random effects analysis of variance, treating the runs as random effects. Prepare graphs that display the parameters within each run with associated 95 percent confidence intervals based on the within-run standard error and the average across-run standard error with the associated 95 percent confidence interval incorporating run-to-run variation. For preparation of the DER, it is not necessary to include all of the graphs that were recommended by the guideline. Rather, the DER should only include example figures for the response of the positive control (see Figure 2.) and test chemical in each run as well as a graph showing the mean response for the test chemical across all runs.

Figure 2. 4-OH ADSN Inhibition.



D. Quality Control-Assay Drift Monitored Using Full Enzyme and Background Activity Controls

Within each run of each test chemical quadruplicate repetitions are made of the full enzyme activity control and background activity control tubes. Half the repetitions are carried out at the beginning of the run and half at the end. If the conditions are consistent throughout the test, the control tubes at the beginning will be equivalent to those at the end.

To assess whether this is the case, the control responses are typically adjusted for background DPMs, divided by the average of the (background adjusted) full enzyme activity control values, and expressed as percent of control. The Guideline recommends that the average of the four background activity controls within a run fall around 0 percent (with an acceptable range of -5 to +6%) and the average of the four full enzyme activity controls within a run fall around 100 percent (with an acceptable range of 90 - 110%). It is recommended that the Reviewer evaluate these values to determine if the assays conditions were consistent throughout the study.

V. REFERENCES

EPA (2007) Integrated Summary Report on Aromatase. December 11, 2007. ://www.epa.gov/endo/pubs/aromatase_isr.