Primary Reviewer: _____ **Signature:** _____
[Insert Name of Organization] **Date:** _____
Secondary Reviewer: _____ **Signature:** _____
[Insert Name of Organization] **Date:** _____

Template version 07/2011

DATA EVALUATION RECORD

STUDY TYPE: Aromatase (Human Recombinant); OCSPP 890.1200

PC CODE: *(if applicable)* **DP BARCODE:** *(if applicable)*

TXR#: *(if applicable)* **CAS No.:** [##]

TEST MATERIAL (PURITY): *(use name of material tested as referred to in the study (common agency chemical name in parenthesis))*

SYNONYMS: *(Other names and codes)*

CITATION: Author *(up to 3, see SOP for exact format)*. ([Study Year]). Title. Laboratory name and location. Laboratory report number, study completion date. MRID *(if applicable) (no hyphen)*. Unpublished. *(OR if published, list Journal name, vol.:pages)*

SPONSOR: *(Name of Study Sponsor)*

EXECUTIVE SUMMARY:

In an *in vitro* aromatase (CYP 19) assay (MRID *(if applicable)* [number]), [Chemical name (% a.i., batch/lot#)] was incubated with human recombinant aromatase and tritiated androstenedione (1-β [^{3}H(N)]-Androst-4-ene-3,17-dione ([^{3}H]ASDN)) in [solvent] at concentrations of 0, [x, x, x, x, x, x, or x] M for 15 minutes to assess the effect of [chemical] on aromatase activity.

Aromatase activity was determined by measuring the amount of tritiated water produced at the end of a 15 minute incubation for each concentration of chemical. Tritiated water was quantified using liquid scintillationcounting (LSC). [X] runs were conducted and each run included a full activity control, a background activity control, a positive control series (10^{-10} – 10^{-5}) using a known inhibitor (4-OH ASDN), and the test chemical series (10^{-X} – 10^{-X}) with [X] repetitions per concentration.

Provide a brief summary of the results and a concise discussion. Be sure to note the adequacy of the data from the full controls and positive controls. Discuss any major deficiencies, failure to meet performance criteria, or any problems encountered in this study.

The IC_{50} of the test material was [X]. Based on the results of this assay, [chemical] was determined to [inhibit, be equivocal, not inhibit or be un-testable at the concentrations used to evaluate] aromatase activity.

The study [satisfies/does not satisfy] the Test Order requirement for an Aromatase Assay (OPPTS/OCSPP 890.1200). *(If it does not satisfy the requirement, concisely list only major deficiencies or refer to deficiency section.)*

COMPLIANCE: Signed and dated Data Confidentiality, GLP Compliance, and Quality Assurance statements [were/were not] provided. *(Discuss deviations from regulatory requirements)*

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Substance:

Common name as used by Agency

Description: *e.g. technical, nature, color, molecular weight*
Source:
Lot/Batch #: *include expiration date*
Purity: %
Volatility:
Storage conditions:
Stability:
Solvent:
Solubility (in test solvent):
Highest Concentration Tested:
Stock Solution Preparation:
Methodology:
Molecular weight:
CAS #: *CAS # or* Not available
Structure: *[Structure] or* Not available

2. Non-Labeled Substrate:

Androstenedione (ASDN)

CAS # : 63-05-8
Source: *include catalog #*
Lot/Batch #: *include expiration date*
Purity: *should be ≥98%*

3. Radiolabeled Substrate:

1-β [^{3}H(N)]-Androst-4-ene-3,17-dione; ([^{3}H]ASDN)

Source: *include catalog #*
Lot/Batch #: *include expiration date*
Radiochemical Purity (Supplier): *should be ≥95%*
Specific activity: *Ci/mmol (on date of use)*
Radiochemical Purity (In-lab determination):

4. Positive Control:

4-hydroxyandrostenedione (4-OH ASDN)

CAS # 566-48-3
Source: *include catalog #*
Lot/Batch #: *include expiration date*
Purity: %

5. Solvent (Vehicle Control):

Lot/Batch #: *include expiration date*
Justification for choice of solvent
Concentration
(% of total volume in assays)

6. Test Microsomes:

Human recombinant aromatase (CYP19) microsomes

Source: *Supplier, include catalog #*
Lot/Batch #: *include expiration date*
Protein concentration: *mg protein per mL*
Cytochrome C reductase activity: *nmole cytochrome c reduced/mg protein/minute*
Aromatase activity: *nmol/mg protein/min*

B. METHODS

1. Assay Components and Preparations: *(Example text is included below and should be altered to apply to the specific methods used by the laboratory)*

A mixture of non-labeled and radiolabeled [^{3}H]ASDN was prepared such that the final concentration of ASDN in the assay was approximately [##] nM, and the amount of tritium added to each incubation tube was [approximately 0.1 µCi in a volume of 100 µL] *(as per the example on pp. 4-5 of the guideline, OCSPP 890.1200)*.

Test chemical(s) stock solutions were prepared such that the total volume of each test chemical formulation used per assay was no more than [x]% of the total assay volume *(test chemical solution should account for ≤1% of the assay volume)* to minimize the potential for the solvent to inhibit the enzyme. Selection of the solvent(s), [name of solvent(s)], was based upon the physical properties of the test chemical's *(e.g., partition coefficient, hydrophobicity, solubility, etc.)*.

A stock solution of the positive control substance, 4-OH ASDN, was formulated in [solvent]. Fresh dilutions of the stock solution were prepared in the same solvent as the stock solution on the day of use. Dilutions were prepared such that the target concentrations of the positive control substance (0.1–10,000 nM; Table 4) were achieved by the addition of [##] µL of the dilution for a final assay volume of [#] mL.

Human recombinant microsomes were purchased from [source], and stored at ≤-[##]ºC for no longer than [##] months. Microsomes were portioned into individual vials based on the protein concentration of the batch *(i.e., approximately 0.4 mg for an 80-90 tube assay, or 0.004 mg/mL microsomal protein per tube)* and stored at ≤-[##]ºC for no longer than [#] months.

Other assay components sodium phosphate buffer, propylene glycol, and NADPH are reported in Table 1. *(If preparations deviated from the guideline, please note the procedure used and the reason(s) for the deviation.)*

TABLE 1. Assay Components and Conditions

Assay Factor	Values
0.1M sodium phosphate buffer (pH 7.4)	
Microsomal Protein	[0.004] mg/mL [a]
NADPH	[0.3] mM
[^{3}H]ASDN	[100] nM
Propylene Glycol	[5%]
Temperature	[37]°C
Incubation Time	[15] min

a The concentration of microsomal protein was optimized for microsomes that produce approximately [1200 pmol product/(min × mg protein) and 5 pmol product/pmol P450/min].

2. Suitability Assessments: The protein concentration was determined on each day the aromatase assay was run. A [six-point] standard curve *(approximately 10-fold; with a suggested range of 0.13-1.5 mg protein/mL)* prepared with bovine serum albumin (BSA) was

analyzed with a standard protein assay kit (*e.g., DC Protein Assay kit, Bio-Rad; BCA Protein Assay, Pierce; or other supplier*).

Aromatase activity in each lot of human recombinant microsomes was determined to demonstrate the presence of sufficient activity for analysis of [test chemical(s)]. The aromatase activity was determined to be [##] nmol/mg-protein/min, which [was/was not] greater than the minimum acceptable aromatase activity of 0.1 nmol/mg-protein/min.

3. **Aromatase Assay:** Each assay run contained 4 tubes for the full enzyme activity and background activity controls, respectively, and a full concentration curve in [triplicate] for the positive control and test substance. The aromatase assay was conducted according to the procedures described in OPPTS 890.1200 (Section h, pp. 9-10).

(If procedures deviate from the guideline procedures, summarize the procedures used in the study, noting the major differences with the guideline procedures, along with any explanation provided to justify the changes.)

The amount of $^{3}H_2O$ in the aqueous fraction was quantified for each assay tube by liquid scintillation counting (LSC), and aromatase activity was reported in units of nmol·mg-protein^{-1}·min^{-1}.

4. **Demonstration of Proficiency:** *State if previously performed, and provide date and study reference. If not performed, provide a rationale. Give a brief synopsis of methods and results including…[summarize from guideline]…. In addition to the basic requirement of demonstrating proficiency of the laboratory in conducting the assay, check to see whether a new laboratory proficiency test was conducted whenever significant changes in personnel at the laboratory have occurred.*

Initial demonstration of laboratory proficiency. Prior to using the assay for evaluation of test chemicals, at least one single run of the positive control experiment and three full scale runs of the proficiency chemicals were conducted to demonstrate assay proficiency of the laboratory. Thereafter, the ability of each new technician to successfully conduct the assay [was/ was not] demonstrated using the same approach.

a. **Positive Control**:

(1) Initial Demonstration of Laboratory Proficiency: The positive control [new/historical data for laboratory] data [met/did not meet] the following criteria:

- Mean aromatase activity in the absence of an inhibitor was at least 0.1 nmol/mg-protein/min.
- Mean background control activity was ≤ 15% of the full activity control.
- Coefficient of variation (CV) for replicates within each sample type and concentration of 4-OH ASDN was <15%.
- Performance criteria (Table 2) [were met/were not met], and served as guidance in identifying runs that provided parameters in the preferred ranges.

(2) Demonstration of Proficiency of New Technician for Conducting Assay (when applicable). The positive control data [met/did not meet] the criteria as listed in section (i) of OPPTS 890.1200.

TABLE 2. Performance Criteria for the Positive Control

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

b. **Proficiency Chemicals**: *(if applicable to demonstrate the proficiency of the laboratory and/or the ability of new technicians to conduct the assay include text similar to the following example)*

(1) The proficiency of the laboratory was demonstrated once prior to running any chemicals using the assay by conducting full scale test runs [3 per proficiency chemical] as listed in Table 3. Historical proficiency data with the aromatase assay from the laboratory/technicians [was/was not] included in lieu of current proficiency data.

(2) Demonstration of proficiency of new technician *(when applicable)*. The data for the proficiency chemicals [met/did not meet] the criteria as listed in Table 3.

TABLE 3. Proficiency Chemicals

Compound	CAS#	Class	Concentrations
Econazole	24169-02-6	Inhibitor	
Fenarimol	60168-88-9	Inhibitor	
Nitrofen	1836-75-5	Inhibitor	
Atrazine	1912-24-9	Non-inhibitor	

5. Determination of Aromatase Activity with Pest Chemical(s): The response of aromatase activity to the presence of [eight] concentrations of a test chemical per run, [in triplicate], was tested during [three] independent runs. After completion of the first run, the data were reviewed and*, if necessary, the concentration of test chemical used in the second and third runs was adjusted (i.e., due to problems with solubility or definition of full concentration-response curve).*

If adjustments were necessary for subsequent runs, describe by using the following guidelines and example text for adjusting the concentrations selection:

If insolubility was observed at the highest concentration (10^{-3} M), the highest concentration for the second and third runs was set at the highest concentration that appeared to be soluble with mid-log concentrations; (i.e., $10^{-3.3}$ M or $10^{-3.5}$ M) to define the lower portion of the curve. *(A concentration lower than 10^{-5} M should not be used for the highest concentration tested).* If the highest concentration to be tested was lowered to 10^{-4} or 10^{-5} M, mid-log concentration(s) [were/were not] added near the lower end of the curve (higher

concentrations) and around the estimated IC_{50} based on the results of the first run in order to keep eight concentrations in the test set. The lowest concentration to be tested was [10^{-10} M]. *(unless lower concentrations were required to obtain the "top of the curve")* The full enzymatic activity [was/was not] obtained at the two lowest concentrations of the test chemical to define the top of the concentration-response curve.

TABLE 4. Test Chemical Study Design for each Test Run

Sample Type	Repetitions (Tubes)	Description	Reference or Chemical (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[b]	3	All test components plus test chemical	[#]
Test Chemical Conc 2[b]	3	All test components plus test chemical	[#]
Test Chemical Conc 3[b]	3	All test components plus test chemical	[#]
Test Chemical Conc 4[b]	3	All test components plus test chemical	[#]
Test Chemical Conc 5[b]	3	All test components plus test chemical	[#]
Test Chemical Conc 6[b]	3	All test components plus test chemical	[#]
Test Chemical Conc 7[b]	3	All test components plus test chemical	[#]
Test Chemical Conc 8[b]	3	All test components plus test chemical	[#]

a The complete assay contained buffer, propylene glycol, microsomal protein, [^{3}H]ASDN, and NADPH
b Proficiency chemicals or tests chemical(s)

C. DATA ANALYSIS

1. **Raw Data:** Raw data were converted to aromatase activity (nmol/mg protein/min) and percent control for the positive control, proficiency chemicals and test chemical(s). The following raw data and calculated endpoints for each run were included in the report (Table 5).

TABLE 5. Raw and Calculated Data

Raw/Calculated Data	Included (X)
DPM/mL for each portion 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 percentage of substrate converted to product	
Total DPM after extraction corrected for background	
Aromatase activity expressed in nmol/mg protein/min	
Average aromatase activity in the full activity control tubes	
Percentage of control activity remaining in the presence of various inhibitor concentrations	

DPM= Disintegrations per minute

2. Statistical Methods: *Describe the statistical methods used and list the parameters that were analyzed. Include a statement as to whether the Reviewer considers the analyses used to be appropriate; if inappropriate, provide alternative/rationale. Example text is included below and should be altered to apply to the specific methods used by the laboratory.*
Statistical analyses and displays such as summary tables, graphical displays, analysis of variance, and multiple comparisons were conducted using the following software programs: [software, version #, company].

The response curve was fitted by weighted least squares nonlinear regression analysis with [software program, version ##; company]. For each run, the individual percent of control values were plotted versus logarithm of the test chemical concentration. The fitted concentration response curve was superimposed on the plot, with individual plots prepared for each run. The average percent of control values versus logarithm of test chemical concentration for the individual runs for each test chemical (with different symbols for each run) were included on the same graph with their respective fitted response curves. In addition, the average percent of control values for each run versus the logarithm of test chemical concentration were plotted on a separate graph along with the average concentration response curve across runs were superimposed on the same plot.

In order to determine the consistency among runs, the slope and $\log_{10}IC_{50}$ for the positive control and test chemical(s) were compared across runs based on one-way random effects analysis of variance (ANOVA), with the runs treated as random effects. The parameters were graphed 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.

3. Interpretation of Results

Interpretation of the assay results was based on the average of three runs, using the categories presented in Table 7.

TABLE 7. 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 classified as equivocal.

b If the test compound was not soluble above 10^{-6} M and the inhibition curve does not cross 50%, the chemical is typically determined to be untestable in the aromatase assay.

II. RESULTS

A. CONTROL ACTIVITY: Aromatase activity in the full activity controls ranged from [## to ##] nmol·mg-protein^{-1}·min^{-1} for the [3] test runs, with a mean and standard deviation of

[## ± ##] nmol·mg-protein^{-1}·min^{-1}. Activity in the background controls averaged [##] nmol·mg-protein^{-1}·min^{-1}, or [##]% of the full control activity. The response of the full activity controls and background controls [were/were not] acceptable for each run.

(To assess the acceptability of the controls, each of the four full activity and background control replicates is adjusted by subtracting out the background DPMs and then expressed as a percent of the average full activity control (background adjusted). Within each run, EPA recommends that the background controls be between -5 to +6% and the full controls be between 90 to 110% of the average fully activity.)

B. POSITIVE CONTROL: For the positive control substance (4-OH ADSN), aromatase activity averaged [## ± ##] nmol·mg-protein^{-1}·min^{-1} at the lowest tested concentration [10^{-10} M] and [## ± ##] nmol·mg-protein^{-1}·min^{-1} at the highest tested concentration [10^{-5}]. The mean aromatase activity of the positive control (expressed as % full control activity) for each concentration tested across all [#] runs is presented in Table 8, along with the overall standard deviation and % CV. An example of the inhibition response curve for the positive control from one run is shown in Figure 1.

Note: A positive control (full concentration curve) is required for each daily run of the test chemical. Whether the data meet the performance criteria presented in the test guideline is a significant factor in evaluating the adequacy of the data.

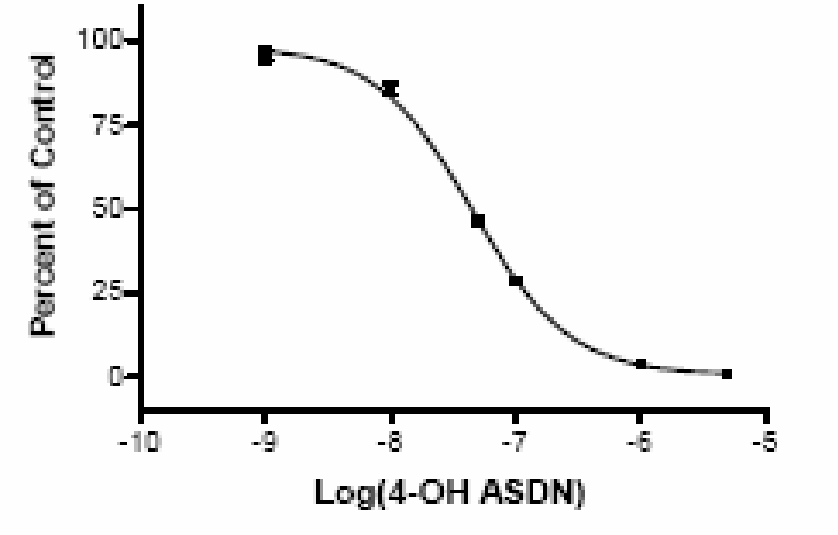
TABLE 8. Effect of [Chemical] on Aromatase Activity (as percent of control) from Independent Runs

Chemical	Concen. Log M	# Runs	Overall Mean	Overall SD	Overall SEM	Overall %CV
4-OH ADSN (positive control)	-5	[3]				
	-6	[3]				
	-6.5	[3]				
	-7	[3]				
	-7.5	[3]				
	-8	[3]				
	-9	[3]				
	-10	[3]				
[Test Substance(s)]	[x]	[3]				
	[x]	[3]				
	[x]	[3]				
	[x]	[3]				
	[x]	[3]				
	[x]	[3]				
	[x]	[3]				
	[x]	[3]				

SD= Standard Deviation
SEM= Standard Error of the Mean
CV= Coefficient of Variance

Figure 1. Inhibition Response Curves for 4-OH ADSN.

Insert the three curves for the positive control data (from the 3 daily runs of the test chemical).



C. <u>TEST SUBSTANCE</u>: For [chemical], aromatase activity averaged [## ± ##] nmol·mg-protein^{-1}·min^{-1} at the lowest tested concentration [10^{-10} M] and [## ± ##] nmol·mg-protein^{-1}·min^{-1} at the highest tested concentration [10^{-3}]. The mean aromatase activity of [chemical] (expressed as %full control activity) for each concentration tested across all [#] runs is presented in Table 8, along with the overall standard deviation and % CV. Inhibition response curves for [chemical] from each run are shown in Figure 2, and the average inhibition response curve across all runs is shown in Figure 3.

Figure 2. Inhibition Response Curves for [chemical] From Each Test Run. *(show data per example figure)*

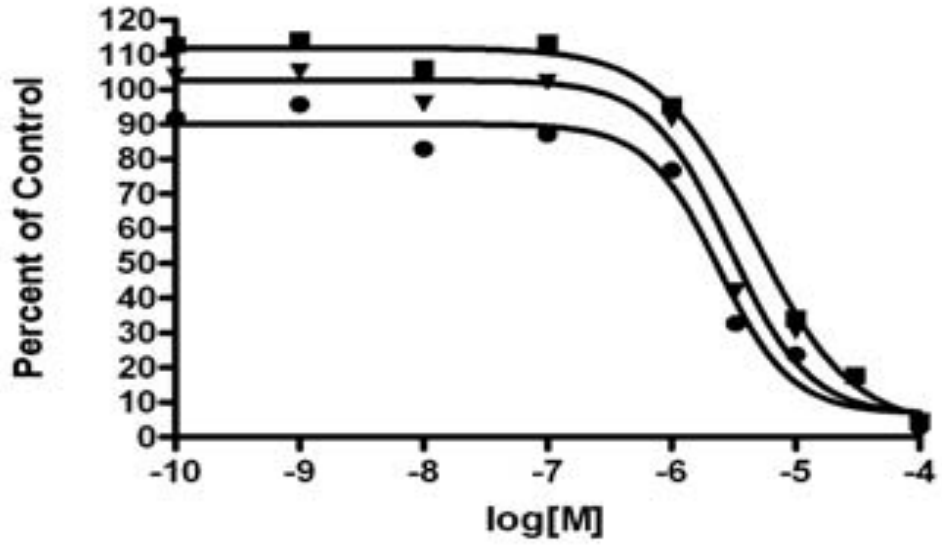
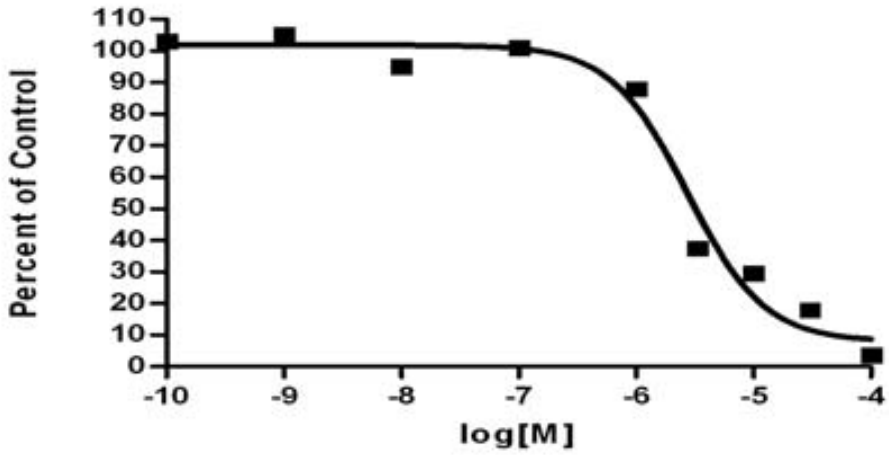


Figure 3. Mean Inhibition Response Curve for [chemical]. *(show data per example figure)*



The effect of [chemical] on inhibition of aromatase activity is presented in Table 9. The estimated Log IC_{50} ranged from [#%] to [#%] for the test material and averaged [##%] compared to the positive control 4OH-ADSN, which averaged [#%]. The average slope of the concentration response curve was [-#] for [chemical] and [-#] for 4OH-ADSN. Confidence in these numbers is [high/low] due to the [small/large] variation.

TABLE 9. Effect of [Chemical] on Aromatase Activity (as Percent of Control) From Independent Runs						
Chemical	**Run 1**	**Run 2**	**Run 3**	**Mean**	**SD**	**%CV**
IC_{50} (µM)						
[Test Chemical]						
4-OH ADSN						
Slope						
[Test Chemical]						
4-OH ADSN						

SD= Standard Deviation

Based on the data from the average response curve and the criteria listed above in Table 7, the results support the conclusion that chemical [inhibits aromatase, does not inhibit aromatase, is equivocal, or is un-testable] in the aromatase assay.

III. DISCUSSION AND CONCLUSIONS

A. INVESTIGATORS CONCLUSIONS: *Provide a brief paragraph of the investigators' conclusions.*

B. REVIEWER COMMENTS:

- *Provide a brief summary of the results and discuss them. In particular, mention the conclusion of the data for the test compound (inhibits aromatase activity, does not inhibit aromatase activity, equivocal, or untestable) and its log(IC_{50}) (range, mean, and standard error).Discuss any discrepancy with investigators' conclusions.*
- *Were the performance criteria met? If not, which criteria were not met? Was a reason provided for the failure? How does the failure impact the study?*
- *If unacceptable, is the study potentially upgradable to acceptable, and how?*

C. STUDY DEFICIENCIES: *List each deviation from the protocol and classify the deviation as major or minor. Also report any rationale provided by the investigator(s) for the deviation. Similarly list, classify, and discuss all other deficiencies with the conduct, results, and reporting of the study. Discuss the possibility of resolving the deficiencies and what would be required. Major deficiencies may be presented and discussed in paragraph form, whereas minor deficiencies can be presented in a bulleted list.*