DRAFT FINAL REPORT

PLACENTAL AROMATASE ASSAY VALIDATION STUDY: PREPARE MICROSOMES IN TWO PARTICIPATING LABORATORIES

VOLUME I (Report and Appendix A)

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Prepared for

GARY E. TIMM
WORK ASSIGNMENT MANAGER
U.S. ENVIRONMENTAL PROTECTION AGENCY
ENDOCRINE DISRUPTOR SCREEING PROGRAM
WASHINGTON, D.C.

BATTELLE
505 KING AVENUE
COLUMBUS, OHIO 43201

PLACENTAL AROMATASE ASSAY VALIDATION STUDY: PREPARE MICROSOMES IN TWO PARTICIPATING LABORATORIES

WA 4-16, Task 6

OVERALL TASK DRAFT FINAL REPORT

Authors (Overall Task Report):

Jerry D. Johnson, Ph.D.
Diplomate, A.B.T.
Work Assignment Leader
Battelle Memorial Institute

Paul I. Feder, Ph.D.
Senior Statistician
EDSP Data Coordination Center
Battelle Memorial Institute

Performing Laboratory:

Battelle Memorial Institute
505 King Avenue
Columbus, Ohio 43201-2693

Sponsor:

Environmental Protection Agency
Endocrine Disruptor Screening Program
Washington, D.C.

Sponsor's Representative:

David P. Houchens, Ph.D.
EDSP Program Manager
Battelle Memorial Institute

Jerry D. Johnson, Ph.D.
Diplomate, A.B.T.
Work Assignment Leader
Battelle Memorial Institute

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Title: Placental Aromatase Assay Validation Study: Prepare Microsomes in Two Participating Laboratories

Overall Task Report Authors/Lab:

Jerry D. Johnson, Ph.D.
Diplomate, A.B.T.
Work Assignment Leader
Battelle Memorial Institute

Paul I. Feder, Ph.D.
Senior Statistician
EDSP Data Coordination Center
Battelle Memorial Institute

Lead Lab: Research Triangle Institute

Participating Labs:

Battelle Memorial Institute
In Vitro Technologies
WIL Research Laboratories

Sponsor:

Environmental Protection Agency
Endocrine Disruptor Screening Program
Washington, D.C.

Sponsor's Representatives:

David P. Houchens, Ph.D.
EDSP Program Manager
Battelle Memorial Institute

Jerry D. Johnson, Ph.D.
Diplomate, A.B.T.
Work Assignment Leader
Battelle Memorial Institute

Authors:

_____
Jerry D. Johnson, Ph.D., D.A.B.T.
Signature / Date

_____
Paul I. Feder, Ph.D.
Signature / Date

Approved:

_____
David P. Houchens, Ph.D.
Signature / Date

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Quality Assurance Unit

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Date

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EXECUTIVE SUMMARY

In this task, human placental microsomes were prepared and analyzed for protein content and aromatase activity. This task was conducted in two stages. In Stage 1, two participating laboratories, Battelle and In Vitro, obtained human placentas and used them to prepare microsomes. Microsomal characterization included protein concentration and aromatase activity (uninhibited and inhibited). The inhibition study determined the response of the microsomes to six concentrations of the known aromatase inhibitor 4-hydroxyandrostenedione (4-OH ASDN) (positive control study). The data were reviewed by the EPA for approval before proceeding to Stage 2. In the second stage, Battelle and In Vitro each shipped their microsomes to each of the other laboratories, RTI and WIL. Three laboratories characterized the Battelle- or In Vitro-prepared microsomes by determining the protein concentration and aromatase activity (uninhibited), which also included a positive control (4-OH ASDN) and a negative control (lindane).

The objectives of this task were to determine whether participating laboratories would be able to procure a human placenta and process it into viable microsomes that could be used to conduct the aromatase assay. In addition, comparisons between microsome preparations were carried out within laboratories and comparisons among laboratories were carried out within microsome preparations.

The overall mean ± SEM (% CV) task microsomal protein concentration for the Battelle-prepared microsomes was 22.5 ± 0.9 mg/mL (16.8 percent) and for the In Vitro-prepared microsomes was 8.4 ± 0.3 mg/mL (13.1 percent).

Aromatase activity decreased with increasing concentration of the inhibitor for both microsomal preparations. At a concentration of 10^{-9} M 4-OH ASDN, approximately 95 to 100 percent of the aromatase activity was present, whereas at a concentration of 10^{-6} M, approximately 6 percent of the aromatase activity was observed for both preparations. The 4-OH ASDN IC_{50} and slope values were similar for both preparations, i.e. 51.7 and 56.9 nM for IC_{50} and -0.9930 and -0.9919 for the slope.

For a given microsomal preparation, the three laboratories obtained aromatase activity values that differed by approximately 50 to 60 percent, i.e. 0.0464 to 0.0708 nmol/mg/min for the Battelle-prepared microsomes and 0.0276 to 0.0443 nmol/mg/min for the In Vitro-prepared microsomes. The overall average ± SEM (% CV) aromatase activity values for the Battelle- and In Vitro-prepared microsomes were 0.0578 ± 0.0051 nmol/mg/min (21.5 percent) and 0.0362 ± 0.0032 nmol/mg/min (21.9 percent). If the aromatase activity values obtained by the source laboratories are used as a benchmark, i.e. 0.0542 and 0.0382 nmol/mg/min for the Battelle- and In Vitro-prepared microsomes, respectively, then the % RE values for In Vitro, RTI, and WIL were -14.3, 3.8 and 30.6 percent, respectively, for the Battelle-prepared microsomes, and for Battelle, RTI, and WIL were -27.7, -4.5 and 16.1 percent, respectively, for the In Vitro-prepared microsomes.

Inhibition by the positive control (4-OH ASDN) ranged from approximately 44 to 56 percent and, for the negative control (lindane), from approximately 95 to 107 percent for both microsomal preparations and all laboratories.

The principal results of the interlaboratory analysis are summarized below.

Aromatase Activity

For the full enzyme activity, negative, and positive controls the mean estimates indicated greater activity for the Battelle microsomes than for the In Vitro microsomes for each control type and at each laboratory. Averaged across laboratories the Battelle microsomes had significantly greater activity than the In Vitro microsomes ($p=0.05$) for each of the control types, however most of the differences within most of the individual laboratories were not significant.

The among laboratory CVs (excluding background activity controls) ranged from 32.5% to 46.2%, depending on control type. The among laboratory variation was comparable to the within laboratory variation.

Protein Concentration

There was strong evidence that the protein concentration was greater for the Battelle microsomes than for the In Vitro microsomes, at each individual laboratory and averaged across laboratories. The among laboratory CV for protein concentration differences was 17.5% – about half the CV for the aromatase activity determinations. The among laboratory variation was comparable to the within laboratory variation.

In conclusion, the results from this task indicated that an inexperienced laboratory should be able to obtain a human placenta and, using the procedure described in the present task, prepare viable microsomes that will have an acceptable level of aromatase activity. Also, this task provided information about the intralaboratory and interlaboratory variability of conducting experiments that can be used to characterize the human placental microsomes for use in the aromatase assay.

1.0 INTRODUCTION

1.1 Background

The Food Quality Protection Act of 1996 was enacted by Congress to authorize the Environmental Protection Agency (EPA) to implement a screening program on pesticides and other chemicals found in food or water sources for endocrine effects in humans. Thus, the U.S. EPA is implementing an Endocrine Disruptor Screening Program (EDSP). In this program, comprehensive toxicological and ecotoxicological screens and tests are being developed for identifying and characterizing the endocrine effects of various environmental contaminants, industrial chemicals, and pesticides. The program's aim is to develop a two-tiered approach, e.g., a combination of *in vitro* and *in vivo* mammalian and ecotoxicological screens (Tier 1) and a set of *in vivo* tests (Tier 2) for identifying and characterizing endocrine effects of pesticides, industrial chemicals, and environmental contaminants. Validation of the individual screens and tests is required, and the Endocrine Disruptor Methods Validation Advisory Committee (EDMVAC) will provide advice and counsel on the validation assays.

Estrogens are sex steroid hormones that are necessary for female reproduction and affect the development of secondary sex characteristics of females. Estrogens are biosynthesized from cholesterol by a series of enzymatic steps, with the last step involving the conversion of androgens into estrogens by the enzyme aromatase. Estrogen biosynthesis occurs primarily in the ovary in mature, premenopausal women. During pregnancy, the placenta is the main source of estrogen biosynthesis and pathways for production change. Small amounts of these hormones are also synthesized by the testes in the male and by the adrenal cortex, the hypothalamus, and the anterior pituitary in both sexes. The major source of estrogens in both postmenopausal women and men occurs in extraglandular sites, particularly in adipose tissue. One potential endocrine target for environmental chemicals is the enzyme aromatase, which catalyzes the biosynthesis of estrogens. An aromatase assay is proposed as one of the Tier 1 Screening Battery Alternate Methods. A detailed literature review on aromatase was performed and encompassed (1) searching the literature databases, (2) contacting individuals to obtain information on unpublished research, and (3) evaluating the literature and personal communications.

Aromatase is a cytochrome $P450_{arom}$ enzyme complex responsible for estrogen biosynthesis and converts androgens, such as testosterone and androstenedione, into the estrogens estradiol and estrone. Aromatase is present in the ovary, placenta, uterus, testis, brain, and extraglandular adipose tissues. Two proteins, cytochrome $P450_{arom}$ and NADPH-cytochrome P450 reductase, are necessary for enzymatic activity, and the enzyme complex is localized in the smooth endoplasmic reticulum. The aromatase gene, designated CYP19, encodes the cytochrome $P450_{arom}$ and consists of ten exons, with the exact size of the gene exceeding 70 kilobases. Aromatase is found in breast tissue, and the importance of intratumoral aromatase and local estrogen production is being unraveled. Effective aromatase inhibitors have been developed as therapeutic agents for estrogen-dependent breast cancer to reduce the growth stimulatory effects of estrogens in

breast cancer. Investigations on the development of aromatase inhibitors began in the 1970's and have expanded greatly in the past three decades.

An *in vitro* aromatase assay could easily be utilized as an alternative screening method in the Tier 1 Screening Battery to assess the potential effects of various environmental toxicants on aromatase activity. Both *in vitro* subcellular (microsomal) assays and cell-based assays are available for measuring aromatase activity. The *in vitro* subcellular assay using human placental microsomes is commonly used to evaluate the ability of pharmaceuticals and environmental chemicals to inhibit aromatase activity. In addition, human JEG-3 and JAR choriocarcinoma cell culture lines, originally isolated from cytotrophoblasts of malignant placental tissues, have been used as *in vitro* systems for measuring the effects of compounds on aromatase activity. These cell lines are also utilized for investigations on the effects of agents in placental toxicology.

Numerous flavonoids and related phytoestrogen derivatives have been extensively evaluated for their ability to inhibit aromatase activity for two primary reasons: (1) these natural plant products can serve as possible leads for the development of new nonsteroidal aromatase inhibitors; and (2) humans and other animals are exposed to these agents through the diet. In general, the flavonoids and related analogs demonstrate aromatase inhibition with IC_{50} values in the micromolar range; however, these compounds lack both the potency and specificity of aromatase inhibitors developed for breast cancer therapy. Several pesticides have also demonstrated inhibition of aromatase activity in the human placental microsomal assay system, with IC_{50} values for aromatase inhibition ranging from 0.04 μM to greater than 50 μM.

The human placental microsomal aromatase assay was recommended as the *in vitro* aromatase screening assay to be included in the Tier 1 Screening Battery. This assay will detect environmental toxicants that possess the ability to inhibit aromatase activity. Prevalidation studies on recombinant aromatase (WA 2-24) were conducted to optimize the microsomal aromatase assay protocol for human placenta, demonstrate the utility of the microsomal assay to detect known aromatase inhibitors, and compare the performance of a recombinant assay system and the placental microsomal assays.

1.2 Task Description and Objectives

In this task, human placental microsomes were prepared, analyzed for protein content and aromatase activity (uninhibited and inhibited) was determined. For the inhibition studies, the known aromatase inhibitor 4-hydroxyandrostenedione (4-OH ASDN) was used to demonstrate the responsiveness of the assay to this known aromatase inhibitor. This task was conducted in two stages as described below.

1.2.1 Stage 1 - Placenta Procurement/Microsomal Preparation and Characterization/Positive Control Study

Two participating laboratories, Battelle and In Vitro, were given the assignment to obtain a human placenta and prepare microsomes. Protein concentration (two independent replicate experiments) and aromatase activity (uninhibited, two independent

replicate experiments) were determined by each of the laboratories using the microsomes that they prepared. In addition, Battelle and In Vitro were given the assignment to conduct two independent replicates of a study to determine the response of the microsomes to six concentrations of the known aromatase inhibitor 4-OH ASDN (positive control study) using their own microsomal preparations. The data from these studies were sent to Battelle's EDSP Program Office and, together with staff from the lead laboratory (RTI), the data was reviewed prior to submission to the EPA for approval.

1.2.2 Stage 2 - Distribution of Microsomes and Conduct of Aromatase Activity Studies

After receiving EPA's approval, Battelle and In Vitro each shipped their microsomes to each of the other laboratories, i.e. Battelle distributed its microsomes to RTI, In Vitro, and WIL, whereas In Vitro distributed its microsomes to RTI, Battelle, and WIL. In this way, each laboratory used microsomes prepared by both laboratories in their tests. Protein concentration and aromatase activity information was included with the shipped microsomes. Each laboratory was given the assignment to determine the protein concentration and aromatase activity (uninhibited) for the microsomal preparations that they received.

1.2.3 Objectives

The objectives of this task were to determine whether participating laboratories would be able to procure a human placenta and process it into viable microsomes that could be used to conduct the aromatase assay. In addition, comparisons between microsome preparations were carried out within laboratories and comparisons among laboratories were carried out within microsome preparations. The preparation and analysis effects were independently estimated. Finally, if viable microsomes were prepared, these microsomes would be used for the next task in this sequence of tasks designed to validate the assay, i.e. testing various reference chemicals.

1.3 Overall Report Content and Format

The overall report includes salient information about the methods used and results obtained by the lead laboratory and three participating laboratories, as well as the interlaboratory statistical analysis narrative. Detailed information about the results obtained by the lead and individual participating laboratories can be found in their reports, which are included in the appendices of the overall report. In addition, there are a few important supplemental documents that were the same for all laboratories, i.e. chemistry reports and QAPP, and others that were laboratory specific, i.e. protocol, spreadsheets, intralaboratory statistical analysis narrative. All of these documents can be found in the appendices of the individual laboratory reports.

2.0 MATERIALS AND METHODS

2.1 Chemistry

2.1.1 Substrate – Androstenedione (ASDN)

The substrate for the assay was androstenedione (ASDN). Non-radiolabeled and radiolabeled ASDN were used. The non-radiolabeled ASDN was obtained from Sigma (St. Louis, MO) by Battelle's Chemical Repository (CR) and, from there, was distributed to each of the laboratories. The non-radiolabeled ASDN had a reported purity of 100%. The radiolabeled androstenedione ([1β-^{3}H]-androstenedione, [^{3}H]ASDN) was obtained from Perkin Elmer Life Science (Boston, MA) by Battelle's Chemical Repository, who distributed it to the other laboratories. The radiolabeled ASDN had a reported specific activity of 25.3 Ci/mmol. Radiochemical purity was reported by the supplier to be > 97%. Radiochemical purity was assessed by high performance liquid chromatography (HPLC) at RTI and the results are included in the individual laboratory report appendices. Centralized procurement and distribution were performed by the Chemical Repository to reduce variability in the conduct of this task. The procedure followed to produce the substrate solution for testing is described in the methods sections of the reports for each laboratory (see appendices).

2.1.2 Control Substances

The Chemical Repository at Battelle was responsible for the chemistry activities associated with using 4-OH ASDN, the positive control, and lindane, the negative control, i.e. chemical procurement, solubility, formulation stability assessment, formulation preparation, formulation analysis and shipment of the stock formulation to the lead and participating laboratories. These chemistry activities and results of the analysis and stability determinations are described in the Chemical Repository chemistry report that is in the appendix of the individual laboratory reports. Table 1 summarizes the salient information for the control substances.

Table 1. Chemistry Information for the Control Substances

Chemical Name	Mfr. Purity	CAS No.	Molecular Formula	Molecular Weight (g/mol)
4-hydroxyandrostenedione	99%	566-48-3	$C_{19}H_{26}O_3$	302.4
Lindane	99.6%	58-89-9	$C_6H_6Cl_6$	290.8

Stock formulations of the control substances were prepared by the Chemical Repository. 4-OH ASDN was prepared as a 0.01 M solution in 95% ethanol, whereas lindane was prepared as a 0.1 M solution in dimethylsulfoxide (DMSO). The stock formulations were shipped to the lead and participating laboratories. In addition to using

the 4-OH ASDN and lindane as control substances, Battelle and In Vitro also used 4-OH ASDN in the positive control assay, which tested six different concentrations of 4-OH ASDN to determine the responsiveness of the assay to a known inhibitor and to estimate the IC_{50}.

The lead and participating laboratories prepared fresh dilutions of the stock formulations using 95% ethanol for 4-OH ASDN or DMSO for lindane, which were supplied by the CR. The dilution schemes used by the laboratories to prepare the 4-OH ASDN used in the positive control assay (Battelle and In Vitro) or to prepare the 4-OH ASDN and lindane for use as positive and negative controls can be found in the individual laboratory reports (see appendices). The six 4-OH ASDN final concentrations used in the positive control assay were 10^{-6}, 10^{-7}, 5 x 10^{-8}, 2.5 x 10^{-8}, 10^{-8}, and 10^{-9} M. The final concentrations used in the assay for 4-OH ASDN as a positive control was 5 x 10^{-8} M and for lindane was 10^{-6} M.

2.2 Human Placental Microsomes

The two participating laboratories assigned to procure a human placenta to prepare microsomes were Battelle and In Vitro. For Battelle, a human placenta was obtained from a 24-year-old healthy Hispanic female with full term delivery. The patient denied usage of tobacco, alcohol and drugs. For In Vitro, a human placenta was obtained from a 26-year-old Caucasian female. The mother had no reported medical history, except to note that she was a non-smoker.

The procedure for preparing microsomes from the placenta is provided by the Battelle and In Vitro reports (see appendices). Briefly, the placenta was kept on ice during dissection to keep the tissue chilled. The membrane and fibrous material was dissected, removed and discarded. The spongy tissue was cut into small pieces, placed in a beaker containing ice-cold Buffer A (0.25 M sucrose; 0.04 M nicotinamide, 0.05 M sodium phosphate, pH 7.0), and homogenized. The homogenate was transferred to centrifuge tubes and centrifuged at the setting of 10,000 g for 30 minutes at 4°C. The supernatant was transferred to ultracentrifuge tubes and centrifuged at 100,000 g for one hour at 4°C to obtain a crude microsomal pellet. The supernatant was decanted and the microsomal pellet dislodged with a few mL of Buffer B (0.1 M sodium phosphate, pH 7.4). The clear pellet on the bottom was left in the tube and disposed of. The microsomal pellet was poured into Potter-Elvejhem homogenizer and resuspended in Buffer B. The suspension was transferred to ultracentrifuge tubes and was centrifuged at 100,000 g for one hour at 4°C to wash the microsomes. This washing procedure (supernatant decanting, pellet resuspension and centrifugation) was repeated one additional time. The supernatant was decanted and the twice-washed microsomal pellet was dislodged from the bottom wall of the tube by gentle swirling in a few mL of ice-cold Buffer C (0.1 M sodium phosphate, 0.25 M sucrose, 20 % glycerol, 0.05 mM dithiothreitol, pH 7.4). All microsomal pellets were combined into a single lot and were resuspended in Buffer C. The microsomal suspension was aliquoted (ca. 200 µL/tube) into labeled cryotubes, flash frozen in liquid nitrogen, and stored at ca. -70°C until

removed for use. In Vitro diluted the microsomal suspension before aliquotting into storage tubes.

The lot number and protein concentration for the Battelle microsomes was 6-041305 and 21 mg protein/mL, and for the In Vitro microsomes was BAA and 8 mg protein/mL.

On the day of use, the microsomes were thawed rapidly in a 37 ± 1°C water bath, rehomogenized using a Potter-Elvejhem homogenizer and then kept on ice until used. For use in the assay, the microsomes were diluted in the assay buffer to approximately 0.025 mg/mL. The final target protein concentration in the incubation mixture was approximately 0.0125 mg/mL.

2.3 Other Assay Components

Information about the other assay components is provided in Table 2. The Chemical Repository obtained the NADPH (β-Nicotinamide Adenine Dinucleotide Phosphate, reduced form), DMSO, and ethanol and distributed it to the lead and participating laboratories.

Table 2. Other Assay Components

Chemical	Suppliers by Lab			
	RTI	Battelle	In Vitro	WIL
NADPH (co-factor)[a]	Sigma	Sigma	Sigma	Sigma-Aldrich
Propylene glycol	J. T. Baker	Spectrum	J. T. Baker	J. T. Baker
Sodium phosphate dibasic (buffer)	J. T. Baker	Sigma	J. T. Baker	J. T. Baker
Sodium phosphate monobasic (buffer)	J. T. Baker	Sigma	J. T. Baker	J. T. Baker
Methylene chloride	Not provided	Not provided	Sigma	Not provided
DMSO (vehicle)	Battelle CR	Battelle CR	Battelle CR	Battelle CR
95% Ethanol (vehicle)	Battelle CR	Battelle CR	Battelle CR	Battelle CR

a. Supplied by the EDSP Chemical Repository at Battelle.

2.4 Protein Determination

The microsomal protein concentration was determined using a DC Protein Assay kit from Bio-Rad (Hercules, CA). The 6-point standard curve was prepared using bovine serum albumin (BSA) reconstituted in Milli-Q water. The standard curve range was from 5 to 250 µg protein/mL. Due to slight non-linearity when the 250 µg/mL standard was used, the standard curve was also analyzed using standards from 5 to 125 µg protein/mL. QC standards for use on this task were prepared by diluting a purchased protein standard in order to prepare QC standards containing 10 and 100 µg protein/mL. The absorbance at a wavelength of 750 nm was measured using a spectrophotometer. The protein

concentration of the microsomal sample was determined from the absorbance value using linear regression to the absorbance of the protein standards.

It is important to note that the Battelle protein assay spreadsheet has units listed as mg/mL but should be µg/mL.

2.5 Aromatase Assay Procedure

In Stage 1, Battelle and In Vitro, the two laboratories that each obtained a human placenta and prepared the microsomes, determined the aromatase activity (uninhibited) of their own preparations. The experimental design involved determining aromatase activity by conducting two independent replications of the assay, which consisted of two types of control samples: full enzyme activity and background activity controls (Table 3).

Table 3 - Aromatase Activity Determination (Stage 1)[a]

Sample Type	Repetitions (test tubes)	Description
Full Enzyme Activity Control	4	Complete assay[b] with vehicle (control)
Background Activity Control	4	Complete assay with vehicle (control), omitting NADPH

a. Performed by Battelle and In Vitro.
b. The complete assay contained buffer, propylene glycol, microsomal protein, [^{3}H]ASDN and NADPH.

Also in Stage 1, Battelle and In Vitro conducted two independent replicates of the aromatase assay to determine the response of the microsomes to six concentrations of the known aromatase inhibitor 4-OH ASDN (positive control study). Each lab used its own microsomal preparations. In each replicate, there were four types of control samples (full enzyme activity control, background activity control, and positive and negative controls) and the graded concentrations of 4-OH ASDN (Table 4).

Table 4 – Positive Control Study (Stage 1)[a]

Sample Type	Repetitions (test tubes)	Description	Final Concentration (M)
Full Enzyme Activity Control	4	Complete assay[b] with vehicle (control)	NA
Background Activity Control	4	Complete assay with vehicle (control), omitting NADPH	NA
Positive Control	4	Complete assay with 4-OH ASDN added	5×10^{-8}
Negative Control	4	Complete assay with lindane added	1×10^{-6}
4-OH ASDN Concentration 1	3	Complete assay with 4-OH ASDN added	1×10^{-6}
4-OH ASDN Concentration 2	3	Complete assay with 4-OH ASDN added	1×10^{-7}
4-OH ASDN Concentration 3	3	Complete assay with 4-OH ASDN added	5×10^{-8}
4-OH ASDN Concentration 4	3	Complete assay with 4-OH ASDN added	2.5×10^{-8}
4-OH ASDN Concentration 5	3	Complete assay with 4-OH ASDN added	1×10^{-8}
4-OH ASDN Concentration 6	3	Complete assay with 4-OH ASDN added	1×10^{-9}

a. Performed by Battelle and In Vitro.
b. The complete assay contained buffer, propylene glycol, microsomal protein, [^{3}H]ASDN and NADPH.

In Stage 2, each of the laboratories analyzed the microsomes prepared by Battelle and In Vitro. (Battelle and In Vitro did not analyze their own microsomes but did analyze each other's microsomes.) The experimental design involved determining aromatase activity by conducting two independent replications of the assay, which consisted of four types of control samples: full enzyme activity control, background activity control, and positive and negative controls (Table 5).

Table 5 - Aromatase Assay Study Design[a]

Sample Type	Repetitions (test tubes)	Description	Chemical Concentration (M)
Full Enzyme Activity Control	4	Complete assay[b] with vehicle (control)	NA
Background Activity Control	4	Complete assay with vehicle (control), omitting NADPH	NA
Positive Control	4	Complete assay with 4-OH ASDN added	5×10^{-8}
Negative Control	4	Complete assay with lindane added	1×10^{-6}

a. Performed by all labs using the Battelle- and In Vitro-prepared microsomes.
b. The complete assay contained buffer, propylene glycol, microsomal protein, [^{3}H]ASDN and NADPH.

Details of how the assay was actually performed by each laboratory are presented in the individual laboratory reports. Briefly, the general procedure was as follows. The assays were performed in test tubes maintained at 37 ± 1° C in a shaking water bath. Propylene glycol (100 µL) and [^{3}H]ASDN, NADPH, control chemical or vehicle, and assay buffer (0.1 M sodium phosphate buffer, pH 7.4) were combined in the test tubes (total volume of 1.0 mL). The total assay volume was 2.0 mL. The volume of the control chemical solutions or vehicle used was 20 µL (1 percent of the total assay volume). The final concentrations for the assay components are presented in Table 6.

The tubes and the microsomal suspension were placed at 37 ± 1° C in the water bath for approximately 5 minutes prior to initiation of the assay by the addition of 1 mL of the diluted microsomal suspension.

Table 6. Aromatase Assay Conditions

Assay Components	Component Volume Added to the Assay	Final Concentration in the Assay
Microsomal Protein	1.0 mL	0.0125 mg/mL
NADPH	100 µL	0.3 mM
[^{3}H]ASDN	100 µL	100 nM
Propylene glycol	100 µL	5% (v/v)
4-OH ASDN or vehicle	20 µL	Varies
Assay buffer	700 µL	~ 0.094 M

The tubes were incubated for 15 minutes at 37 ± 1°C. The incubations were stopped by the addition of methylene chloride (2 mL); the tubes were vortex-mixed for ca. 5 seconds and placed on ice. The tubes were then vortex-mixed an additional 20-25 seconds to extract unreacted ASDN, then centrifuged for 10 minutes to facilitate separation of the organic and aqueous layers. The methylene chloride layer was removed and discarded; the aqueous layers were extracted two more times, each time with 2 mL of methylene chloride. The aqueous layers were transferred to vials and duplicate aliquots (0.5 mL) were transferred to 20 mL liquid scintillation counting vials. Liquid scintillation cocktail was added to each counting vial and the vials shaken to mix.

Analysis of the samples was performed using liquid scintillation spectrometry (LSS). Radioactivity found in the aqueous fractions represented ^3H_2O formed from the hydrolysis of [^{3}H]-ASDN. One H_2O molecule was released per molecule of ASDN converted to estrogen in a stereospecific reaction. Thus, the amount of estrogen product formed was determined by dividing the total amount of ^3H_2O formed by the specific activity of the [^{3}H]ASDN substrate (expressed in dpm/nmol). Results are presented as the activity (velocity) of the enzyme reaction and expressed in nmol (mg protein)$^{-1}$min^{-1}.

2.6 Data Analysis

Relevant data were entered into an Excel spreadsheet for calculation of aromatase activity and percent of control.

For each repeat tube (full enzyme activity control, background activity control, positive and negative controls, and each control substance concentration), the Excel spreadsheet included total observed (uncorrected) disintegration per minute (dpm) per tube and total aromatase activity per tube. The dpm and aromatase activity values were corrected for the background dpm, as measured by the average of the background activity control tubes. The aromatase activity was calculated as the corrected dpm, normalized by the specific activity of the [^{3}H]ASDN, the mg of protein of the aromatase, and the incubation time. The average (corrected) dpm and aromatase activity across the four background activity control repeat tubes were necessarily equal to 0 (zero) within each replicate.

2.7 Statistical Analysis

2.7.1 Intralaboratory Statistical Analysis

The intralaboratory statistical analysis was done by the Data Coordination Center at Battelle for two of the three participating laboratories (Battelle and In Vitro) and the reports for these laboratories are included in their respective reports that can be found in the appendices. For the lead laboratory (RTI) and third participating laboratory (WIL), the intralaboratory statistical analysis was done by their statistician according to the unified statistical analysis plan. Their statistical analysis report is included in their report, which can also be found in the appendices.

The principal objectives of the statistical analysis were to:

1. Fit concentration response models within each of the two replicates of the inhibition curve studies with Battelle or In Vitro microsomes to describe the trend in percent of control activity across varying inhibition concentrations of the positive control inhibitor 4-OH ASDN. Estimate the IC_{50} concentration, the slope, and associated standard errors within each replicate. Combine the results across replicates to determine the average IC_{50} concentration, the average slope, and associated standard errors across replicates.

2. Determine whether there were differences between the beginning and the end of each replicate for the full enzyme activity, background activity, positive, and negative control results within each replicate of the inhibition curve test.

3. Compare the aromatase activity values (nmol/mg protein/min) of the full enzyme activity, background activity, positive, and negative controls between the microsomes prepared by In Vitro and the microsomes prepared by Battelle.

4. Compare the protein concentrations (mg/mL) between the microsomes prepared by In Vitro and the microsomes prepared by Battelle.

2.7.1.1 Concentration Response Inhibition Curves

Within each replicate a concentration response inhibition curve was fitted to the percent of aromatase activity values at the three repetitions at each of the six graded 4-OH ASDN inhibitor concentrations.

For purposes of response curve fitting, concentration was expressed on the log scale. In agreement with past convention, common logarithms (i.e. base 10) were used. Let X denote the logarithm of the concentration of inhibitor compound (e.g. if concentration = 10^{-5} then X = -5). Let

Y ≡ (background corrected) percent of control in the inhibitor tube
X ≡ logarithm (base 10) of the concentration
DAVG ≡ average (not corrected for background) DPMs across the repeat tubes with the same inhibitor concentration
β ≡ slope of the concentration response curve (β is negative)
$\mu \equiv \log_{10}IC_{50}$ (IC_{50} is the concentration corresponding to percent of control equal to 50%)

The following two parameter concentration response curve was fitted to relate percent of aromatase activity to logarithm of concentration within each replicate

$$Y = 100/[1 + 10^{(\mu - X)\beta}] + \epsilon$$

where ϵ was the variation among repetitions, distributed with mean 0 and variance approximately proportional to DAVG (based on the Poisson distribution theory for radiation counts) and also approximately proportional to the response Y.

The response curve was fitted by weighted least squares nonlinear regression analysis with weights equal to 1/Y. This weighting system gives greater weight to the lower end of the concentration response curve, where greater inhibition occurs. Observed percent of control values above 100% were set to 99.5%. Observed percent of control values below 0% were set to 0.5%. This adjustment tacitly assumes an upper bound of 100% on the concentration response curve and a lower bound of 0%.

For each replicate the estimated $\log_{10}IC_{50}$ (μ) and its associated standard error, the IC_{50} and its associated geometric standard error, the slope (β) and its associated standard error, and the "Status" of each response curve are reported. The "Status" of each response curve is indicated as "C", complete, if the concentration response curve inhibition ranges from essentially 0 percent to 100 percent of control. Otherwise it is indicated as "I", incomplete.

For each replicate the individual percent of control values were plotted versus logarithm of inhibitor compound concentration. The fitted concentration response curve was superimposed on the same plot. These plots display the data, the fitted response curves in relation to these data, and deviations from the fits.

One-way random effects analysis of variance models with heterogeneous variances among the replicates were fitted to the parameter estimates, $\log_{10}IC_{50}$ (μ) and slope (β), from the concentration response curve fits within each replicate, using weights incorporating within replicate variances. The random effect was replicate. The within replicate variances were estimated as the squares of the standard errors for each replicate. The analysis of variance fits provide estimated weighted averages (means) across the replicates and their associated standard errors. Degrees of freedom associated with the mean effects were calculated based on Satterthwaite's approximation.

The estimated IC_{50} for the control substance was calculated as 10 to the power mean $\log_{10}IC_{50}$. The geometric standard error associated with the estimated IC_{50} was calculated as 10 to the power standard error associated with mean $\log_{10}IC_{50}$.

Slope (β) and $\log_{10}IC_{50}$ (μ) were each compared across replicates based on the one-way random effects analysis of variance model fit. For each of β and μ, plots were prepared that displayed the parameters within each replicate with associated 95% confidence intervals based on the within replicate standard error, and the average across replicates with associated 95% confidence interval incorporating replicate-to-replicate variation.

Concentration response curves were also fitted to the averages of the three repetitions within each replicate and estimates and associated standard errors (or geometric standard error) for $\log_{10}IC_{50}$ (μ), IC_{50}, and slope (β) were displayed. The averages of the three repetitions for each of the three replicates were plotted in the same plot with plotting symbols distinguishing among replicates. The concentration response curves for each replicate, fitted to the average data, were superimposed on the same plot to compare the percent of aromatase activity values across replicates.

On a separate plot the average percent of control values for each of the replicates were plotted versus logarithm of inhibitor concentrations. The average concentration response curve across replicates was superimposed on the same plot. The average response curve was defined as

$$Y_{avg} = 100/[1 + 10^{\beta avg(\mu avg - X)}]$$

where β_{avg} and μ_{avg} were the mean values across the replicates, based on the random effects one-way analysis of variance model discussed above.

All concentration response curves were fitted to the data using the nonlinear regression analysis features in the PRISM statistical analysis package, Version 4. Supplemental statistical analyses and displays such as summary tables, graphical

displays, analysis of variance, and multiple comparisons were carried out using PRISM and the SAS statistical analysis system- Version 8 or higher.

Within each replicate, quadruplicate repetitions were made for the full enzyme activity, background activity, positive, and negative controls responses. Half the repetitions were carried out at the beginning of the replicate and half at the end. If the test conditions were consistent throughout the replicate, the control tube responses at the beginning should have been equivalent to those at the end.

The control responses were expressed as percent of full enzyme activity control. The full enzyme activity, background activity, positive, and negative control percent of control responses associated with the inhibitor concentration tests were plotted across replicates, with plotting symbol distinguishing between beginning and end, and with reference line at 0% (background activity control), at 100% (full enzyme activity control) at 50% (positive control), or at 100% (negative control). These plots indicate the extent of consistency across replicates with respect to average value and variability, and provide comparisons of beginning versus end of each replicate. Additional plots were prepared displaying the differences of the averages of the first two percent of control values (i.e. those based on the "beginning" tubes) and the averages of the last two percent of control values (i.e. those based on the "end" tubes) across replicates (end minus beginning). Each plot has a reference line of 0.

Mixed effects analysis of variance models were fitted to the full enzyme activity, background activity, positive, and negative control data. The response was percent of control. The fixed effect factor in the analysis of variance was portion (beginning or end). The random effects were replicate and portion by replicate interaction. The residual error variation was based on the variation among repetitions within replicate and portion. For the background activity and full enzyme activity controls, the average of the repetitions within a replicate were constrained to be 0 and 100 respectively, which implies that the variation associated with the replication effect is necessarily constrained to be 0.

2.7.1.2 Aromatase Activity Data

Each of the four types of aromatase activity responses (full enzyme activity, background activity, positive, and negative controls) were fitted with mixed effects analysis of variance models. The response was aromatase activity (nmol/mg protein/min). The fixed effect was microsome source (the laboratory which prepared the microsomes) and the random effect was replicate within microsome source. Analysis of variance tests were performed to determine if the microsome source effect was significant. Summary statistics (N, mean, and standard deviation) were calculated. Scatter plots were also prepared with different plotting symbols for each microsome source.

2.7.1.3 Protein Concentration Data

A two-sample t-test was performed to compare protein concentrations between the two microsome sources. The response was protein concentration (mg/mL). Summary statistics (N, mean, and standard deviation) were calculated by microsome source. A scatter plot was also prepared, having different plotting symbols for each microsome source.

2.7.1.4 Round Off

Some derived values in the results tables may differ from those in the computer printouts or from those obtained using hand calculations by several units in the least significant digit due to round off in intermediate numbers or in intermediate calculations.

2.7.2 Interlaboratory Statistical Analysis

The interlaboratory statistical analysis was done by the Data Coordination Center at Battelle and the full statistical analysis report is included as an appendix to this overall report.

Aromatase activity determinations were carried out in conjunction with the inhibition curve analyses (at Battelle and In Vitro Technologies) and in separate aromatase activity tests (at all four laboratories). Protein concentration determinations were carried out in conjunction with the inhibition curve analyses (at Battelle and In Vitro Technologies), in separate aromatase activity tests, and in separate protein concentration determination tests (at all four laboratories). For each replicate of the aromatase activity tests four repetitions were carried out. For each replicate of the protein concentration determination tests a single determination was made. Table 7 displays the number of replicates carried out for each response type at each test laboratory.

The inter-laboratory statistical analysis combines summary results from each of the intra-laboratory analyses to assess relationships among the results at each laboratory, the extent of laboratory-to-laboratory variation, and overall consensus estimates among the laboratories with associated variability estimates (incorporating laboratory-to-laboratory variability).

Table 7. Number of Replicates at Each Test Laboratory for Each Response Type

	Microsome Source	Test Laboratory			
		RTI	WIL	Battelle	In Vitro
Background Activity Controls[1]	Battelle	2	2	4	2
	In Vitro	2	2	2	4
Full Enzyme Activity Controls[1]	Battelle	2	2	4	2
	In Vitro	2	2	2	4
Negative Controls[1]	Battelle	2	2	2	2
	In Vitro	2	2	2	4
Positive Controls[1]	Battelle	2	2	2	2
	In Vitro	2	2	2	4
Protein Concentration[2]	Battelle	4	4	6	4
	In Vitro	4	4	4	6

The objectives of the interlaboratory statistical analysis were to:

- Determine the average values and the variabilities among laboratories for the above parameters.
- Determine the coefficients of variation among laboratories for the above parameters.
- Estimate the ratio of the among laboratory variation to the average within laboratory variation for the parameters mentioned above.

Statistical analyses were carried out for each of the five endpoints displayed in Table 7: source effects (Battelle minus In Vitro) for background activity, full enzyme activity, negative, positive controls, and protein concentration.

For each endpoint a one-way random effects analysis of variance model with heterogeneous variances among the participating laboratories was fitted to the summary microsome source effects differences within laboratories. Laboratory was treated as a random effect. The within laboratory variances were based on the squares of the standard errors associated with the endpoint estimates in each of the intralaboratory analyses. The analysis of variance resulted in a weighted average across all the laboratories and its associated standard error as well as an estimate of the laboratory-to-laboratory component of variation. The weights included in the weighted averages incorporated

[1] Four repeat determinations (in separate tubes) per replicate.
[2] One determination per replicate.

both laboratory-to-laboratory variation and within laboratory variation. The degrees of freedom associated with the overall weighted averages were calculated based on Satterthwaite's approximation as

$$2*[((1/K)*\sum(S_L^2 + S_i^2))^2]/[(var(S_L^2)+(2/K^2)*\sum(S_i^4/df_i))]$$

where S_L^2 is the random laboratory to laboratory variance, S_i^2 and df_i are the reported within laboratory variance and degrees of freedom for the i^{th} laboratory, $var(S_L^2)$ is the variance of S_L^2, and K is the number of laboratories (Hartung and Makambi, 2001).

For each endpoint, the estimated overall average and its associated standard error (incorporating both within laboratory and among laboratory components of variation) and associated degrees of freedom were used to construct a 95% confidence interval based on the t-distribution. For each laboratory the individual effect and associated 95% confidence interval (based on the within laboratory standard error) were also determined. These were plotted side-by-side to provide a graphical comparison among the laboratories.

To describe the variability among the individual laboratory values relative to the overall average value, coefficients of variation (CV) were calculated. The coefficient of variation is defined as the standard deviation of the effect response divided by its mean. The CV is expressed as

$$CV=(S/d_{avg}) \times 100\%$$

where d_{avg} is the weighted average Battelle minus In Vitro microsome source difference across the four laboratories, S^2 is the total variance among the four laboratories, and $S = \sqrt{S^2}$. S^2 is approximated by $4(se)^2$ where se is the standard error of the pooled average. This would be exact if the within laboratory variances were equal across laboratories.

To describe the variability among laboratories relative to variability within laboratories the ratio of the standard deviation of the among laboratories component of variation to the unweighted average standard error within laboratories was calculated as

$$R=S_{lab}/ [⅓(s_1 + s_2 + s_3+ s_4)] \times 100\%$$

where S_{lab} is the square root of the component of variance among the three laboratories and (s_1, s_2, s_3, s_4) are the within laboratory standard errors at the four laboratories. This ratio was calculated for each of the five parameters shown in Table 7.

In several places entries in the tables in the interlaboratory analysis report tables may differ from corresponding entries in the intralaboratory analysis report tables by one or a small number of trailing digits in the last decimal place. This is often due to differences between the intralaboratory analyses and the interlaboratory analysis in rounding in intermediate calculations.

2.8 Good Laboratory Practices

The toxicology laboratories at RTI, Battelle, and WIL Research Laboratories and the chemistry laboratories at Battelle conducted this task in compliance with the U.S. EPA FIFRA Good Laboratory Practices Standards. In Vitro Technologies operated in compliance with the U.S. FDA Good Laboratory Practices Standards.

2.9 Personnel

The personnel involved in the conduct of this task are listed in their respective laboratory reports that are included in the appendices.

3.0 RESULTS

3.1 [^{3}H]-ASDN Radiochemical Purity

The radiochemical purity for the substrate was 97 percent. The radiochemical purity report is included as an appendix of the individual laboratory reports.

3.2 Stock Formulation Analyses

The 4-OH ASDN and lindane stock formulations were prepared and analyzed by Battelle's Chemical Repository. The target and actual 4-OH ASDN concentrations were 3.02 and 3.08 mg/mL and for lindane were 29.08 and 29.37 mg/mL, respectively. Thus, the actual concentrations were within 2 and 1 percent of their respective target concentrations. The 4-OH ASDN and lindane stock formulations were shown to be stable for at least 173 and 168 days, respectively, when stored refrigerated.

3.3 Microsomal Protein Analysis

Microsomal protein concentration determinations were made at different times by different laboratories using different microsomes according to the experimental design. The results are presented by experimental stage and, for a given stage, the experiments performed by the laboratories involved in that stage. In addition, the overall results for a given microsomal preparation by laboratory are presented.

In Stage 1, Battelle and In Vitro determined the microsomal protein concentration of their respective microsomes following preparation of the microsomes. The results of the initial determination are summarized in Table 8. Battelle's microsomal preparation was more concentrated than In Vitro's preparation but both preparations were found to have acceptable concentrations of protein and enzyme activity to conduct the aromatase assay.

Table 8. Initial Human Placental Microsomal Protein Concentration Determinations by Laboratory (Stage 1)[a]

Laboratory	Replicate	Protein Concentration (mg/mL)	
		Individual Value	Average
Battelle	1	22.58	21.40
	2	20.22	
In Vitro	2	8.202	8.068
	3	7.934	

a. Independent replicates.

Also in Stage 1, Battelle and In Vitro determined the microsomal protein concentration of their respective microsomes in the process of performing the aromatase activity experiment (uninhibited) and the positive control experiments with 4-OH ASDN (inhibited). The protein concentration determination results from these experiments are summarized in Table 9. These results were in good agreement with the initial protein concentrations determined by each laboratory.

Table 9. Human Placental Microsomal Protein Concentration Determinations Obtained During the Aromatase Activity Experiments (Stage 1)[a]

Laboratory	Experiment Type	Replicate	Protein Concentration (mg/mL)	
			Individual Value	Average
Battelle	Aromatase Activity (uninhibited)	1	20.27	19.17
		2	17.72	
	Aromatase Activity (inhibited)	1	20.07	
		2	18.61	
In Vitro	Aromatase Activity (uninhibited)	1	8.951	7.689
		2	8.086	
	Aromatase Activity (inhibited)	2	6.172	
		3	7.545	

a. Independent replicates were performed for the aromatase activity experiments (uninhibited and inhibited).

The average ± standard error of the mean (SEM) (%CV) microsomal protein concentration that each laboratory reported for their respective microsomal preparations using all of the determinations in Stage 1 (n=6) was 19.91 ± 0.68 mg/mL (8.4 percent) for Battelle and 7.82 ± 0.38 mg/mL (11.9 percent) for In Vitro.

In Stage 2, Battelle distributed its microsomes to the other three laboratories and In Vitro did likewise. Each laboratory performed a protein concentration determination experiment and an aromatase activity (uninhibited) experiment, which included a protein determination (Table 10). The protein concentrations determined by the laboratories were in good agreement with the concentrations as reported by the laboratory that prepared the microsomes. The % RE values for analysis of the Battelle-prepared microsomes by In Vitro, RTI, and WIL were 23.3, 20.9, and 11.0 percent, respectively. The % RE values for analysis of the In Vitro-prepared microsomes by Battelle, RTI, and WIL were 9.6, 19.9, and 3.1, respectively.

In order to calculate an overall task protein concentration for the Battelle- and In Vitro-prepared microsomes, the determinations from Tasks 1 and 2 from all laboratories were used. The overall mean ± SEM (% CV) task protein concentration for the Battelle-prepared microsomes was 22.5 ± 0.9 mg/mL (16.8 percent) and for the In Vitro-prepared microsomes was 8.4 ± 0.3 mg/mL (13.1 percent).

Table 10. Human Placental Microsomal Protein Concentration Determinations (Stage 2)[a]

Source of the Microsomes	Laboratory	Experiment Type	Replicate	Protein Concentration (mg/mL)		
				Individual Value	Mean (± sd, SEM, %CV)	% RE[b]
Battelle	In Vitro	Protein Determination	1	31.19	24.6 (± 5.0, ± 2.5, 20.4)	23.3
			2	25.14		
		Aromatase Activity	1	22.48		
			2	19.38		
	RTI[c]	Protein Determination	1	23.6	24.1 (± 3.5 ± 1.7, 14.4)	20.9
			2	24.7		
		Aromatase Activity	3	19.8		
			4	28.2		
	WIL	Protein Determination	1	19.69	22.1 (± 3.0, ± 1.5, 13.7)	11.0
			2	25.63		
		Aromatase Activity	1	19.48		
			2	23.58		
In Vitro	Battelle	Protein Determination	1	8.800	8.57 (± 0.53, ± 0.26, 6.2)	9.6
			2	9.181		
		Aromatase Activity	1	7.987		
			2	8.304		
	RTI[c]	Protein Determination	1	9.2	9.4 (± 1.3, ± 0.66, 14.0)	19.9
			2	7.8		
		Aromatase Activity	3	9.5		
			4	11.0		
	WIL	Protein Determination	1	8.689	8.06 (± 0.75, ± 0.37, 9.3)	3.1
			2	8.345		
		Aromatase Activity	1	6.978		
			2	8.229		

a. Independent replicates were performed for the protein determination and aromatase activity experiments (uninhibited).
b. % RE – calculated by comparing the protein concentration determined by the source laboratories for both experiments in Stage 1 (19.91 mg/mL for Battelle and 7.82 mg/mL for In Vitro) to the mean value determined by the participating laboratory.
c. RTI reported values to 0.1 mg/mL, whereas the other laboratories reported values to at least four significant figures.

3.4 QCs for the Protein Concentration Assay

QC standards were included in the protein determination assay in order to evaluate day-to-day results for a given laboratory and laboratory-to-laboratory results. Two QC standards were used (10 and 100 µg/mL). The precision (% CV) and accuracy (% RE) are summarized in Table 11 for the laboratories. For the low QC standard, precision ranged from 9.1 to 137 percent with two of four laboratories attaining % CV values of less than approximately 30 percent. Accuracy was within 20 percent, except for two laboratories that had % RE values of -60 and -34 percent. For the high QC standard, precision was less than approximately 20 percent and accuracy was within approximately 10 percent for all laboratories. With the exception of one laboratory, the QC standards indicated that the protein concentration determinations were similar from day-to-day

within a laboratory and from laboratory-to-laboratory, with the exception of two laboratories that had poor precision and accuracy using the low QC standard.

Table 11. Summary Results for Protein Assay QC Standards

QC Standard (mg/mL)	Laboratory	Mean	sd	SEM	% CV	% RE
0.010	Battelle	0.009	0.001	0.000	9.1	-10.0
	In Vitro	0.004	0.006	0.002	137	-60.0
	RTI	0.008	0.002	0.001	28.2	-17.1
	WIL	0.007	0.002	0.001	33.2	-33.8
0.100	Battelle	0.092	0.003	0.001	3.2	-8.5
	In Vitro	0.107	0.016	0.007	15.1	7.3
	RTI	0.096	0.007	0.002	6.8	-4.2
	WIL	0.091	0.003	0.001	3.3	-9.3

3.5 Microsomal Activity Characterization by Source Laboratories (Stage 1)

Battelle and In Vitro characterized the microsomes that they prepared by determining the aromatase activity in the absence (uninhibited) and in the presence (inhibited) of 4-OH ASDN. This latter experiment (also referred to as a positive control study) generated aromatase activity that was both uninhibited and inhibited. Thus, the results from the first experiment, which only generated uninhibited aromatase activity data, and the uninhibited aromatase activity data generated from the second experiment will be included in the first subsection below. The second subsection will present the percent of control results, thereby focusing on the inhibition characterization of the microsomes by both laboratories.

3.5.1 Aromatase Activity (Uninhibited)

The aromatase activity as determined by the laboratories that prepared the microsomes is summarized in Table 12. Both laboratories prepared microsomes from a human placenta with aromatase activity that was able to be measured and that met the acceptance criteria value of greater than 0.03 nmol/mg/min.

Table 12. Aromatase Activity of the Human Placental Microsomes as Determined by the Source Laboratories (Stage 1)[a]

Laboratory	Experiment Type	Replicate	Aromatase Activity (nmol/mg/min)				
			Individual Value	Mean	sd	SEM	%CV
Battelle	Aromatase Activity (uninhibited)	1	0.0444	0.0542	0.0094	0.0047	17.4
		2	0.0528				
	Aromatase Activity (inhibited)	1	0.0671				
		2	0.0524				
In Vitro	Aromatase Activity (uninhibited)	1	0.0338	0.0382	0.0054	0.0014	14.1
		2	0.0342				
	Aromatase Activity (inhibited)	2	0.0462				
		3	0.0385				

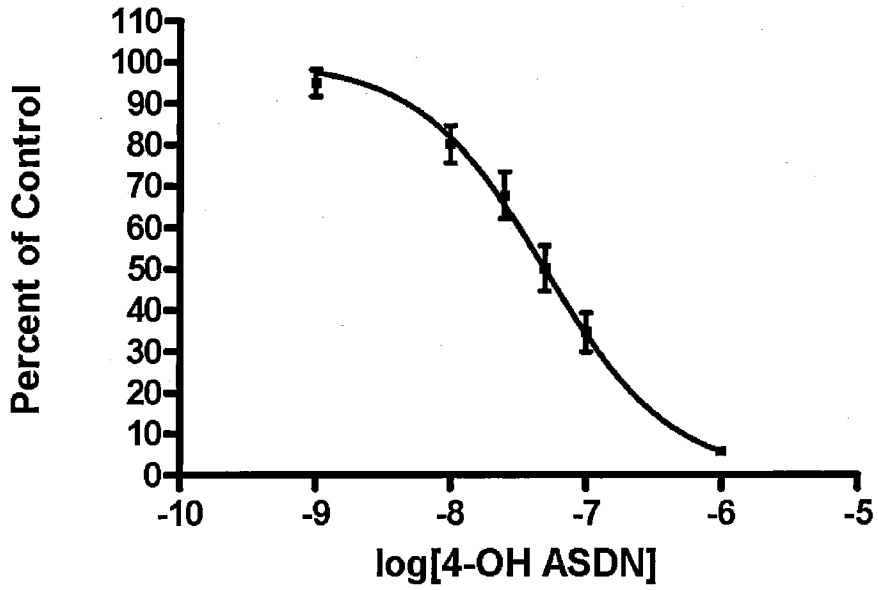
a. Independent replicates were performed for the aromatase activity experiments (uninhibited and inhibited).

3.5.2 Aromatase Activity (Inhibited) – Positive Control Study

The aromatase activity in the presence of graded concentrations of 4-OH ASDN for each of the microsomal preparations as determined by the laboratory that prepared the microsomes is summarized in Table 13. Aromatase activity decreased with increasing concentration of the inhibitor for both microsomal preparations. At a concentration of 10^{-9} M 4-OH ASDN, approximately 95 to 100 percent of the aromatase activity was present, whereas at a concentration of 10^{-6} M, approximately 6 percent of the aromatase activity was observed for both preparations. An example of one of the laboratory's concentration response curves and corresponding Prism output is shown in Figure 1.

Table 13. Aromatase Activity in the Presence of 4-OH ASDN as Determined by the Source Laboratories (Stage 1)

Laboratory	Log [4-OH ASDN]	Percent of Control			
		Overall Mean	sd	SEM	%CV
Battelle	-6.00	5.79	0.95	0.39	16.5
	-7.00	34.66	4.74	1.94	13.7
	-7.30	50.14	5.43	2.22	10.8
	-7.60	67.69	5.56	2.27	8.2
	-8.00	80.01	4.44	1.81	5.5
	-9.00	95.22	3.86	1.57	4.1
In Vitro	-6.00	6.03	0.84	0.34	14.0
	-7.00	36.70	3.35	1.37	9.1
	-7.30	54.62	1.87	0.76	3.4
	-7.60	69.06	2.21	0.90	3.2
	-8.00	86.31	2.85	1.16	3.3
	-9.00	100.96	2.49	1.02	2.5



Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.0
TOP	100.0
LOGEC50	-7.304
HILLSLOPE	-0.9307
EC50	4.964e-008
Std. Error	
LOGEC50	0.01809
HILLSLOPE	0.03134
95% Confidence Intervals	
LOGEC50	-7.341 to -7.267
HILLSLOPE	-0.9944 to -0.8669
EC50	4.561e-008 to 5.403e-008
Goodness of Fit	
Degrees of Freedom	34
R^2 (unweighted)	0.9794
Weighted Sum of Squares (1/Y)	12.02
Absolute Sum of Squares	658.3
Sy.x	4.400
Constraints	
BOTTOM	BOTTOM = 0.0
TOP	TOP = 100.0
Data	
Number of X values	6
Number of Y replicates	6
Total number of values	36
Number of missing values	0

Figure 1. 4-OH ASDN Concentration Response Curve and Prism Output

Based on the curve-fit for the concentration response graphs, the IC_{50} and slope values for 4-OH ASDN were calculated (Table 14). The 4-OH ASDN IC_{50} and slope values were similar for both preparations, i.e. 51.7 and 56.9 nM for IC_{50} and -0.9930 and -0.9919 for the slope. Also, these values were in good agreement with results obtained in previous experiments.

Table 14. IC_{50} and Slope Values for the 4-OH ASDN Concentration Response Curves as Determined by the Source Laboratories (Stage 1)

		Parameters			
Laboratory	Replicate	Individual IC_{50} (nM)	Mean IC_{50} (nM)	Individual Slope	Mean Slope
Battelle	1	40.4	51.7	-0.9075	-0.9330
	2	63.0		-0.9584	
In Vitro	2	53.6	56.9	-1.027	-0.9919
	3	60.2		-0.9567	

3.6 Microsomal Activity Characterization by Other Laboratories (Stage 2)

Battelle and In Vitro distributed their characterized microsomes to each of the other laboratories for characterization, i.e. determine the aromatase activity (uninhibited). The Battelle-prepared microsomes were analyzed by In Vitro, RTI, and WIL; whereas the In Vitro-prepared microsomes were analyzed by Battelle, RTI, and WIL (Table 15). For a given microsomal preparation, the three laboratories obtained aromatase activity values that differed by approximately 50 to 60 percent, i.e. 0.0464 to 0.0708 nmol/mg/min for the Battelle-prepared microsomes and 0.0276 to 0.0443 nmol/mg/min for the In Vitro-prepared microsomes. The overall average ± SEM (% CV) aromatase activity values for the Battelle- and In Vitro-prepared microsomes were 0.0578 ± 0.0051 nmol/mg/min (21.5 percent) and 0.0362 ± 0.0032 nmol/mg/min (21.9 percent). If the aromatase activity values obtained by the source laboratories are used as a benchmark, i.e. 0.0542 and 0.0382 nmol/mg/min for the Battelle- and In Vitro-prepared microsomes, respectively, then the % RE values for In Vitro, RTI, and WIL were -14.3, 3.8, and 30.6 percent for the Battelle-prepared microsomes, respectively, and for Battelle, RTI, and WIL were -27.7, -4.5, and 16.1 percent, respectively, for the In Vitro-prepared microsomes.

Table 15. Aromatase Activity of the Human Placental Microsomes as Determined by the Other Laboratories (Stage 2)[a]

Laboratory	Replicate	Aromatase Activity (nmol/mg/min)				
		Individual Value	Mean	sd	SEM	%CV
Battelle-Prepared Microsomes						
In Vitro	1	0.0423	0.0464	0.0047	0.0017	10.1
	2	0.0506				
RTI[b]	1	0.0645	0.0563	0.0116	0.0082	20.7
	2	0.0480				
WIL	1	0.0721	0.0708	0.0094	0.0033	13.3
	2	0.0695				
In Vitro-Prepared Microsomes						
Battelle	1	0.0288	0.0276	0.0019	0.0009	6.8
	2	0.0264				
RTI[b]	1	0.0370	0.0365	0.0007	0.0005	1.8
	2	0.0360				
WIL	1	0.0481	0.0443	0.0048	0.0017	10.8
	2	0.0406				

a. Independent replicates were performed for the aromatase activity experiments (uninhibited).
b. N = 2

3.7 Positive and Negative Controls (Stage 2)

The experimental design used in Stage 2 included analyzing the effect of 4-OH ASDN (positive control) and lindane (negative control) on aromatase activity (Table 16). Inhibition by the positive control ranged from approximately 44 to 56 percent and, for the negative control, from approximately 95 to 107 percent for both microsomal preparations and all laboratories.

Table 16. Effect of 4-OH ASDN (Positive Control) and Lindane (Negative Control) on Aromatase Activity as Determined by the Other Laboratories (Stage 2)[a]

Laboratory	Aromatase Activity (nmol/mg/min)				
	Full Enzyme Activity	4-OH ASDN	% Inhibition	Lindane	% Inhibition
Battelle-Prepared Microsomes					
In Vitro	0.0464	0.0259	55.8	0.0496	106.9
RTI	0.0563	0.0268	47.6	0.0550	97.7
WIL	0.0708	0.0309	43.6	0.0674	95.2
In Vitro-Prepared Microsomes					
Battelle	0.0276	0.0153	55.4	0.0288	104.3
RTI	0.0365	0.0167	45.8	0.0353	96.7
WIL	0.0443	0.0208	47.0	0.0440	99.3

a. The 4-OH ASDN concentration was 5×10^{-8} M and, for lindane, it was 10^{-8} M.

3.8 Intralaboratory Statistical Analysis

The full individual laboratory statistical analysis reports are included in their respective laboratory reports, which can be found in the appendices.

3.8.1 Battelle Intralaboratory Statistical Analysis

A summary of the results are as follows.

For the inhibition curve fits, the $\log_{10}IC_{50}$ replicate-to-replicate variation was two orders of magnitude larger than the individual replicate within replicate variation. The within-replicate variations were close to zero. For slope, the replicate-to-replicate variation was about three times the individual replicate within-replicate variances.

For the full enzyme activity controls and the positive controls, in the inhibition curve tests, the averages of the two percent of control measurements at the end were lower than the averages at the beginning for both replicates. The average difference was significant for the full enzyme activity controls and borderline significant (p=0.055) for the positive controls. For the background activity controls and for the negative controls, the averages of the measurements at the end were lower than the average at the beginning, but the differences were not statistically significant. In general the aromatase activity at the end of each replicate was lower than at the beginning.

For the aromatase activity results, significant laboratory effects were found for the full enzyme activity controls, positive controls, and negative controls. The activity levels were lower for the In Vitro prepared microsomes than for the Battelle prepared microsomes. (The background adjusted background activity controls are by definition constrained to have on average 0 activity within each replicate within each laboratory.) Variance estimates for replicate and for repetition within replicate were small.

A highly significant microsome source effect was identified for the protein concentration results. The Battelle prepared microsomes had more than 2.3 times higher protein concentration than the In Vitro prepared microsomes.

3.8.2 RTI Intralaboratory Statistical Analysis

According to the two-sample t-test results, there was a very significant difference (p= 0.0002) between the protein concentrations provided by the two labs, with the higher concentration of protein appearing in the microsomes provided by Battelle.

The t-test results indicated that there were statistically very significant differences between the aromatase activity values from each of the laboratories for the full activity control (p= <0.0001), negative control (p= <0.0001) and positive control (p= <0.0001). The background control aromatase activity values showed very little statistical significance in the difference of the means from each of the two laboratories.

3.8.3 In Vitro Intralaboratory Statistical Analysis

A summary of the results are as follows.

For the inhibition curve fits, the $\log_{10}IC_{50}$ replicate-to-replicate variation was more than nine times the repetition within-replicate variation. For the slope the replicate-to-replicate variation and the repetition within-replicate variation were both close to zero.

For the full enzyme activity controls and the positive controls in the inhibition curve tests, the averages of the two percent of control measurements at the end were lower than the averages at the beginning for both replicates. However, the differences were not significant. For the background activity controls the averages of the two measurements at the end were higher than the averages at the beginning for both replicates. However, the differences were also not significant. For the negative controls, the averages of the two measurements at the end were lower than the averages at the beginning for both replicates and the differences were statistically significant. In general the aromatase activity at the end of each replicate was lower than at the beginning.

For the aromatase activity results, no significant microsome source effects were found for any of the four types of controls (full enzyme activity control, background activity control, positive control, and negative control). Variance estimates for replicate and for repetition within replicate were small.

A highly significant microsome source effect was identified for the protein concentration results. The Battelle prepared microsomes had more than three times higher protein concentration than the In Vitro prepared microsomes.

3.8.4 WIL Intralaboratory Statistical Analysis

The protein concentration and enzyme activity data were subjected to two types of statistical analysis to determine if the results showed significant differences between the microsomes.

A two-sample T-test was performed on the protein concentration data to determine if the concentration of protein in the microsomes from Battelle was the same as that in the microsomes from In Vitro based on the experimentally determined concentration and the number of sample analyzed from each preparation. The likelihood that the protein concentration in the two preparations was the same was very small (T-test result = 0.0018).

The full enzyme activity, background activity, positive control activity and negative control activity values were analyzed by two-way analysis of variance (ANOVA) with aromatase activity (nmol/mg protein/min) as the response variable. The aromatase activity values were analyzed for comparison of the aromatase activity in the control values between microsomes from Battelle and In Vitro. Average activity of the microsomes from Battelle and In Vitro Technologies were compared by ANOVA with a fixed term for microsomes source and a random term for replicates within the microsome source. Results of the ANOVA indicated a high random term for replicates within the microsome source. Results of the ANOVA indicated a high probability that the difference in the mean activity of the samples tested from the two microsome preparations represented a true and statistically significant difference in activity. For the full enzyme activity, positive control activity and negative control activity the probability is less than 10% (p-values = 0.0214, 0.0803, and 0.0690, respectively) that the difference was a result of chance based on the sample size. Thus, the enzymatic activity of the microsomes prepared at Battelle was significantly different than those prepared by In Vitro. Because no activity was expected in the absence of NADPH, the background activity controls were the same in all assays regardless of the source of the microsomes. This was reflected in the p-value of 1.0000.

3.9 Interlaboratory Statistical Analysis

The full interlaboratory statistical analysis report is included in the appendices.

3.9.1 Aromatase Activity

Table 17 displays the estimated within laboratory mean differences and their associated within laboratory standard errors, degrees of freedom, and 95% confidence intervals about these values for the background activity, full enzyme activity, negative, and positive controls. These values are based on the least squares means, standard errors, and degrees of freedom reported in the intra-laboratory analyses. It also displays the overall mean differences averaged across laboratories and their associated standard errors, degrees of freedom, and 95% confidence intervals, incorporating among laboratory variation based on the random effects analysis of variance. These mean differences and confidence intervals are graphically displayed in Figures 2 to 5. Each figure includes reference lines corresponding to the overall average.

Table 18 displays the total standard deviation (square root of the total variance) across laboratories, the pooled average mean difference, and the among laboratory coefficient of variation for the background activity, full enzyme activity, negative, and positive controls. The coefficient of variation is not displayed for the background activity controls because the mean difference is 0.

Table 19 displays the within laboratory standard errors for each laboratory for the background activity, full enzyme activity, negative, and positive controls. Table 19 also displays the laboratory to laboratory variance component standard deviation, and the

ratios of the among laboratory standard deviations to the unweighted average of the within laboratory standard errors.

Table 17 shows that for the background activity controls there is no source effect, either within laboratories or averaged across laboratories. By definition the average background corrected aromatase activity for the background activity controls must be 0 within each replicate. For the full enzyme activity, negative, and positive controls the mean estimates indicate greater activity for the Battelle microsomes than for the In Vitro microsomes for all control types and at all laboratories. For RTI and In Vitro the differences are not significantly greater than 0 (p=0.05) for any of the control types since the confidence intervals include 0. For WIL Laboratories the differences are not significantly greater than 0 (p=0.05) for the negative or positive controls. For Battelle the differences are significantly greater than 0 (p=0.05) for each of the control types. Averaged across laboratories the Battelle microsomes have significantly greater activity than the In Vitro microsomes (p=0.05) for each of the control types.

Table 18 shows among laboratory CVs (excluding background activity controls) in the range from 32.5% to 46.2%, depending on control type. Table 19 shows that the among laboratory variation is comparable to the within laboratory variation. The ratio of the among laboratory standard deviation to the average within laboratory standard error (excluding background activity controls) is between 106.6% and 125.2%.

3.9.2 Protein Concentration

Tables 20 to 22 display the same summary information as Tables 17 to 19 for protein concentration. The mean differences and confidence intervals are graphically displayed in Figure 6.

Table 20 shows very strong evidence that the protein concentration was determined to be greater for the Battelle microsomes than for the In Vitro microsomes, at each individual laboratory and averaged across laboratories. Table 21 shows an among laboratory CV for protein concentration differences of 17.5%. This is about half the CV for the aromatase activity determinations shown in Table 18. Table 22 shows that the among laboratory variation is comparable to the within laboratory variation. The ratio of the among laboratory standard deviation to the average within laboratory standard error is 105.9%

Table 17. Difference in Aromatase Activity (nmol/mg protein/min) Between Battelle Microsomes and In Vitro Technologies Microsomes (Battelle Minus In Vitro). Background Activity, Full Enzyme Activity, Negative, and Positive Controls

RTI	WIL	Battelle	In Vitro	Average
Background Activity Controls				
-0.00000[1](0.00006[2]) 2.00[3] df (-0.000249, 0.000249)[4]	0.00000(0.00003) 2.00 df (-0.000122, 0.000122)	-0.00000(0.00004) 4.00 df (-0.000100, 0.000100)	0.00000(0.00021) 4.00 df (-0.000572, 0.000572)	-0.00000[5](0.00002[6]) 5.02[7] df (-0.000051, 0.000051)[8]
Full Enzyme Activity Controls				
0.01977(0.00634) 2.00 df (-0.007502, 0.047042)	0.02647(0.00393) 2.00 df (0.009554, 0.043386)	0.02596(0.00778) 4.00 df (0.004373, 0.047547)	0.00823(0.00500) 4.00 df (-0.005655, 0.022115)	0.01987(0.00459) 3.61 df (0.006560, 0.033180)
Negative Controls				
0.01969(0.00477) 2.00 df (-0.000846, 0.040226)	0.02339(0.00648) 2.00 df (-0.004479, 0.051259)	0.03135(0.00394) 2.00 df (0.014402, 0.048298)	0.01012(0.00699) 4.00 df (-0.009298, 0.029538)	0.02215(0.00440) 3.08 df (0.008372, 0.035928)
Positive Controls				
0.01001(0.00310) 2.00 df (-0.003346, 0.023366)	0.01012(0.00305) 2.00 df (-0.003023, 0.023263)	0.01464(0.00081) 2.00 df (0.011155, 0.018125)	0.00723(0.00344) 4.00 df (-0.002321, 0.016781)	0.01147(0.00187) 4.49 df (0.006508, 0.016432)

[1]Within laboratory mean difference (Battelle minus In Vitro)
[2]Within laboratory standard error of mean
[3]Within laboratory degrees of freedom
[4]Within laboratory 95 percent confidence interval on mean difference.
[5]Pooled average mean difference (Battelle minus In Vitro)
[6]Pooled average standard error of mean
[7]Pooled average degrees of freedom
[8]Pooled average 95 percent confidence interval on mean difference.

Table 18. Total Standard Deviation, Pooled Average Mean Aromatase Activity (nmol/mg protein/min), and Among Laboratory Coefficient of Variation.

Control Type	Total Standard Deviation[1]	Pooled Average Mean[2]	Among Laboratory CV(%)[3]
Background Activity Controls	0.000040	-0.00000	.
Full Enzyme Activity Controls	0.009184	0.01987	46.2204
Negative Controls	0.008794	0.02215	39.7020
Positive Controls	0.003730	0.01147	32.5196

1. Square root of 4 (number of laboratories) times the pooled average standard error of mean.
2. Pooled average mean difference (Battelle minus In Vitro)
3. Ratio of total standard deviation to pooled average mean times 100%

Table 19. Ratio (%) of Among Laboratory Standard Deviation to Unweighted Average of Within Laboratory Standard Errors. Aromatase Activity (nmol/mg protein/min).

Within Laboratory Standard Errors[1]				Unweighted Average of Within Laboratory Standard Errors	Random Among Laboratory Standard Deviation[2] (df=3)	Ratio of Among Laboratory Standard Deviation to Unweighted Average of Within Laboratory Standard Errors(%)
RTI	WIL	Battelle	In Vitro			
Background Activity Controls						
0.00006	0.00003	0.00004	0.00021	0.00008	0	0.000
Full Enzyme Activity Controls						
0.00634	0.00393	0.00778	0.00500	0.00576	0.0072	125.160
Negative Controls						
0.00477	0.00648	0.00394	0.00699	0.00555	0.0069	123.620
Positive Controls						
0.00310	0.00305	0.00081	0.00344	0.00260	0.0028	106.607

[1]Standard error of within laboratory difference (Battelle minus In Vitro).
[2]Square root of among laboratory component of variation.

Table 20. Difference in Protein Concentration (mg/mL) Between Battelle Microsomes and In Vitro Technologies Microsomes (Battelle Minus In Vitro).

RTI	WIL	Battelle	In Vitro	Average
14.6987[1](1.85856[2])	14.0340(1.55508)	11.3420(0.72900)	16.7300(2.53540)	13.5370[5](1.18610[6])
6.00[3] df	3.37 df	6.39 df	3.14 df	4.22[7] df
(10.1510, 19.2464)[4]	(9.3763, 18.6917)	(9.5840, 13.1000)	(8.8574, 24.6026)	(10.3097,16.7643)[8]

[1]Within laboratory mean difference (Battelle minus In Vitro)
[2]Within laboratory standard error of mean
[3]Within laboratory degrees of freedom
[4]Within laboratory 95 percent confidence interval on mean difference.
[5]Pooled average mean difference (Battelle minus In Vitro)
[6]Pooled average standard error of mean
[7]Pooled average degrees of freedom
[8]Pooled average 95 percent confidence interval on mean difference.

Table 21. Total Standard Deviation, Pooled Average Mean Protein Concentration (mg/mL), and Among Laboratory Coefficient of Variation.

Total Standard Deviation[1]	Pooled Average Mean[2]	Among Laboratory CV(%)[3]
2.3722	13.5370	17.5238

[1]Square root of 4 (number of laboratories) times the pooled average standard error of mean.
[2]Pooled average mean difference (Battelle minus In Vitro)
[3]Ratio of total standard deviation to pooled average mean times 100%

Table 22. Ratio (%) of Among Laboratory Standard Deviation to Unweighted Average of Within Laboratory Standard Errors. Protein Concentration (mg/mL).

Within Laboratory Standard Errors[1]				Unweighted Average of Within Laboratory Standard Errors	Random Among Laboratory Standard Deviation[2] (df=3)	Ratio of Among Laboratory Standard Deviation to Unweighted Average of Within Laboratory Standard Errors(%)
RTI	WIL	Battelle	In Vitro			
1.8586	1.5551	0.7290	2.5354	1.6695	1.7672	105.853

[1]Standard error of within laboratory difference (Battelle minus In Vitro).
[2]Square root of among laboratory component of variation.

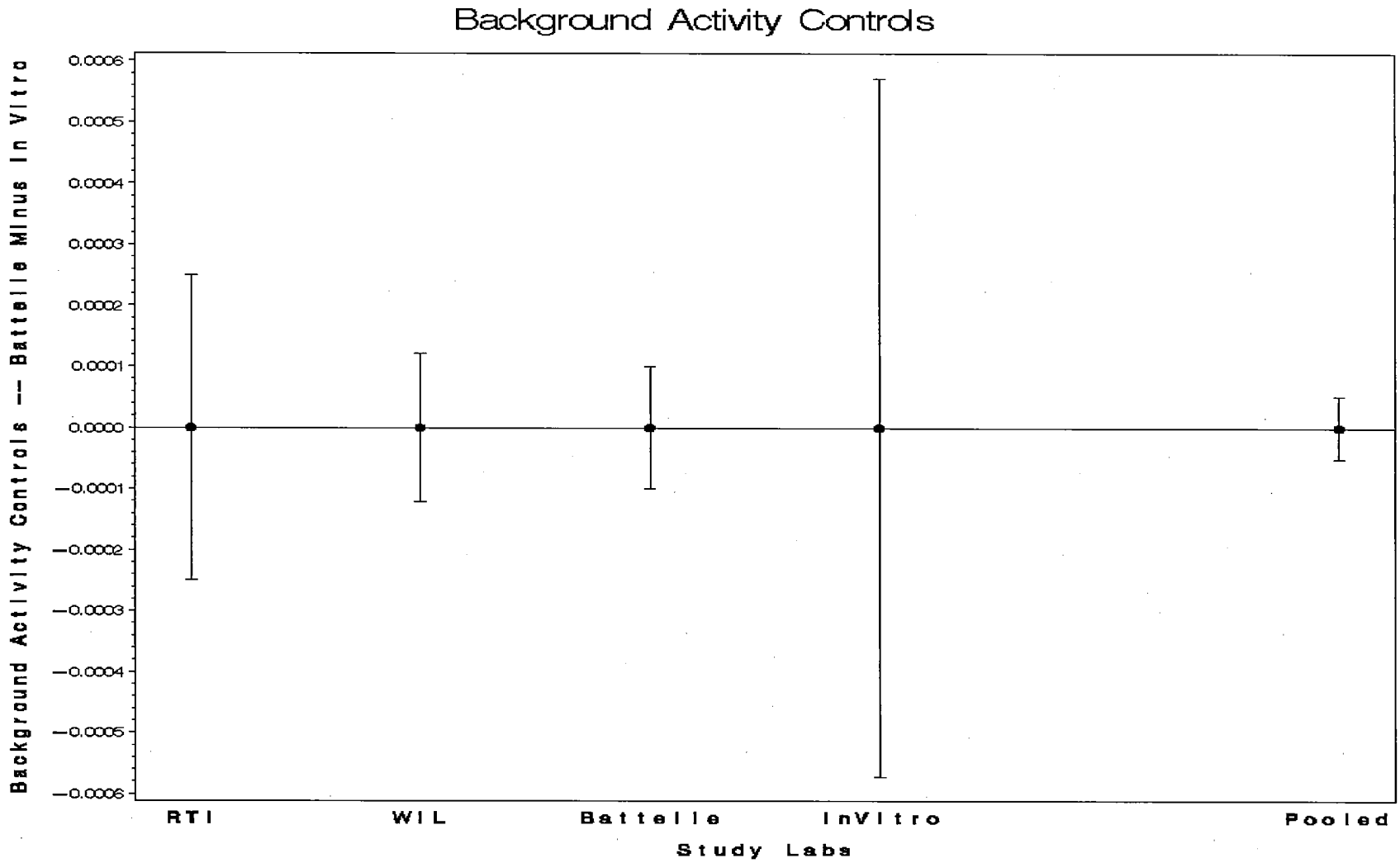


Figure 2. **Background Activity Controls (nmol/mg protein/min). Parameter Estimates and Their Associated 95% Confidence Intervals for Microsome Source Difference (Battelle Minus In Vitro) in the Placental Aromatase Assay. By Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to theAverage Across Laboratories.**

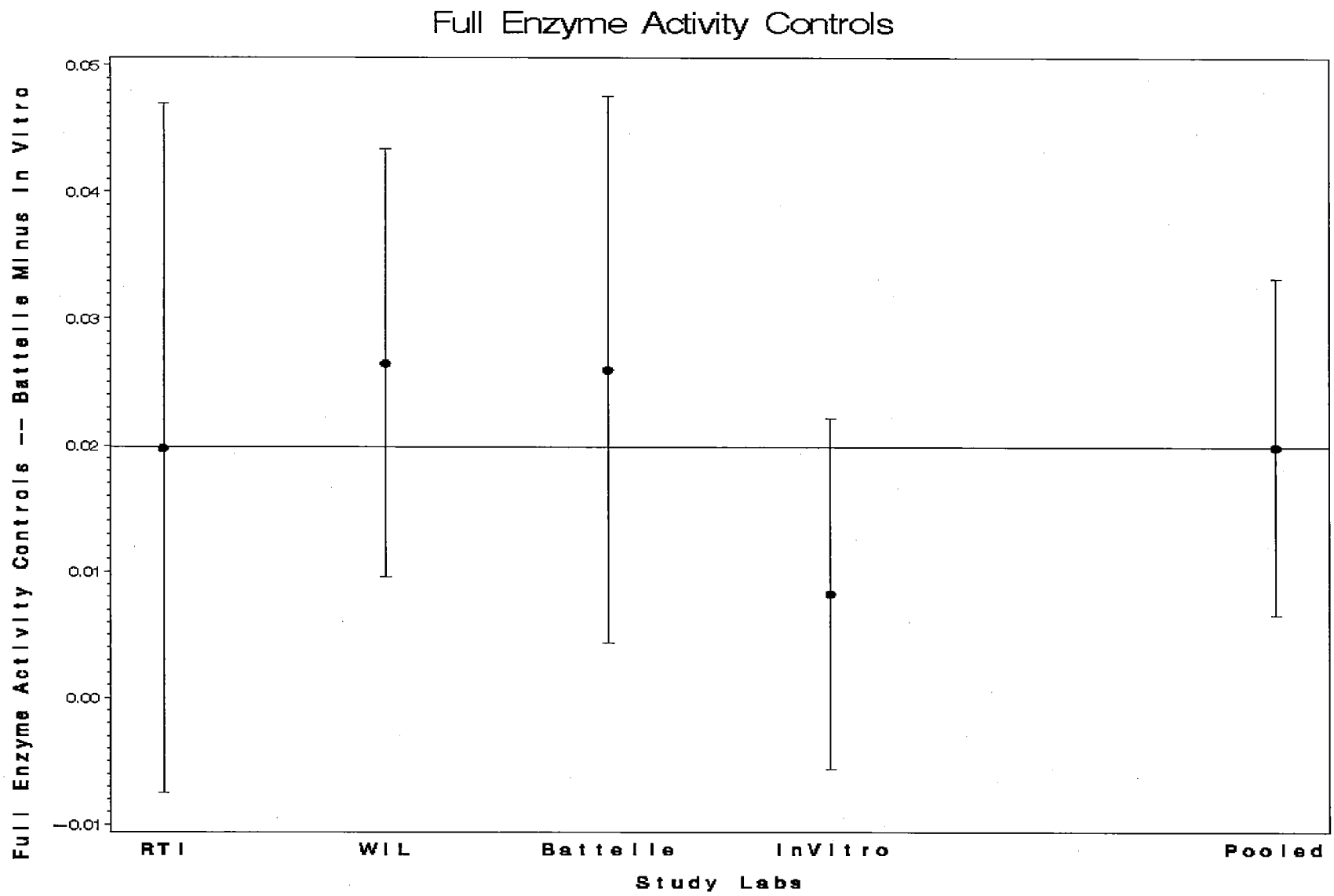


Figure 3. **Full Enzyme Activity Controls (nmol/mg protein/min). Parameter Estimates and Their Associated 95% Confidence Intervals for Microsome Source Difference (Battelle Minus In Vitro) in the Placental Aromatase Assay. By Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average Across Laboratories.**

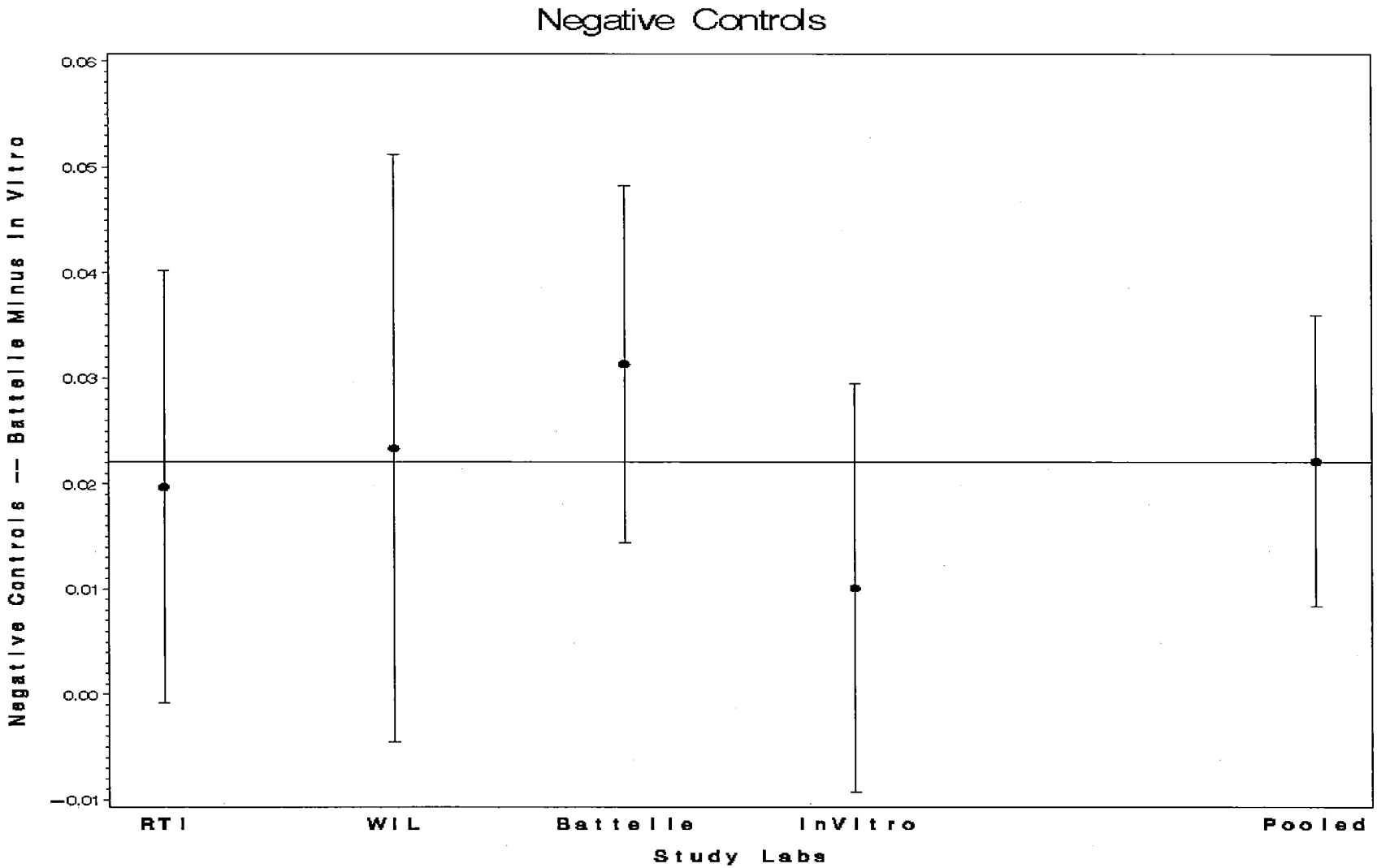


Figure 4. **Negative Controls (nmol/mg protein/min). Parameter Estimates and Their Associated 95% Confidence Intervals for Microsome Source Difference (Battelle Minus In Vitro) in the Placental Aromatase Assay. By Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average Across Laboratories.**

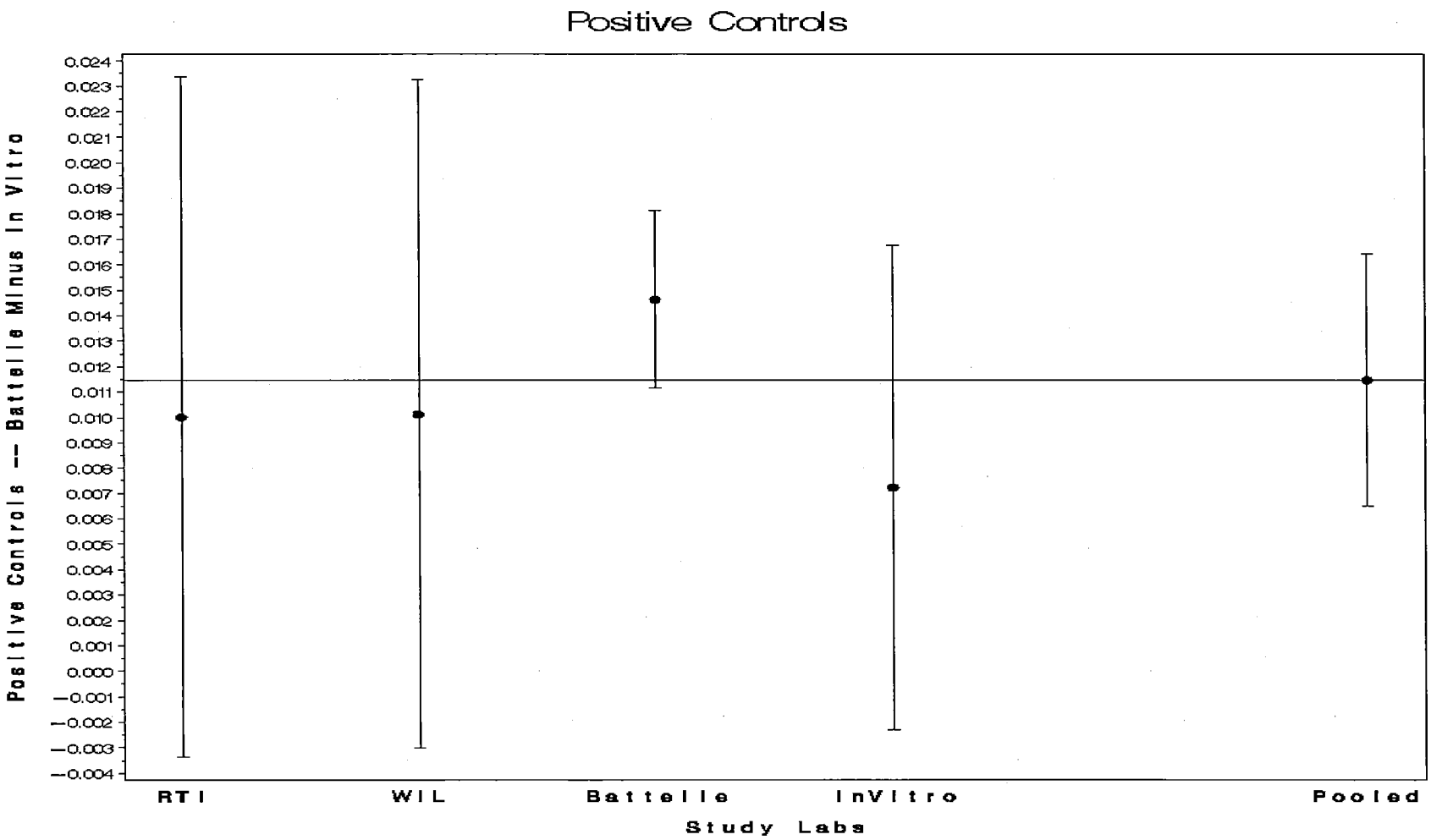


Figure 5. Positive Controls (nmol/mg protein/min). Parameter Estimates and Their Associated 95% Confidence Intervals for Microsome Source Difference (Battelle Minus In Vitro) in the Placental Aromatase Assay. By Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average Across Laboratories.

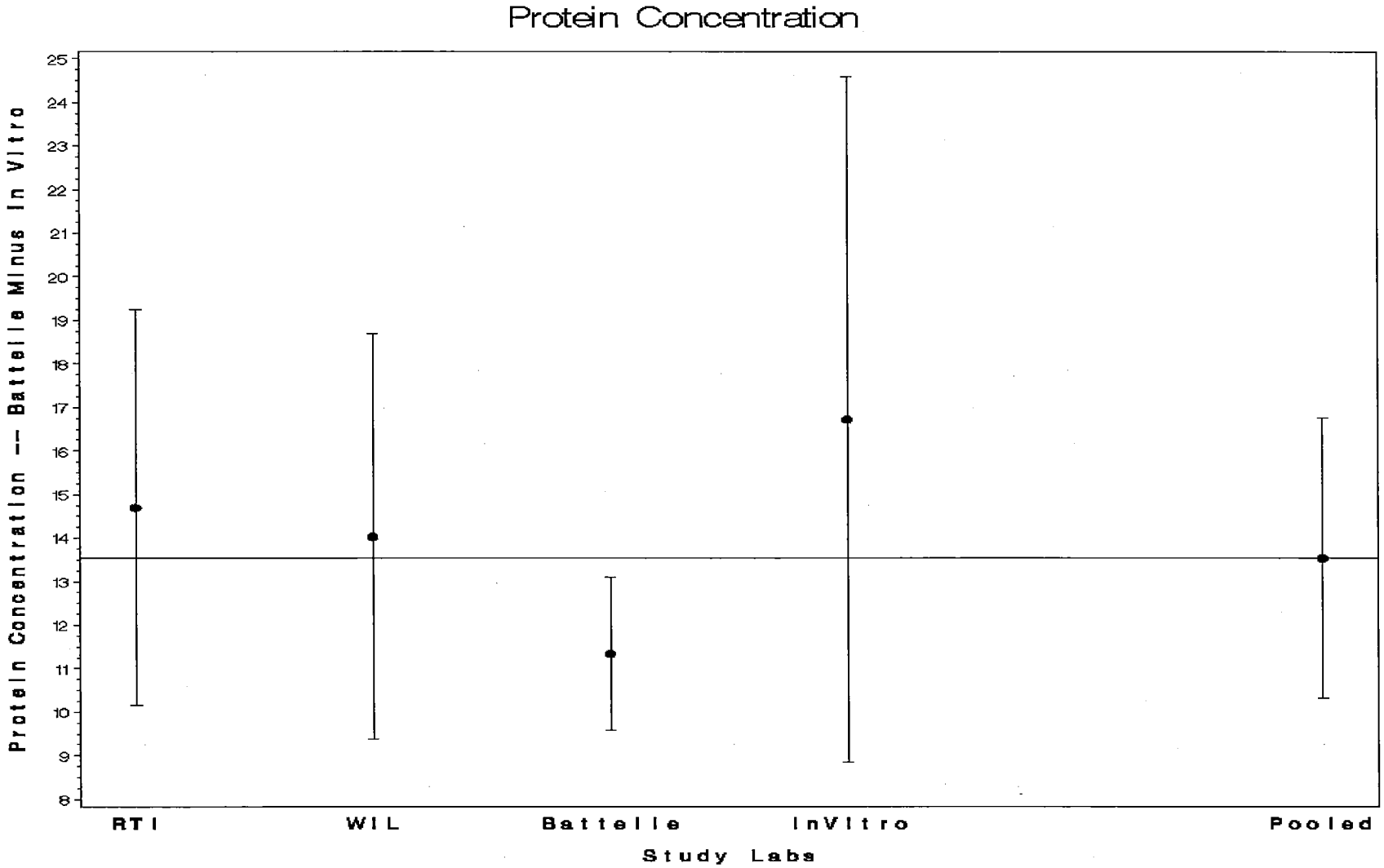


Figure 6. Protein Concentration (mg/mL). Parameter Estimates and Their Associated 95% Confidence Intervals for Microsome Source Difference (Battelle Minus In Vitro) in the Placental Aromatase Assay. By Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average Across Laboratories.

4.0 DISCUSSION

The results of this task demonstrated that two laboratories, without prior experience to do so, were able to procure a human placenta and process it into viable microsomes that could be used to conduct the aromatase assay. Battelle and In Vitro obtained a viable human placenta through contacting the appropriate medical workers in their respective areas. Neither laboratory reported any difficulties in obtaining a placenta. However, it should be noted that the first placenta obtained by In Vitro was not used in this task because of the time delay in getting the placental microsomes prepared after delivery of the placenta, e.g. approximately 11 hours. However, this same time delay occurred with the second placenta but it was processed into placental microsomes and they were used on this task. The protocol specified processing the placenta within 2 hours after delivery. For Battelle, preparation of the placental microsomes began within 2 hours after delivery. The earlier processing of the placenta may explain the higher aromatase activity of the Battelle-prepared microsomes when compared to the In Vitro-prepared microsomes. Although an unplanned event, the results from the In Vitro-prepared microsomes demonstrated that a delay of approximately 11 hours does not necessarily preclude obtaining viable microsomes with acceptable aromatase activity, i.e. 0.03 nmol/mg/min.

The results of this task also provided data for making comparisons between microsome preparations within laboratories and comparisons among laboratories within microsome preparations. The preparation and analysis effects were independently estimated. In addition, the outcome of this task resulted in the production of a sufficient number of vials that could be distributed to all of the laboratories involved in conducting the follow-on task designed to further validate the assay, i.e. testing various reference chemicals (WA 4-16, Task 7).

The 4-OH ASDN results obtained by the laboratories in the present study were in good agreement with previous results reported by RTI (Work Assignment 4-10, Task 3; Work Assignment 2-24; and Work Assignment 4-16, Task 4), Battelle and WIL (Work Assignment 4-16, Task 4), and in the literature. In the present study, the 4-OH ASDN IC_{50} values were 51.7 and 56.9 nM. In WA 4-10, Task 3, RTI reported an average (± sd) IC_{50} value for 4-OH ASDN to be 65.2 ± 10.5 nM (range 54.7 – 83.5 nM) and in WA 4-16, Task 4 they reported an average ± SEM to be 57.9 ± 5.9 nM. In WA 4-16, Task 4, Battelle and WIL reported an average (± SEM) IC_{50} value for 4-OH ASDN of 81.1 ± 5.5 and 47.3 ± 2.6 nM. Literature citations have reported the 4-OH ASDN IC_{50} to range from approximately 30 – 50 nM (WA 2-24 protocol).

5.0 CONCLUSIONS

In conclusion, the results from this task indicated that an inexperienced laboratory should be able to obtain a human placenta and, using the procedure described in the present task, prepare viable microsomes that will have an acceptable level of aromatase activity. Also, this task provided information about the intralaboratory and interlaboratory variability of conducting experiments that can be used to characterize the human placental microsomes for use in the aromatase assay.

6.0 REFERENCES

Brueggemeier, R. W. and Sloan, C. S. Detailed Review Paper on Aromatase. EPA Contract Number 68-W-01-023, Work Assignment 2-7. Battelle, Columbus, OH.

Hartung, J. and Makambi, K.H. *Simple non-iterative t-distribution based tests for meta-analysis.* South African Statistical Journal, 2001, Vol. 35, p. 1-17.

Work Assignment 4-10, Task 3 (2004). "Microsomal Aromatase Prevalidation Supplementary Study: WA 4-10, Task 3: Determine Day-to-Day and Technician Variability" EPA Contract Number 68-W-01-023.

Work Assignment 2-24 (2003). "Microsomal Aromatase Assay Optimization and Comparison Study" EPA Contract Number 68-W-01-023.

Work Assignment 4-16, Task 4 (2005). "Placental Aromatase Assay Validation Sudy: Positive Control Study". EPA Contract Number 68-W-01-023.

APPENDIX A

BATTELLE REPORT

DRAFT REPORT

AROMATASE ASSAY VALIDATION:

PREPARATION AND CHARACTERIZATION OF HUMAN PLACENTAL MICROSOMES

Prepared By:
Battelle Memorial Institute
505 King Avenue
Columbus, Ohio 43201-2693

Prepared For:
EPA Contract: 68-W-01-023
Work Assignment: WA 4-16, Task 6

Battelle Study No. G608316

DRAFT REPORT

Title:	AROMATASE ASSAY VALIDATION: PREPARATION AND CHARACTERIZATION OF HUMAN PLACENTAL MICROSOMES Task 6
Author:	Bozena D. Lusiak, Ph.D.
Performing Laboratory:	Battelle Memorial Institute 505 King Avenue Columbus, OH 43201-2693
Sponsor:	Battelle Memorial Institute 505 King Avenue Columbus, OH 43201-2693
Sponsor's Representatives:	David P. Houchens, Ph.D. EDSP Program Manager Battelle Jerry D. Johnson, Ph.D. Diplomate, A.B.T. Work Assignment Leader Battelle
Study Initiation Date:	March 24, 2005
Experimental Dates:	April 13, 14, 15, 22, 25 and June 16, 17, 2005

Author:

Signature Date

Approved:

Signature Date

QUALITY ASSURANCE STATEMENT

The Quality Assurance Unit (QAU) inspected this study and reports were submitted to the Study Director and management as follows:

Phase Inspected	Date Inspected	Date of Report to Study Director/Management
Protocol review	3/18/2005	3/18/2005
Solution preparation	4/15/2005	4/15/2005
Standard preparation	4/15/2005	4/15/2005
Dilution	4/15/2005	4/15/2005
Test system preparation	4/15/2005	4/15/2005
Sample preparation	4/15/2005	4/15/2005
Solution preparation	4/25/2005	4/26/2005
Standard preparation	4/25/2005	4/26/2005
Dilution	4/25/2005	4/26/2005
Test system preparation	4/25/2005	4/26/2005
Protein analysis	4/25/2005	4/26/2005
Extraction	4/25/2005	4/26/2005
Spectrophotometer absorbance readings	4/25/2005	4/26/2005
Dispensing	4/25/2005	4/26/2005
Protocol Amendment Review	5/23/2005	5/23/2005
Audit draft report	1/30/2006	1/30/2006
Audit study file	1/30/2006	1/30/2006
Protocol Amendment Review	2/10/2006	2/10/2006

This report provides an accurate record of the results obtained

Quality Assurance Unit Date

COMPLIANCE STATEMENT

This study, Battelle Study No. G608316, was conducted in compliance with the United States (U.S.) Environmental Protection Agency (EPA) Good Laboratory Practice (GLP) Standards (40 CFR, Part 160), October 16, 1989; the standard operating procedures (SOPs) of Battelle Memorial Institute; and the protocol as approved by the Sponsor.

Bozena D. Lusiak, Ph.D.
Study Director

Date

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1.0 EXECUTIVE SUMMARY

During the course of this task, human microsomes were prepared from fresh human placenta using a classical differential centrifugation procedure. Six independent protein concentration measurements and four independent aromatase activity determinations were carried out using in-house prepared placental microsomes. The overall mean (± SD and % CV) full aromatase activity control value was 0.0542 nmol/mg protein/min (± 0.0094 and 17.4%, for all four independent enzyme activity determinations). The overall mean (± SD and % CV) protein concentration was 19.91 mg/mL (± 1.66 and 8.36%). Additionally, two independent determinations of the aromatase response to six concentrations of the known aromatase inhibitor 4-hydroxyandrostenedione (4-OH ASDN) were performed. Briefly, 4-OH ASDN, at six different concentrations, was incubated with human placental microsomes in the presence of ^{3}H-androstenedione (substrate for aromatase), propylene glycol, and NADPH in a 0.1 M sodium phosphate buffer solution (pH = 7.4) at 37 ± 1°C for 15 minutes. Concentration response curves were fitted within each replicate to describe the relation between 4-OH ASDN concentration and extent of inhibition. 4-OH ASDN produced a concentration-dependent inhibition in aromatase activity. At the lowest (10^{-9} M) and highest (10^{-6} M) concentration tested, the overall mean percent of control aromatase activity were 95.2 and 5.79%, respectively. The overall mean IC_{50} value for 4-OH ASDN was 51.68 nM (calculated as an overage of two independent replicates).

After receiving the Environmental Protection Agency (EPA) approval, the in-house prepared microsomes were distributed to the three laboratories: In Vitro Technologies, Inc., RTI International, and WIL Laboratories, along with information about protein concentration and estimated aromatase activity.

The placental microsomes sent to Battelle from In Vitro Technologies, Inc. were used to determine the protein concentration (four independent determinations) and aromatase activity of the microsomes that In Vitro prepared from the placenta that they obtained. The overall mean (± SD and % CV) protein concentration was 8.568 mg/mL (± 0.528 and 6.16%) and the overall mean (± SD and % CV) aromatase activity was 0.0276 nmol/mg protein/min (± 0.0019 and 6.77%).

Both microsomal preparations (from Battelle and from In Vitro Technologies, Inc.) showed good assay-to-assay consistency and responded appropriately to the presence of both known aromatase inhibitors and non-inhibitors. Both preparations are suitable for use in the next task of this work assignment.

2.0 INTRODUCTION

2.1 Background

The Food Quality Protection Act of 1996 was enacted by Congress to authorize the EPA to implement a screening program on pesticides and other chemicals found in food or water sources for endocrine effects in humans. Thus, the U.S. EPA is implementing an Endocrine Disruptor Screening Program (EDSP). In this program, comprehensive toxicological and ecotoxicological screens and tests are being developed for identifying and characterizing the endocrine effects of various environmental contaminants, industrial chemicals, and pesticides. The aim of the program is to develop a two-tiered approach, e.g., a combination of *in vitro* and *in vivo* mammalian and ecotoxicological screens (Tier 1) and a set of *in vivo* tests (Tier 2) for identifying and characterizing endocrine effects of pesticides, industrial chemicals, and environmental contaminants. Validation of the individual screens and tests is required, and the Endocrine Disruptor Methods Validation Advisory Committee (EDMVAC) will provide advice and counsel on the validation assays.

Estrogens are sex steroid hormones that are necessary for female reproduction and affect the development of secondary sex characteristics of females. Estrogens are biosynthesized from cholesterol by a series of enzymatic steps, with the last step involving the conversion of androgens into estrogens by the enzyme aromatase. Estrogen biosynthesis occurs primarily in the ovary in mature, premenopausal women. During pregnancy, the placenta is the main source of estrogen biosynthesis and pathways for production change. Small amounts of these hormones are also synthesized by the testes in the male and by the adrenal cortex, the hypothalamus, and the anterior pituitary in both sexes. The major source of estrogens in both postmenopausal women and men occurs in extraglandular sites, particularly in adipose tissue. One potential endocrine target for environmental chemicals is the enzyme aromatase, which catalyzes the biosynthesis of estrogens. An aromatase assay is proposed as one of the Tier 1 Screening Battery Alternate Methods. A detailed literature review on aromatase was performed and encompassed (1) searching the literature databases, (2) contacting individuals to obtain information on unpublished research, and (3) evaluating the literature and personal communications.

Aromatase is a cytochrome P450 enzyme complex responsible for estrogen biosynthesis and converts androgens, such as testosterone and androstenedione, into the estrogens estradiol and estrone. Aromatase is present in the ovary, placenta, uterus, testis, brain, and extraglandular adipose tissues. Two proteins, cytochrome P450arom and NADPH-cytochrome P450 reductase, are necessary for enzymatic activity, and the enzyme complex is localized in the smooth endoplasmic reticulum. The aromatase gene, designated CYP19, encodes the cytochrome P450 and consists of ten exons, with the exact size of the gene exceeding 70 kilobases. Aromatase is found in breast tissue, and the importance of intratumoral aromatase and local estrogen production is being unraveled. Effective aromatase inhibitors have been developed as therapeutic agents for estrogen-dependent breast cancer to reduce the growth stimulatory effects of estrogens in breast cancer. Investigations on the development of aromatase inhibitors began in the 1970's and have expanded greatly in the past three decades.

An *in vitro* aromatase assay could easily be utilized as an alternative screening method in the Tier 1 Screening Battery to assess the potential effects of various environmental toxicants on aromatase activity. Both *in*

vitro subcellular (microsomal) assays and cell-based assays are available for measuring aromatase activity. The *in vitro* subcellular assay using human placental microsomes is commonly used to evaluate the ability of pharmaceuticals and environmental chemicals to inhibit aromatase activity. In addition, human JEG-3 and JAR choriocarcinoma cell culture lines, originally isolated from cytotrophoblasts of malignant placental tissues, have been used as *in vitro* systems for measuring the effects of compounds on aromatase activity. These cell lines are also utilized for investigations on the effects of agents in placental toxicology.

Numerous flavonoids and related phytoestrogen derivatives have been extensively evaluated for their ability to inhibit aromatase activity for two primary reasons: (1) these natural plant products can serve as possible leads for the development of new nonsteroidal aromatase inhibitors; and (2) humans and other animals are exposed to these agents through the diet. In general, the flavonoids and related analogs demonstrate aromatase inhibition with IC_{50} values in the micromolar range; however, these compounds lack both the potency and specificity of aromatase inhibitors developed for breast cancer therapy. Several pesticides have also demonstrated inhibition of aromatase activity in the human placental microsomal assay system.

The human placental microsomal aromatase assay was recommended as the *in vitro* aromatase screening assay to be included in the Tier 1 Screening Battery. This assay will detect environmental toxicants that possess the ability to inhibit aromatase activity. Prevalidation studies on recombinant aromatase (WA 2-24) were conducted to optimize the microsomal aromatase assay protocol for human placenta, demonstrate the utility of the microsomal assay to detect known aromatase inhibitors, and compare the performance of a recombinant assay system and the placental microsomal assays.

2.2 Task Description and Objectives

In this task, human placental microsomes were prepared, analyzed for protein content and uninhibited aromatase activity, and studies were conducted with the known aromatase inhibitor 4-hydroxyandrostenedione (4-OH ASDN) to demonstrate the responsiveness of the assay to aromatase inhibitor. This task was conducted in two stages as described below.

2.2.1. Stage 1 - Placenta Procurement/Microsomes Preparation and Characterization

A human placenta was obtained from The Ohio State University Tissue Procurement Center and placental microsomes were prepared. Protein concentration (two independent replicates) and aromatase activity (two independent replicates) were determined. Two independent determinations of aromatase activity responsiveness to six concentrations of known inhibitor 4-OH ASDN were performed. The obtained data were approved by the EPA.

2.2.2. Stage 2 - Distribution of Microsomes and Conduct of Aromatase Activity Studies

After receiving EPA's approval, the prepared microsomes were distributed to three laboratories: In Vitro Technologies, RTI International, and WIL Research Laboratories, along with information about protein concentration and estimated aromatase activity. The placental microsomes sent from In Vitro Technologies were used to determine the protein concentration and aromatase activity.

The objectives of the presented study were to prepare human placental microsomes from fresh human placenta obtained from The Ohio State University Tissue Procurement Center, to analyze them for protein content and aromatase activity and demonstrate the responsiveness of the assay to 4-OH ASDN. Additional aim of the study was to generate data for intra- and interlaboratory variability estimations. The study protocol and Quality Assurance Project Plan (QAPP) can be found in Appendix A and B, respectively.

3.0 MATERIALS AND METHODS

3.1 Preparation of Substrate Solution

The substrate for the aromatase assay was androstenedione (ASDN). Non-radiolabeled and radiolabeled ASDN were used. The non-radiolabeled ASDN (Lot No. 024K0809) was obtained from Sigma, St. Louis, MO by the Sponsor's Chemical Repository (CR) and was then distributed to the participating laboratories. It had a reported purity of 100%. The radiolabeled androstenedione ([1β-^{3}H]-androstenedione, [^{3}H]ASDN, Lot No. 3538496), was obtained from PerkinElmer Life Sciences, Inc., Boston, MA and had a reported specific activity of 25.3 Ci/mmol. Radiochemical purity was reported by the supplier to be > 97%. Radiochemical purity was assessed by high performance liquid chromatography (HPLC) by the lead laboratory (see Results section.)

Preparing the substrate solution involved mixing of non-radiolabeled and radiolabeled [^{3}H]ASDN in order to achieve a 100 nM final concentration of ASDN in the assay. The amount of tritium added to each incubation was about 0.1 µCi. This substrate solution had a concentration of 2 µM with a radiochemical content of about 1 µCi/mL.

The following describes the preparation of a substrate solution using a stock of [^{3}H]ASDN with a specific activity of 25.3 Ci/mmol and a concentration of 1 mCi/mL. A 1:100 dilution of the radiolabeled stock solution in buffer and a 1 mg/mL solution of ASDN in ethanol were prepared. Subsequently, the 1 mg/mL ASDN in ethanol solution was diluted in buffer to a final concentration of 1 µg/mL. Four-and-one half (4.5) mL of the 1 µg/mL solution of ASDN, 800 µL of the [^{3}H]ASDN buffer dilution and 2.7 mL buffer to make 8 mL were combined. The weight of each component added to the substrate solution was recorded. After mixing the solution, five aliquots of ca. 20 µL were weighed out and combined with scintillation cocktail for radiochemical content analysis.

3.2 Control Substances

The Sponsor's Chemical Repository was responsible for chemistry activities required to perform this study. Their responsibilities included chemical procurement, solubility, formulation stability assessment, formulation preparation, formulation analysis, and shipment of stock formulation to the participating laboratories (see Results section.)

The known aromatase inhibitor, 4-OH ASDN, was used as a positive control and the known aromatase non-inhibitor, lindane, was used as a negative control (Table 1). Stock solutions of both compounds were supplied by Battelle's CR. Dilutions were made fresh each day of use in the same vehicle (with the same lot number) that was used to prepare the stock solutions (see Table 6, Section 3.4 for details). Tables 2 and 3 describe the dilution scheme for 4-OH ASDN (positive control) and lindane (negative control), respectively.

Table 1 - Control Substances

Test Substance Name	Mfr. Purity	CAS No.	Molecular Formula	Molecular Weight (g/mol)	Stock Solution ID	Stock Solution Con. (M)	Vehicle	Storage Conditions (°C)
4- hydroxyandrostenedione	99%	566-48-3	$C_{19}H_{26}O_3$	302.4	2-ASDN-1	0.01	95% ethanol	2-8
Lindane	99.6%	58-89-9	$C_6H_6Cl_6$	290.8	1-LIN-1	0.1	DMSO	2-8

Table 2 - Dilution Scheme for 4-OH ASDN Control

Dilution ID	EtOH Volume (µL)	Solution Volume (µL)	Solution Used	Solution Conc. (M)	Final Concentration in Assay (M)
1	9900	100	Stock	1×10^{-4}	NA
2	1900	100	1	5×10^{-6}	5×10^{-8}

Table 3 - Dilution Scheme for Lindane Control

Dilution ID	DMSO Volume (µL)	Solution Volume (µL)	Solution Used	Solution Conc. (M)	Final Concentration in Assay (M)
1	9900	100	Stock	1×10^{-3}	NA
2	900	100	1	1×10^{-4}	1×10^{-6}

4-OH ASDN was used as a positive control but was also used at six different concentrations to determine the aromatase assay responsiveness to a known inhibitor. Appropriate stock solution dilutions were made fresh on the day of use as presented in Table 4 in the same vehicle (and the same lot number) that was used to prepare the stock solutions (see Table 6, Section 3.4 for details).

Table 4 - Dilutions Scheme for 4-OH ASDN for Study Aromatase Response

Solution Name Concentration (mM)		Volume of Solution (µL)	Volume of Ethanol (µL)	Dilution Name Concentration (mM)		Final Concentration in the Assay (M)
Stock Sol	(10 mM)	100	900	Sol.1	(1.0 mM)	N/A
Sol 1	(1.0 mM)	100	900	Sol 2	(0.1 mM)	1×10^{-6}
Sol 2	(0.1 mM)	100	900	Sol 3	(0.01 mM)	1×10^{-7}
Sol 2	(0.1 mM)	50	950	Sol 4	(0.005 mM)	5×10^{-8}
Sol 2	(0.1 mM)	25	975	Sol 5	(0.0025 mM)	2.5×10^{-8}
Sol 3	(0.01 mM)	100	900	Sol 6	(0.001 mM)	1×10^{-8}
Sol 6	(0.001 mM)	100	900	Sol 7	(0.0001 mM)	1×10^{-9}

3.3 Human Placental Microsomes

3.3.1 Preparation

3.3.1.1 Source of Placenta

A human placenta was obtained from the Ohio State University Tissue Procurement Center from a 24-year old healthy Hispanic female with full term delivery. The patient denied usage of tobacco, alcohol and drugs. Weight of the placenta was 0.45 kg. The freshly delivered placenta was placed in a tissue container, sealed and placed on wet ice in an insulated shipping container. The placenta was transported to the laboratory and preparation of microsomes started within 25 minutes of obtaining the placenta.

3.3.1.2 Microsome Preparation Buffers

Buffer A: 0.25 M Sucrose, 0.04 M nicotinamide, 0.05 M sodium phosphate (pH 7.0) - 6.02 g of sodium phosphate monobasic (NaH_2PO_4) was dissolved in 1 L of Milli-Q water to obtain a 0.05 M solution; 7.09 g of sodium phosphate dibasic was dissolved in 1 L of Milli-Q water to prepare a 0.05 M solution. Both mono and dibasic 0.05 M sodium phosphate solutions were combined to a final pH of 7.0. To complete preparation of Buffer A, 85.52 g of sucrose and 4.89 g of nicotinamide were dissolved in 1 L 0.05 M sodium phosphate buffer, pH 7.0.

Buffer B: 0.1 M sodium phosphate (pH 7.4) - 12.00 g sodium phosphate monobasic was dissolved in 1 L Milli-Q water to prepare a 0.1 M NaH_2PO_4 solution; 14.21 g sodium phosphate dibasic was dissolved in 1 L Milli-Q water to prepare a 0.1 M Na_2HPO_4 solution. Both mono and dibasic solutions were combined to a final pH of 7.4.

Buffer C: 0.1 M sodium phosphate (pH 7.4) with 0.25 M sucrose, 20% of glycerol and 0.05 mM dithiothreitol - 17.13 g of sucrose and 1.58 mg of dithiothreitol were dissolved in ca. 100 mL of 0.1 M sodium phosphate buffer, pH 7.4 (prepared as described above), and was diluted to 160 mL with an additional volume of 0.1 M sodium phosphate buffer, pH 7.4. Glycerol was added to obtain a total volume of 200 mL.

Supplier and lot numbers for the components used for buffer preparations are presented in Table 5.

Table 5 - Supplier and Lot Numbers for Buffer Components (Microsomes Preparation Procedure)

Chemical	Supplier	Lot Number
Sucrose	Sigma	014K0010
Nicotinamide	Sigma	084K0031
Sodium phosphate dibasic	Sigma	083K0120
Sodium phosphate monobasic	Sigma	054K0144
Glycerol	Sigma	114K0111
Dithiothreitol	Sigma	044K3486

3.3.1.3. Placental Microsomes Preparation

In the laboratory, the placenta was transferred from the shipping container to a tray which was set over ice to keep the tissue chilled during dissection operations. While keeping placenta on ice, the membrane and fibrous material was dissected, removed and discarded. The spongy tissue was cut into small pieces and placed on ice in a beaker with ice-cold Buffer A. Approximately 800 mL of Buffer A was added to the minced tissue and the tissue-buffer mixture was homogenized. The homogenate was transferred to ice-cold centrifuge tubes and centrifuged at the setting of 10,000 g for 30 minutes at 4°C. Next, the supernatant was transferred to ultracentrifuge tubes and was centrifuged at a setting of 35,000 rpm (equal to 100,000 g) in an ultracentrifuge for one hour at 4°C to obtain the crude microsomal pellet. Obtained supernatant was decanted, and the microsomal pellet was dislodged from the bottom wall of the tube by gentle swirling with 2 to 3 mL of ice-cold Buffer B (the clear pellet on the bottom was left in the tube and disposed). The microsomal pellet (along with the Buffer B) was poured into a Potter-Elvejhem homogenizer and resuspended in ice-cold Buffer B. Subsequently, the suspension was transferred to ultracentrifuge tubes and was centrifuged at a setting of 35,000 rpm for one hour at 4°C to wash the microsomes. This washing procedure (supernatant decanting, pellet resuspension and centrifugation) was repeated one additional time. Next, the supernatant was decanted and the twice-washed microsomal pellet was dislodged from the bottom wall of the tube by gentle swirling in a 2 to 3 mL of ice-cold Buffer C. All microsomal pellets were combined into a single lot and were resuspended in approximately 20 mL of ice-cold Buffer C. The microsomal suspension was aliquoted (ca. 200 µL/tube) into labeled cryotubes and was flash frozen in liquid nitrogen and stored at ca. -70°C until removed for use.

3.3.1.4 Use of Microsomes

Human placental microsomes (Lot No. 6-041305, protein concentration approximately 21 mg/mL, prepared at Battelle, and Lot No. BAA, protein concentration approximately 8 mg/ mL, prepared at In Vitro Technologies) were used during this study. The microsomes were stored at approximately -70°C. Prior to the assay, microsomes were thawed rapidly in a 37 ± 1°C water bath, rehomogenized by brief vortexing and kept on ice until used. The microsomes stock was diluted with buffer (1:900 and 1:950 overall for Battelle and 1:440 for In Vitro Technologies microsomes) and maintained on ice until used. The time between thawing of the microsomes and their use in the assay was limited to less than 1 hour; in most cases the delay was about 30 minutes.

3.4 Other Assay Components

In addition to substrate, control substances or vehicle, and microsomes, the aromatase assay contained beta - nicotinamide adenine dinucleotide phosphate, reduced form (β- NADPH), propylene glycol and phosphate buffer. Supplier and lot numbers for other aromatase assay components are presented in Table 6.

Table 6 - Supplier and Lot Numbers for Aromatase Assay Components

Chemical	Supplier	Lot Number
NADPH	Sigma	103K7046
Propylene glycol	Spectrum Chemical	SQ0397
Sodium phosphate dibasic	Sigma	083K0120
Sodium phosphate monobasic	Sigma	054K0144
Ethanol, 95% (vehicle)	Sponsor	04B10UB, 05C14GB
DMSO (vehicle)	Sponsor	2969A24437, 04H23QB

3.4.1 β-Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form (β-NADPH)

β-NADPH was the required co-factor for aromatase. The final concentration in the assay was 0.3 mM. Typically, a 6 mM stock solution was prepared by dissolving ca. 20 mg of NADPH in 4 mL of assay buffer.

3.4.2 Assay Buffer

The assay buffer was 0.1 M sodium phosphate buffer, pH 7.4. One liter of 0.1 M solution of sodium phosphate monobasic (NaH_2PO_4) in Milli-Q water and one liter of 0.1 M solution of sodium phosphate dibasic (Na_2HPO_4) in Milli-Q water were prepared. The solutions were combined in the approximate ratio 80:20 (dibasic: monobasic sodium phosphate) to achieve a pH of 7.4.

3.5 Protein Determination

The protein concentration of microsomal preparations was determined in six independent replicates for the Battelle preparation and in four replicates for the In Vitro preparation.

The protein concentration in the microsomes was determined each day the microsomes were used with a DC Protein Assay kit from BioRad (Hercules, CA). The 6-point standard curve was prepared using bovine serum albumin (BSA) reconstituted in Milli-Q water. During the first part of the study (using Battelle prepared microsomes) a 6-point standard curve ranging from 0.11 to 1.0 mg protein/mL was used. Quality control (QC) standards (0.125, 0.5 and 1.0 mg protein/mL) obtained from Pierce (Rockford, IL) were run in duplicate with each assay. Briefly, to a 25 µL aliquot of the microsome solution, standard or QC sample 125 µL of BioRad DC Protein Kit Reagent A was added and mixed. Next, 1.0 mL of BioRad DC Protein Kit Reagent B was added and gently mixed. During the second part of the study (using In Vitro prepared microsomes), a 6-point standard curve ranging from 5 to 250 µg protein/mL was prepared. QC standards were prepared by diluting a purchased protein standard to prepare samples containing 10 and 100 µg protein/mL. Unknown and curve standards were run in triplicate and QC samples were run in duplicate. To a 200 µL aliquot of unknown, standard or QC sample, 100 µL of BioRad DC Protein Kit Reagent A was added and mixed. Next, 800 µL of BioRad DC Protein Kit Reagent B was added and the samples were vortexed. The samples were incubated at room temperature for at least 15 minutes. Each sample (standards and unknown) was transferred to disposable polystyrene cuvettes and the absorbance at 750 nm was measured using a spectrophotometer. The protein concentration of the microsomal sample was determined by interpolation, reading the protein concentration on the standard curve that corresponded to its absorbance.

The change of the range of the standard curve and the QC sample concentrations was done as per Sponsor request in order to improve the accuracy of the protein determination.

3.6 Cytochrome P450 Aromatase (CYP19) Activity

As the first part of the presented study, two independent determinations of aromatase activity were performed using Battelle prepared microsomes to assess if newly prepared microsomes were meeting established criteria for minimum acceptable aromatase activity. The minimum acceptable aromatase activity was set at 0.03 nmol/mg protein/min (See QAPP, Appendix B). To verify the aromatase activity, two types of control samples were conducted: full enzyme activity and background activity controls (Table 7).

Table 7 - Aromatase Activity Verification Study Design

Sample Type	Repetitions (test tubes)	Description
Full Enzyme Activity Control	4	Complete assay[a] with inhibitor vehicle control
Background Activity Control	4	Complete assay with inhibitor vehicle control, omitting NADPH

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

To assess the responsiveness of Battelle prepared microsomes to a known aromatase inhibitor, two independent replicates of the aromatase assay with six concentration of 4-OH ASDN were conducted. In each replicate/test run four types of control samples were included: full enzyme activity, background activity, positive and negative controls as presented in Table 8.

Table 8 - Aromatase Assay Inhibition Study Design

Sample Type	Repetitions (test tubes)	Description	Final Control Substance Concentration (M)
Full Enzyme Activity Control	4	Complete assay[a] with inhibitor vehicle control	NA
Background Activity Control	4	Complete assay with inhibitor vehicle control, omitting NADPH	NA
Positive Control	4	Complete assay with 4-OH ASDN added	5×10^{-8}
Negative Control	4	Complete assay with lindane added	1×10^{-6}
4-OH ASDN Concentration 1	3	Complete assay with 4-OH ASDN added	1×10^{-6}
4-OH ASDN Concentration 2	3	Complete assay with 4-OH ASDN added	1×10^{-7}
4-OH ASDN Concentration 3	3	Complete assay with 4-OH ASDN added	5×10^{-8}
4-OH ASDN Concentration 4	3	Complete assay with 4-OH ASDN added	2.5×10^{-8}
4-OH ASDN Concentration 5	3	Complete assay with 4-OH ASDN added	1×10^{-8}
4-OH ASDN Concentration 6	3	Complete assay with 4-OH ASDN added	1×10^{-9}

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

Two independent assay replicates were conducted (by two different technicians) for the In Vitro microsomal preparation. A single replicate study of an example microsomal preparation is described in Table 9.

Four types of control samples were included for each replicate. Four test tubes were run for each type of control. The controls sets were split so that two tubes (of each control type) were run at the beginning and two at the end of each replicate set.

Table 9 - Aromatase Assay Study Design

Sample Type	Repetitions (test tubes)	Description	Final Control Substance Concentration (M)
Full Enzyme Activity Control	4	Complete assay[a] with inhibitor vehicle control	NA
Background Activity Control	4	Complete assay with inhibitor vehicle control, omitting NADPH	NA
Positive Control	4	Complete assay with 4-OH ASDN added	5×10^{-8}
Negative Control	4	Complete assay with lindane added	1×10^{-6}

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

The assays were performed in 13 × 100 mm test tubes maintained at 37 ± 1°C in a shaking water bath. Propylene glycol, [^{3}H]ASDN, NADPH, and assay buffer were combined in the test tubes with or without inhibitor (as described below) to the total volume of 1.0 mL. The final concentrations for the assay major components are presented in Table 10. The tubes and the microsomal suspension were placed at 37 ± 1°C in the water bath for approximately 5 minutes prior to initiation of the assay by the addition of 1 mL of the diluted microsomal suspension.

Table 10 - Aromatase Assay Conditions using Human Placental Microsomes

Assay Components	Component Volume Added to the Assay	Final Concentration in the Assay
Microsomal Protein	1.0 mL	0.0125 mg/mL
NADPH or assay buffer	100 µL	0.3 mM
[^{3}H]ASDN	100 µL	100 nM
Propylene glycol	100 µL	5 %(v/v)
Control Substance or vehicle	20 µL	Varies[a]
Assay buffer	700 µL	~ 0.094 M

[a] See Table 8 for details.

The total assay volume was 2.0 mL and the tubes were incubated for 15 minutes. The incubations were stopped by the addition of methylene chloride (2.0 mL); the tubes were vortex-mixed for ca. 5 seconds and placed on ice. The tubes were then vortex-mixed an additional 20 to 25 seconds, then centrifuged using a Beckman GS-6 centrifuge with GH-3.8 rotor for 10 minutes at a setting of 1000 rpm. After centrifugation, the methylene chloride layer was removed and discarded; the aqueous layers were extracted again with methylene chloride (2.0 mL). This extraction procedure was performed one additional time, each time discarding the methylene chloride layer. The aqueous layers were transferred to vials and duplicate aliquots (0.5 mL) were transferred to 20-mL liquid scintillation counting vials. Liquid scintillation cocktail (Ultima Gold, Packard, 10 mL) was added to each counting vial and shaken to mix the solution.

Analysis of the samples was performed using liquid scintillation spectrometry (LSS). Radioactivity found in the aqueous fractions represented amount of formed ^3H_2O.

Results are presented as the activity (velocity) of the enzyme (aromatase). The amount of the estrogen product formed was determined by dividing the total amount of ^3H_2O formed (the aromatization of one mole of ASDN resulted in the production of one mole of estrone and one mole of water) by the specific activity of the [^{3}H]ASDN substrate (expressed in dpm/nmol). The activity of the enzyme was expressed in nmol

(mg protein)$^{-1}$ min^{-1} and was calculated by dividing the amount of estrogen formed by the amount of microsomal protein used (in mg) times the incubation time (15 minutes).

3.7 Data Analysis

3.7.1 Aromatase Activity and Percent of Control Calculation

Relevant data were entered into an Excel spreadsheet for calculation of aromatase activity and percent of control. The master spreadsheet was titled Aromatase_Master_Version 1.2.xls.

For each repeat tube (full, background activity controls, positive and negative controls and each control substance concentration), the Excel spreadsheet included total observed (uncorrected) disintegration per minute (dpm) per tube and total aromatase activity per tube. The dpm and aromatase activity values were corrected for the background dpm, as measured by the average of the background activity control tubes. The aromatase activity was calculated as the corrected dpm, normalized by the specific activity of the [^{3}H]ASDN, the mg of protein of the aromatase, and the incubation time. The average (corrected) dpm and aromatase activity across the four background activity control repeat tubes necessarily were equal to 0 (zero) within each replicate.

3.7.2. Concentration Response Fits for the Control Substance

For the 4-OH ASDN, two independent replicates of the concentration response curve fit were carried out.

For each replicate two repeat tubes of the full enzyme activity controls, the background activity controls and positive and negative controls were run prior to the repetition of the graded concentrations of 4-OH ASDN and two repeat tubes of each control were run following the repetition of 4-OH ASDN. Three repetitions were prepared for each concentration of 4-OH ASDN.

For each tube, percent of control was determined by dividing the background corrected aromatase activity for each tube by average background corrected aromatase for activity for the four full enzyme activity tubes and multiplying by 100.

Concentration response trend curves were fitted to the percent of control activity values within each of the repeat tubes at each 4-OH ASDN concentration. Concentration was expressed on the log scale. Let:

Y= percent of control activity in the inhibitor tube

X= logarithm (base 10) of the concentration

DAVG = average dpms across the repeat tubes with the same 4-OH ASDN concentration

β = slope of the concentration response curve (β was negative)

$\mu = \log_{10}IC_{50}$ (IC_{50} is the concentration corresponding to percent of control activity equal 50%).

The following response curve was fitted to relate percent of control activity to logarithm of concentration within each replicate:

$$Y = 100/[1 + 10^{(\mu-X)\beta}] + \epsilon$$

where ϵ was the variation among repetition, distributed with mean 0 and variance proportional to DAVG (based on the Poisson distribution theory for radiation counts). The variance was approximated by Y.

The response curves were fitted by weighted least squares nonlinear regression analysis with weights equal to 1/Y. Observed individual percent activity values above 100% were set to 99.5%. Observed individual percent activity values below 0% were set to 0.5%.

Concentration response models were fitted for each replicate test. Based on the results of the fit within each replicate the extent of aromatase inhibition was summarized as IC_{50} (10μ) and slope (β) (see Appendix G for full statistical analysis).

3.7.3. Statistical Software

Concentration response curves were fitted to the data using nonlinear regression analysis features in the Prism statistical analysis package, Version 4.0. Supplemental statistical analyses were carried out using the SAS statistical analysis system, Version 9.

3.8 Retention of Records

All study records, including the final report, are retained in the archives as specified in the study protocol.

4.0 RESULTS

4.1 Radiochemical Purity

The radiochemical purity for the ^{3}H-androstenedione was 97% as reported by RTI International (Appendix C).

4.2 Stock Formulation Analysis

The formulation stability and formulation analysis results for lindane and 4-OH ASDN from the Battelle CR are included in the reports presented in Appendix D. Some of the formulation analysis data are summarized in Table 11.

Table 11 - Formulation Analysis Data

Chemical ID	Stock Solution ID	Stock Solution Preparation Date	Manufacturer's Purity (%)	Number of Days Known Stable	Date of Last Use on Task 6
Lindane	1-LIN-1	1/24/2005	99.6	168	6/17/2005
4-OH ASDN	2-ASDN-1	1/25/2005	99	173	6/17/2005

4.3 Protein Analysis

Protein content of the human placental microsomes were measured each day of the aromatase assay and at two other times per each microsomes preparation as described in Section 3.6. As it was noticed during the second part of the study, the protein standards from 5 to 250 μg/mL were not producing a linear standard curve. However, when the standard curve was constructed using only five standards from 5 to 125 μg/mL, then the standard curve was linear. The Sponsor accepted usage of 5-point standard curve ranging from 5 to 125 μg/mL.

The results of measuring the protein concentration are provided in Table 12.

Table 12 - Protein Concentration Data

Microsomes	Replicate	Protein Concentration (mg/mL)	Mean	SD	SEM	% CV
Battelle[a]	1	22.58	19.91	1.66	0.68	8.36
	2	20.22				
	1	20.27				
	2	17.72				
	1	20.07				
	2	18.61				
In Vitro[b]	1	8.800	8.568	0.528	0.26	6.16
	2	9.181				
	1	7.987				
	2	8.304				

[a] 6-point standard curve ranging from 0.11 to 1.0 mg/mL was used.
[b] 5-point standard curve ranging from 5 to 125 µg/mL was used.

In order to better characterize the protein assay, QC standards were included on all runs (see Section 3.5). The results for QC standards are presented in Table 13.

Table 13 - Protein QC Sample Data

Known Concentration (mg/mL)	Measured Concentration (mg/mL)	Mean	SD	SEM	% CV	% Difference from Known
0.125[a]	0.059	0.073	0.016	0.007	22.09	-52.8
	0.070					-44.0
	0.084					-32.8
	0.056					-55.2
	0.099					-20.8
	0.070					-44.0
0.5[a]	0.458	0.474	0.023	0.009	4.88	-8.4
	0.457					-8.6
	0.517					3.4
	0.460					-8.0
	0.483					-3.4
	0.471					-5.8
1.0[a]	0.915	0.920	0.027	0.011	2.93	-8.5
	0.888					-11.2
	0.970					-3.0
	0.916					-8.4
	0.915					-8.5
	0.913					-8.7
0.010[b]	0.009	0.009	0.001	0.000	9.07	-10.0
	0.010					0.0
	0.008					-20.0
	0.009					-10.0
0.100[b]	0.092	0.092	0.003	0.001	3.15	-8.0
	0.095					-5.0
	0.088					-12.0
	0.091					-9.0

[a] The QC samples were assayed with the 6-point standard curve ranging from 0.11 to 1.0 mg/mL and with Battelle prepared microsomes.
[b] The QC samples were assayed with the 5-point standard curve ranging from 5 to 125 µg/mL and with In Vitro Technologies prepared microsomes.

The determined concentrations of the 0.125 mg/mL QC samples measured with 6-point standard curve ranging from 0.11 to 1.0 mg/mL were between approximately 21 - 53% lower than the known values in all performed assays. The data indicate a high level of uncertainty for all protein concentration measurements close to the low end of the standard curve. Those data also indicated the need for a change of the standard curve range to improve the accuracy of the protein determination.

After changing the standard curve range (5 to 250 µg/mL) and the QC sample concentrations the improvement of the accuracy of protein determination was observed, although the uncertainty in the protein values at the lower end of the curve still exist.

4.4 Aromatase Activity

Aromatase activity was measured in Battelle prepared microsomes in four independent replicates as described in Section 3.6. In the first set of two replicates, full enzyme and background activity was measured to verify aromatase activity in newly prepared microsomes. The results are presented in Table 14.

Table 14 - Aromatase Activity Determination

Microsomes Source	Replicate	Assay Date	Technician	Aromatase Activity (nmol/mg/min)
Battelle	1	4/15/05	LR	0.0444
	2	4/15/05	TD	0.0528

The aromatase activity was calculated by normalizing the radioactivity present in each tube by the amount of microsomal protein and the reaction time and has the units nmol/mg protein/min.

The aromatase activities determined for human placental microsomes prepared at Battelle met the minimum activity set at 0.03 nmol/mg/min.

Information regarding assay dates, technicians, protein concentration and substrate specific activity is presented in Table 15.

Table 15 - Aromatase Assay Summary

Microsomes Source	Replicate	Assay Date	Technician	Protein Stock Concentration (mg/mL)	Substrate Solution Specific Activity (µCi/µg ASDN)
Battelle	1	4/15/05	LR	20.27	1.132
Battelle	2	4/15/05	TD	17.72	1.152
Battelle	1	4/22/05	TD/LR	20.07	1.066
Battelle	2	4/25/05	TD/LR	18.61	1.078
In Vitro	1	6/17/05	TD	7.987	1.158
In Vitro	2	6/17/05	LR	8.304	1.234

4.4.1 Control Results

Each replicate set included four types of controls, each run in quadruplicate. The control types were full aromatase activity, background activity, positive and negative controls. The positive control tubes contained the known aromatase inhibitor, 4-OH ASDN, at a concentration of 5×10^{-8} M and the negative control tubes contained

the known aromatase non-inhibitor, lindane, at the concentration of 1×10^{-6} M. The control tubes were divided so that two of each type were run at the beginning of the set and two were run at the end of the set. The aromatase activity in full aromatase activity controls represented 100% activity and since all aromatase activities were corrected for background, the background activity controls necessarily were set to 0%. The mean activity for each type of control (except background) for the beginning and end groups and the overall mean, SD, SEM and % CV across replicates are presented in Table 16.

Table 16 - Aromatase Activity in Controls

Microsomes Source	Replicate	Mean Beginning Control[a]	Mean End Control[a]	Overall Mean[a]	SD	SEM	% CV	Overall (by microsomes source) Mean[a]	SD	SEM	% CV
Full Aromatase Activity Controls											
Battelle	1	0.0686	0.0655	0.0671	0.0020	0.0010	2.98	0.0597	0.0089	0.0044	14.86
	2	0.0553	0.0495	0.0524	0.0040	0.0020	7.63				
In Vitro	1	0.0288	0.0289	0.0288	0.0003	0.0002	1.04	0.0276	0.0019	0.0009	6.77
	2	0.0278	0.0249	0.0264	0.0019	0.0010	7.20				
Positive Controls											
Battelle	1	0.0311	0.0299	0.0305	0.0010	0.0005	3.28	0.0299	0.0009	0.0005	3.14
	2	0.0299	0.0288	0.0294	0.0008	0.0004	2.72				
In Vitro	1	0.0159	0.0158	0.0159	0.0003	0.0002	1.89	0.0153	0.0007	0.0004	4.64
	2	0.0150	0.0144	0.0147	0.0004	0.0002	2.72				
Negative Control											
Battelle	1	0.0640	0.0639	0.0639	0.0012	0.0006	1.88	0.0601	0.0047	0.0023	7.77
	2	0.0580	0.0545	0.0562	0.0020	0.0010	3.56				
In Vitro	1	0.0299	0.0293	0.0296	0.0004	0.0002	1.35	0.0288	0.0010	0.0005	3.52
	2	0.0279	0.0279	0.0279	0.0004	0.0002	1.43				

[a] Units are nmol/mg protein/min.

The inherent aromatase activity varied between the two preparations, with the mean full aromatase activity for the Battelle microsomes at 0.0597 nmol/mg/min while the In Vitro microsomes were 0.0276 nmol/mg/min. Both sets of microsomes responded as expected to the presence of the known aromatase inhibitor, 4-OH ASDN and the known non-inhibitor, lindane. Generally, positive control activities were close to 50% of control (50.1% for the Battelle preparation and 55.4% for In Vitro) and negative control activities were near 100% of control (101% for the Battelle preparation and 104% for the In Vitro preparation).

For all four independent full aromatase activity determinations the average calculated value (± SD, ± SEM and % CV) was 0.0542 nmol/mg/min (± 0.0094, ± 0.0047 and 17.44%) (Table 17).

Table 17 - Summary of Aromatase Activity for Battelle Prepared Microsomes

Aromatase Activity (nmol/mg/min)	Average Aromatase Activity (nmol/mg/min)	SD	SEM	% CV
0.0444	0.0542	0.0094	0.0047	17.44
0.0528				
0.0671				
0.0524				

4.4.2. Percent of Control

Two independent determinations of the aromatase responsiveness to six concentrations of 4-OH ASDN were performed.

Table 18 summarizes aromatase activity (expressed as a percent of full activity) detected in assays with various inhibitor (4-OH ASDN) concentrations. Increasing the 4-OH ASDN concentration affected the aromatase activity in a concentration-dependent manner. The highest applied concentration of 4-OH ASDN (1×10^{-6} M) inhibited aromatase activity to approximately 94% of full enzyme activity (approximately 94% inhibition); the lowest concentration of 4-OH ASDN (1×10^{-9} M) inhibited aromatase activity only by approximately 4.8% (approximately 95.2% of aromatase activity remains intact, Table 19). Table 19 presents overall mean percent of control values for two replicates across six repetitions/tubes.

Table 18 - Individual Percent of Control Values by Tube and Replicate

Replicate	Log [4-OH ASDN]	Percent of Control			Mean	SD	SEM	% CV
		Tube 1	Tube 2	Tube 3				
1	-6.00	4.80	5.33	4.74	4.96	0.32	0.19	6.55
	-7.00	30.45	30.00	30.70	30.38	0.35	0.20	1.17
	-7.30	44.59	46.14	45.11	45.28	0.79	0.46	1.74
	-7.60	62.60	62.79	62.78	62.72	0.11	0.06	0.17
	-8.00	77.21	75.47	75.56	76.08	0.98	0.57	1.29
	-9.00	92.74	91.52	93.85	92.70	1.17	0.67	1.26
2	-6.00	6.30	6.62	6.93	6.62	0.32	0.18	4.76
	-7.00	37.74	39.79	39.29	38.94	1.07	0.62	2.74
	-7.30	53.82	54.41	56.74	54.99	1.54	0.89	2.81
	-7.60	73.87	70.62	73.48	72.66	1.77	1.02	2.44
	-8.00	84.61	82.38	84.84	83.94	1.36	0.78	1.62
	-9.00	93.42	98.25	101.56	97.74	4.09	2.36	4.19

Table 19 - Overall Mean Percent of Control Values

Log [4-OH ASDN]	Mean Percent of Control	SD	SEM	% CV
-6.00	5.79	0.95	0.39	16.47
-7.00	34.66	4.74	1.94	13.68
-7.30	50.14	5.43	2.22	10.83
-7.60	67.69	5.56	2.27	8.21
-8.00	80.01	4.44	1.81	5.54
-9.00	95.22	3.86	1.57	4.05

4.5 IC_{50}

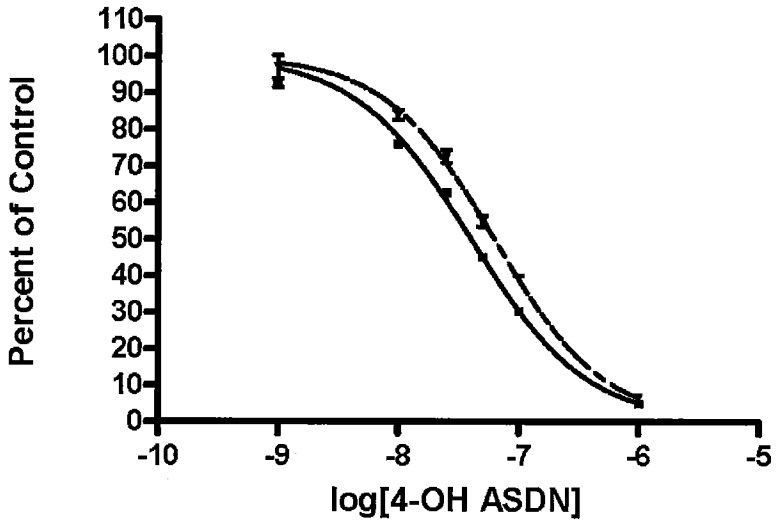
Based on the curve-fit of the percent of control aromatase activity across six concentrations of 4-OH ASDN, the calculated IC_{50} values are presented in Table 20.

Table 20 - Calculated IC_{50} Values

Replicate	Log [IC_{50}]	SE Log [IC_{50}]	IC_{50} (nM)	Slope	SE Slope
1	-7.394	0.01109	40.39	-0.9075	0.01835
2	-7.201	0.00964	62.96	-0.9584	0.01728

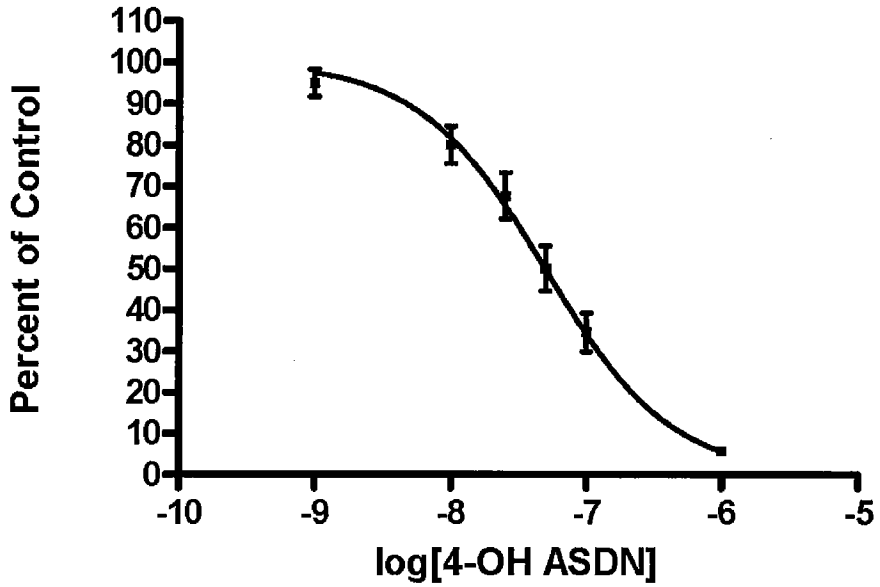
The following figures (Figures 1 and 2) present concentration response curves. Figure 1 presents the individual dose-response curve for each replicate, and Figure 2 presents the fitted curve averaged across two replicates (six repetitions/tubes).

The average IC_{50} calculated as the average value from two replicates (40.39 and 62.96 nM, respectively) is 51.68 nM. The IC_{50} obtained by fitting all experimental percent of control mean values (Table 18) into one curve is 49.64 nM (see Figure 2).



	Data Set-A	Data Set-B
Sigmoidal dose-response (variable slope)		
Best-fit values		
BOTTOM	0.0	0.0
TOP	100.0	100.0
LOGEC50	-7.394	-7.201
HILLSLOPE	-0.9075	-0.9584
EC50	4.039e-008	6.296e-008
Std. Error		
LOGEC50	0.01109	0.009644
HILLSLOPE	0.01835	0.01728
95% Confidence Intervals		
LOGEC50	-7.417 to -7.370	-7.221 to -7.180
HILLSLOPE	-0.9464 to -0.8686	-0.9950 to -0.9218
EC50	3.826e-008 to 4.264e-008	6.007e-008 to 6.600e-008
Goodness of Fit		
Degrees of Freedom	16	16
R^2 (unweighted)	0.9949	0.9963
Weighted Sum of Squares (1/Y)	1.027	0.8077
Absolute Sum of Squares	77.58	59.70
Sy.x	2.202	1.932
Constraints		
BOTTOM	BOTTOM = 0.0	BOTTOM = 0.0
TOP	TOP = 100.0	TOP = 100.0
Data		
Number of X values	6	6
Number of Y replicates	3	3
Total number of values	18	18
Number of missing values	0	0

Figure 1 - Concentration Response Curves for Two Averaged (Between Repetitions) Replicates



Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.0
TOP	100.0
LOGEC50	-7.304
HILLSLOPE	-0.9307
EC50	4.964e-008
Std. Error	
LOGEC50	0.01809
HILLSLOPE	0.03134
95% Confidence Intervals	
LOGEC50	-7.341 to -7.267
HILLSLOPE	-0.9944 to -0.8669
EC50	4.561e-008 to 5.403e-008
Goodness of Fit	
Degrees of Freedom	34
R^2 (unweighted)	0.9794
Weighted Sum of Squares (1/Y)	12.02
Absolute Sum of Squares	658.3
Sy.x	4.400
Constraints	
BOTTOM	BOTTOM = 0.0
TOP	TOP = 100.0
Data	
Number of X values	6
Number of Y replicates	6
Total number of values	36
Number of missing values	0

Figure 2 - Concentration Response Curve for Overall Mean of Two Replicates

4.6 Statistical Analysis

The full statistical analysis report is presented in Appendix G. There are some small differences in data obtained from the Prism output and data presented in the statistical report obtained applying the SAS statistical analysis system.

5.0 DISCUSSION

The presented study involved assaying human placental microsomes (from two sources) for protein content and aromatase activity. The microsomes used in this task were prepared at Battelle and In Vitro Technologies, Inc. laboratories. The average measured protein content for microsomal preparations was 19.91 ± 1.66 mg/mL and 8.57 ± 0.53 mg/mL for the Battelle and In Vitro Technologies, Inc. preparations, respectively. The average measured full aromatase activity for microsomal preparations was 0.0597 ± 0.0089 nmol/mg/min and 0.0276 ± 0.0019 nmol/mg/min for Battelle and In Vitro Technologies, Inc., respectively. The determined value of aromatase activity for the In Vitro Technologies microsomal preparation was approximately only 73% of the value reported by the supplying laboratory (reported value 0.038 nmol/mg/min).

Four types of controls were used for the aromatase assay; a full activity control, which served as the 100% activity control, a background activity control which was used to correct for non-enzymatic product formation and other artifactual radiochemical content in the assay mixture, a positive control which employed a known aromatase inhibitor and a negative control which employed a known aromatase non-inhibitor. Both sets of microsomes responded as expected to the presence of the known aromatase inhibitor, 4-OH ASDN and the known non-inhibitor, lindane.

Additionally, two independent replicates using the Battelle prepared microsomes were performed to determine the response of aromatase to six different concentrations of 4-OH ASDN. Based on the obtained data the calculated overall IC_{50} was 51.68 nM. This IC_{50} value is in good agreement with the previously reported IC_{50} for 4-OH ASDN (see Task 4 report).

6.0 CONCLUSION

Both microsomal suspensions, prepared from human placentas at Battelle and In Vitro Technologies, Inc. laboratories, appear to be acceptable for usage in the future work assignments, although the aromatase activity in In Vitro Technologies, Inc. preparation is very low.

There are significant differences between both preparation in terms of protein concentrations and inherent aromatase activity.

For both microsomal preparations protein content and aromatase activity show good assay-to- assay consistency.

Both preparations show expected responsiveness of the aromatase assay to the known aromatase inhibitor (4-OH ASDN) and non-inhibitor (lindane).

APPENDIX A

BATTELLE STUDY PROTOCOL

AROMATASE ASSAY VALIDATION: PREPARATION AND CHARACTERIZATION OF HUMAN PLACENTAL MICROSOMES

Testing Facility:

Battelle Memorial Institute
505 King Avenue
Columbus, Ohio 43201-2693

Sponsor:

Endocrine Disruptor Screening Program Office
Battelle Memorial Institute
505 King Avenue
Columbus, Ohio 43201-2693

EPA Contract: 68-W-01-023
EPA Work Assignment: WA 4-16, Task 6

Battelle
The Business *of* Innovation

Experiment Start Date: March 25, 2005
Experiment End Date: April 30, 2005

AROMATASE ASSAY VALIDATION: PREPARATION AND CHARACTERIZATION OF HUMAN PLACENTAL MICROSOMES

APPROVED, BATTELLE:

Bozena D. Lusiak — 03-24-05
Bozena D. Lusiak, Ph.D. — Date
Study Director, Battelle Memorial Institute

Steven W. Graves — 3/24/05
Steven W. Graves — Date
CTC Manager, Battelle Memorial Institute

REVIEWED BY, BATTELLE:

Kathleen E. Reed for HAG — 3-24-05
Hillary A. Glover — Date
Quality Assurance Auditor, Battelle Memorial Institute

APPROVED, SPONSOR:

Jerry D. Johnson — 3-24-05
Jerry D. Johnson, Ph.D., D.A.B.T. — Date
Work Assignment Leader
Endocrine Disruptor Screening Program
Battelle Memorial Institute

for David P. Houchens — 3-24-05
David P. Houchens, Ph.D. — Date
Program Manager
Endocrine Disruptor Screening Program
Battelle Memorial Institute

REVIEWED BY, SPONSOR :

Terri L. Pollock
EDSP Quality Assurance Manager
Battelle Memorial Institute

3-28-05
Date

AROMATASE ASSAY VALIDATION: PREPARATION AND CHARATERIZATION OF HUMAN PLACENTAL MICROSOMES

1.0 OBJECTIVES

The objectives of this protocol are to describe procedures for the preparation of human placental microsomes, the analysis of microsomal preparation for protein content and aromatase activity, and the conduct of a study with known aromatase inhibitor 4-hydroxyandrostenedione (4-OH ASDN) to demonstrate responsiveness of the assay to aromatase inhibitors.

This Task will be conducted in two stages as described below.

1.1 Stage 1- Placenta Procurement/Microsomes Preparation and Characterization

A human placenta will be obtained from The Ohio State University Tissue Procurement Center and placental microsomes will be prepared. Protein concentration (two independent replicates) and aromatase activity (two independent replicates) will be determined. Two independent replicates of a study will be performed to determine the response of aromatase to six concentrations of 4-OH ASDN.

The data from these studies will be sent to Battelle EDSP Office and will be reviewed and submitted to the Environmental Protection Agency (EPA). Approval from the EPA Work Assignment Manager (WAM) will be obtained before proceeding to Stage 2.

1.2 Stage 2- Distribution of Microsomes and Conduct of Aromatase Activity Studies

After receiving EPA's approval, the microsomes prepared at Battelle will be distributed to three laboratories: In Vitro Technologies, RTI International, and WIL Research Laboratories along with information about protein concentration and estimated aromatase activity. The placental microsomes sent to Battelle from In Vitro Technologies, will be used to determine the protein concentration and aromatase activity of the microsomes that In Vitro prepared from the placenta that they obtained.

1.3 Justification for Test System

The test system for this study is human placental microsomes. This test system was selected because it provides a biological source of the aromatase enzyme and since the assay is being evaluated for its potential to serve as a screening assay, the use of human tissue enhances its predictive potential.

1.4 Test Method

This *in vitro* test method involves combining microsomes, substrate, appropriate cofactors and test substances in a common reaction vessel. The effect of the test substances on microsomal enzyme activity will be evaluated by measuring the amount of the product

formation.

There is no applicable route of administration in the sense of a dose administration route for this *in vitro* test.

2.0 MATERIALS RECEIPT AND/OR PREPARATION

A sufficient supply of chemical reagents, radiolabeled and non-radiolabeled androstenedione and human placental microsomes will be obtained prior to initiation of the first set of experiments to ensure that sufficient quantities are available to conduct the studies.

2.1 Substrate

2.1.1 Substrate Name/Supplier

The substrate for the aromatase assay is androstenedione (ASDN). Non-radiolabeled and radiolabeled ASDN will be used. The non-radiolabeled ASDN and the radiolabeled androstenedione ([1β-^{3}H]-androstenedione, [^{3}H]ASDN) will be provided to the laboratories by Battelle's Chemical Repository (CR). The CR will forward all applicable information regarding supplier, lot numbers and reported/measured purity for the substrate to the laboratories and this information will be included in study reports. The radiochemical purity of the [^{3}H]ASDN (of each lot that is used) was assessed by the lead laboratory (RTI) in a previous Task and was found to be 97%.

2.1.2 Preparation of Substrate Solution for use in Aromatase Assay

A solution containing a mixture of non-radiolabeled and radiolabeled [^{3}H] ASDN will be prepared to achieve 100 nM final concentration of ASDN in the assay and the amount of tritium added to each incubation about 0.1 µCi. This substrate solution should have a concentration of 2 µM with a radiochemical content of about 1 µCi/mL.

The following illustrates the preparation of a substrate solution using a stock of [^{3}H]ASDN with a specific activity of 25.3 Ci/mmol and a concentration of 1 mCi/mL. Prepare a 1:100 dilution of the radiolabeled stock in buffer. Prepare a 1 mg/mL solution of ASDN in ethanol and then prepare dilutions in buffer to a final concentration of 1 µg/mL. Combine 4.5 mL of the 1 µg/mL solution of ASDN, 800 µL of the [^{3}H]ASDN dilution and 2.7 mL buffer to make 8 mL of substrate solution (enough for 80 tubes). Record the weight of each component added to the substrate solution. After mixing the solution well, weigh aliquots (ca ~~20 µL~~) and combine with scintillation cocktail for radiochemical content analysis. The addition of 100 µL of the substrate solution to each 2 mL assay volume yields a final [^{3}H]ASDN concentration of 100 nM with 0.1 µCi/tube.

2.2 Test Substances

4-Hydroxyandrostenedione (4-OH ASDN) is a known aromatase inhibitor.

2.2.1 4-Hydroxyandrostenedione (4-OH ASDN)

CAS No.: 566-48-3
Molecular Formula/Weight: $C_{19}H_{26}O_3$; 302.4 g/mol
Supplier: Sigma
Lot No: 063K4069
Purity: 99%
Storage Conditions: 2- 8°C (for bulk chemical and solutions)

2.2.2 Test Substance Formulation and Analysis

Test substance stock solutions will be prepared and analyzed by the CR and distributed to the laboratories. 4-OH ASDN will be formulated in 95% ethanol. The total volume of the test substance formulation used in each assay should be no more than 1% of the total assay volume (i.e., 20 µL in a 2 mL assay) in order to minimize the potential of the solvent to inhibit the enzyme. Dilutions of the stock solution will be prepared in ethanol on the day of use such that the target concentration of inhibitor can be achieved by the addition of 20 µL of dilution to a 2 mL assay volume.

2.3 Control Substances

The known aromatase inhibitor, 4-hydroxyandrostenedione (4-OH ASDN), will be used as the positive control substance. A known aromatase non-inhibitor, lindane, will be used as the negative control substance. Table 1 contains identity and property information for these substances.

Table 1. Control Substances

Test Substance	CAS Number	Molecular Formula	Molecular Weight (g/mol)	Target Concentration in Assay (M)	Basis for Selection
4-OH ASDN	566-48-3	$C_{19}H_{26}O_3$	302.4	5×10^{-8}	Known aromatase inhibitor
Lindane	58-89-9	$C_6H_6Cl_6$	290.8	1×10^{-6}	Affects StAR and cholesterol metabolism; no aromatase activity

2.3.1 Control Substance Formulation and Analysis

Control substances stock solutions will be prepared and analyzed by the CR and distributed to the laboratories. Control substances will be formulated in ethanol or DMSO . The total volume of control substance formulation used in each assay should be no more than 1 % of the total assay volume (i.e., 20 µL in a 2 mL assay) in order to minimize the potential of the solvent to inhibit the enzyme. Fresh dilutions of the stock solution will be prepared in the same solvent as the stock solution on the day of use. Dilutions will be prepared such

that the target concentration of control substance (Table 1) can be achieved by the addition of 20 µL of the dilution to a 2 mL assay volume. Information on storage conditions for control substance stock solution will be provided by the CR.

2.4 Human Placental Microsomes

2.4.1 Preparation

Appropriate precautions must be taken in the handling of human placenta, which should be considered potentially infectious. These precautions should extend to the handling of human placental microsomes as well.

2.4.1.1 Source of Placenta

A human placenta will be obtained from The Ohio State University Tissue Procurement Center. The source of placenta will be documented in the study records. Human placenta will be from a non-smoking, 21-40 year-old-mother with a full term delivery. Within 30 minutes of the delivery of the placenta by the mother, it will be placed in a tissue bag, sealed, and packed on wet ice in an insulated shipping container. The placenta tissue bag will be labeled with date and time of delivery. Battelle laboratory personnel will be on-call and will be responsible for transporting the placenta to their laboratory for processing into microsomes, as described below. Efforts will be made to minimize the time from delivery to the initiation of microsomes preparation. Ideally, microsome preparation should begin within 2 hours of obtaining the placenta.

2.4.1.2 Microsome Preparation Buffers

Buffer A:
0.25 M Sucrose, 0.04 M nicotinamide, 0.05 M sodium phosphate (pH 7.0)

First, the 0.05 M sodium phosphate solution, pH 7.0 will be prepared. 6.00 ± 0.48 g of sodium phosphate monobasic (Sigma, 119.98 g/mol; or equivalent) will be dissolved in 1L of Milli-Q water to prepare 0.05 M NaH_2PO_4. 7.10 ± 0.57 g of sodium phosphate dibasic (Sigma, 141.96 g/mol; or equivalent) will be dissolved in 1 L of Milli-Q water to prepare 0.05 M Na_2HPO_4. These solutions will be combined to a final pH of 7.0. The buffer may be stored for up to one month in the refrigerator (ca. 2-8°C).

To complete preparation of Buffer A 85.53 ± 1.35 g of sucrose (Sigma, 342.1 g/mol; or equivalent) and 4.88 ± 0.48 g nicotinamide (Sigma, 122.1 g/mol; or equivalent) will be dissolved in 1L 0.05 M sodium phosphate buffer, pH 7.0. The buffer may be stored up to one month in the refrigerator (ca. 2-8°C).

Buffer B:
0.1 M sodium phosphate (pH 7.4)

Sodium phosphate monobasic and sodium phosphate dibasic will be used in the preparation of the buffer. 12.0 ± 0.30g sodium phosphate monobasic (Sigma, 119.98 g/mol; or equivalent) will be dissolved in 1 L Milli-Q water to prepare 0.1 M NaH_2PO_4. 14.20 ± 0.56 g sodium phosphate dibasic (Sigma, 141.96 g/mol; or equivalent) will be dissolved in 1L Milli-Q water to prepare 0.1 M Na_2HPO_4. These solutions will be combined to a final pH of 7.4. The assay buffer may be stored for up to one month in the refrigerator (ca. 2-8°C).

Buffer C:

0.1 M sodium phosphate (pH 7.4) with 0.25 M sucrose, 20% of glycerol and 0.05 mM dithiothreitol

17.12 ± 0.27 g of sucrose and 1.54 ± 0.12 mg of dithiothreitol (Sigma, 154.3 g/mol; or equivalent) will be dissolved in ca. 100 mL of 0.1 M sodium phosphate buffer, pH 7.4 (prepared as described above), and will be diluted to 160 mL with additional 0.1 M sodium phosphate buffer, pH 7.4. Glycerol (Sigma, 92.0 g/mol; or equivalent) will be added to obtain a total volume of 200 mL.

2.4.1.3 Placental Microsomes Preparation

Caution: Microsomes can be denatured by detergents. Therefore, it is important to ensure that all glassware, etc. used in the preparation or usage of microsomes is free of detergent residue. New disposable test tubes, bottles, vials, pipets and pipet tips may be used directly in the assay. Durable labware that may have been exposed to detergents should be rinsed with water and/or buffer prior to use in the assay.

Human placentas are discoid in shape and have a fetal surface (with umbilicus attached) and a maternal surface. Each of these surfaces is covered with a fibrous, vascularized membrane. It is important for the preservation of aromatase activity to keep the tissue as well chilled on ice as possible and to work quickly. The placenta will be placed on a tray that is set over/in a pan of ice to aid in keeping the tissue chilled during dissection operation. While keeping the placenta chilled on ice, the membrane and fibrous material will be dissected, removed and discarded. The spongy tissue will be cut into small portions and placed on ice in prechilled Buffer A. Batches of the tissue will be sequentially removed to a beaker and minced with scissors. Buffer A will be added to an approximate 2:1 (w:v) ratio and the mixture will be homogenized using a homogenizer. Some fibrous material may be resistant to homogenization and this tissue will be removed from homogenate or it may be allowed to remain with the knowledge that it will be removed in the centrifugation step to follow. The homogenate will be transferred to centrifuge tubes (recommended approximately 40 mL capacity) and kept on ice until all of the tissue is processed or until the capacity of the centrifuge rotor is reached. Tissue

homogenization will continue in batches as described until all tissue is processed. The tissue homogenate will be centrifuged (in batches, as necessary, dependent on rotor capacity and the number of tubes to be processed) at the setting of 10,000g for 30 minutes at ca.4°C. The supernatant will be removed by pipetting and transferred to ultracentrifuge tubes (recommended capacity is 26 mL) and will be centrifuged at a setting of 35,000 rpm (or another speed as necessary to produce approximately 100,000g) in an ultracentrifuge for one hour at about 4°C to obtain the crude microsomal pellet. The supernatant will be decanted and the microsomal pellet will be dislodged from the wall of the tube by gentle swirling with a few mL of Buffer B. Care will be taken to not dislodge the clear pellet that often is visible under the microsomal pellet. The microsomal pellet (along with the buffer) will be poured into a (suggested 15-mL size) Potter-Elvejhem homogenizer and resuspended in Buffer B Next, the suspension will be transferred to ultracentrifuge tubes and will be centrifuged at a setting of 35,000 rpm (ca. 100,000g) for one hour to wash the microsomes. This washing procedure (supernatant decanting, pellet resuspension and centrifugation) will be repeated one additional time. Then the supernatant will be decanted and the twice-washed microsomal pellet will be dislodged from the wall of the tube by gentle swirling in a few mL of Buffer C. All microsomal pellets will be combined into a single lot and will be resuspended in Buffer C using Potter-Elvejhem homogenizer. It is suggested that an appropriate final volume of suspended microsomes may range from 20-30 mL, dependent on the amount of protein that will be isolated from placenta. The concentration of microsomes in the final suspension will be at least 15 mg/mL, which may be measured at this point using the protein assay. The microsomes will be aliquoted (ca. 200 µL/tube) into labeled tubes (cryotubes) and will be flash frozen in liquid nitrogen and then will be stored at approximately -60° to -80°C until removed for use.

2.4.2 Use of Microsomes

On the day of use, microsomes will be thawed quickly in a 37 ± 1°C water bath and then will be immediately transferred to an ice bath. The microsomes will be rehomogenized using a Potter-Elvejhem homogenizer (about 5-10 passes) or vortexed to mix prior to use. The microsomes will be diluted in buffer (serial dilutions may be necessary) to an approximate protein concentration of 0.025 mg/mL. The addition of 1 mL of the microsomes dilution will result in a final approximate protein concentration of 0.0125 mg/mL in the assay tubes. All microsomes samples will be kept on ice until they are placed in the water bath just prior to their addition to the aromatase assay. It is recommended that microsomes not be left on ice for longer than approximately 1 h before proceeding with the assay or microsomal enzyme activity may be decreased.

Under no conditions should thawed or diluted microsomes be refrozen for later use in the assay.

2.5 Other Assay Components

2.5.1 Buffer

The assay buffer is 0.1 M sodium phosphate buffer, pH 7.4. It is prepared as described in Section 2.4.1.2 above for Buffer B. The assay buffer may be stored for up to one month in the refrigerator (ca. 2-8 °C).

2.5.2 Propylene Glycol

Propylene glycol will be added to the assay directly as described below.

2.5.3 NADPH

NADPH (β-nicotinamide adenine dinucleotide phosphate, reduced form), is the required co-factor for CYP19. The final concentration in the assay will be 0.3 mM. Typically, a 6 mM stock solution will be prepared in assay buffer and then 100 µL of the stock will be added to the 2 mL assay volume. NADPH solution must be prepared fresh each day and kept on ice until use.

3.0 PROTEIN ASSAY

The protein concentration in the microsomes will be determined each day of microsome use in the aromatase assay (and at other times as appropriate) by using a DC Protein Assay kit purchased from BioRad (Hercules, CA).

A 6-point standard curve will be prepared; target range will be from 0.11 to 1.0 mg protein/mL. The protein standards will be made from bovine serum albumin (BSA). QC standards (0.125, 0.5 and 1.0 mg protein/mL), obtained from Pierce (Rockford, IL) will be run in duplicate with each assay. To a 25 µL aliquot of microsomes solution (1:50 dilution of microsomes may be required) or standard, 125 µL of BioRad DC Protein Kit Reagent A will be added and mixed. Next, 1 mL of BioRad DC Protein Kit Reagent B will be added to each standard or microsomes solution and the samples will be gently mixed. The samples will be allowed to sit at room temperature for at least 15 min to allow color development. (The absorbances are stable for about 1 hour.) Each sample (unknown and standards) will be transferred to disposable polystyrene cuvettes and the absorbance (750 nm) will be measured using a spectrophotometer. The protein concentration of the microsomal sample will be determined.

4.0 AROMATASE ASSAY METHOD

This procedure will be used to measure the aromatase activity in the microsomal preparations. Four types of control samples will be included for each replicate. These include:

- full enzyme (aromatase) activity controls (substrate, NADPH, propylene glycol, buffer, vehicle [used for preparation of test substance solutions] and microsomes)

- background activity controls (all components that are in the full aromatase activity controls, except NADPH)

- positive controls (all components that are in the full aromatase activity controls, except vehicle, and with the addition of 4-OH ASDN at a single concentration, i.e. 5×10^{-8} M)

- negative controls (all components that are in the full aromatase activity controls, except vehicle, and with the addition of lindane at a single concentration, i.e. 1×10^{-6} M).

Four test tubes of each type of control will be included with each replicate and will be treated the same as the other samples. The controls sets will be split so that two tubes (of each control type) are run at the beginning and two at the end of each set.

The assays will be performed in 13x100 mm test tubes maintained at 37 ± 1°C in a shaking water bath. Each test tube will be uniquely identified by applying a label or writing directly on the test tube. Propylene glycol (100 µL), [^{3}H]ASDN, NADPH, and buffer (0.1 M sodium phosphate, pH 7.4) will be combined in the test tubes (total volume 1.0 mL). The final concentrations for the assay components are presented in Table 2. The tubes and the microsomal suspension will be placed at 37 ± 1°C in the water bath for approximately five minutes prior to initiation of the assay by the addition of 1 mL of the diluted microsomal suspension. The total assay volume will be 2.0 mL, and the tubes will be incubated for 15 min. The incubations will be stopped by the addition of methylene chloride (2.0 mL); the tubes will be vortex-mixed for ca. 5 s and placed on ice. The tubes will be then vortex-mixed an additional 20-25 s. The tubes will then be centrifuged using a Beckman GS-6 centrifuge with GH-3.8 rotor for 10 minutes at a setting of 1000 rpm. The methylene chloride layer will be removed and discarded; the aqueous layers are extracted again with methylene chloride (2.0 mL). This extraction procedure will be performed one additional time, each time discarding the methylene chloride layer. The aqueous layers will be transferred to vials and duplicate aliquots (0.5 mL) will be transferred to 20-mL liquid scintillation counting vials. Liquid scintillation cocktail (Ultima Gold, Packard, 10 mL) will be added to each counting vial and shaken to mix the solution.

Table 2. Optimized Aromatase Assay Conditions

Assay Factor (units)	Human Placental
Microsomal Protein (mg/mL)[a]	0.0125
NADPH (mM)[a]	0.3
[^{3}H]ASDN (nM)[a]	100
Incubation Time (min)	15

[a] Final concentrations

Analysis of the samples will be performed using liquid scintillation spectrometry (LSS). Radioactivity found in the aqueous fractions represents amount of formed ^3H_2O.

5.0 DETERMINATION OF THE RESPONSE OF AROMATASE ACTIVITY TO 4-HYDROXYANDROSTENEDIONE (4-OH ASDN)

Each replicate will test the response of aromatase activity to the presence of six concentrations of 4-OH ASDN. This task will be conducted in two independent replicates (per microsomes preparation). Each concentration of 4-OH ASDN will be run in triplicate tubes in each Study. See Table 3 for the study design. The four types of control samples described in Section 4.0 will be included in each replicate set. Each control type will be run in quadruplicate with controls sets split so that two tubes (of each control type) are run at the beginning and two at the end of each replicate set.

The assay will be conducted as described in Section 4.0 with the following modification. 4-OH ASDN solution (or vehicle) will be added to the mixture of propylene glycol, substrate, NADPH, and buffer in a volume not to exceed 20 µL prior to preincubation of that mixture. The volume of buffer used will be adjusted so the total incubation volume remains at 2 mL.

Table 3. Study Design –Aromatase Response to 4-OH ASDN

Sample type	Repetitions (test tubes)	Description	Control or Test Chemical concentration (M)
Full Activity Control	4	Complete assay[a] with inhibitor vehicle control	N/A
Background Activity Control	4	Complete assay with inhibitor vehicle control omitting NADPH	N/A
Positive Control	4	Complete assay with positive control chemical (4-OH ASDN) added	5×10^{-8}
Negative Control	4	Complete assay with negative control chemical (lindane) added	1×10^{-5}
4-OH ASDN Concentration 1	3	Complete assay with 4-OH ASDN added	1×10^{-6}
4-OH ASDN Concentration 2	3	Complete assay with 4-OH ASDN added	1×10^{-7}
4-OH ASDN Concentration 3	3	Complete assay with 4-OH ASDN added	5×10^{-8}
4-OH ASDN Concentration 4	3	Complete assay with 4-OH ASDN added	2.5×10^{-8}
4-OH ASDN Concentration 5	3	Complete assay with 4-OH ASDN added	1×10^{-8}
4-OH ASDN Concentration 6	3	Complete assay with 4-OH ASDN added	1×10^{-9}

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

6.0 DATA ANALYSIS

6.1 Aromatse Activity and Percent of Control Calculations

Relevant data will be entered into the latest version of the spreadsheet Aromatase_Master_Versionx.y.xls (where x and y denote version number designation) for calculation of aromatase activity and percent of control. The version of the spreadsheet used will be included in the report. A working document detailing the use of this spreadsheet has been issued in a previous task on this work assignment.

6.2 Statistical Analyses

6.2.1 Concentration Response Fits for the Test Substance

For the 4-OH ASDN, two independent replicates of the concentration response curve fit will be carried out.

For each replicate two repeat tubes of the full enzyme activity controls, the background activity controls and the positive and negative controls will be run prior to the repetition of the graded concentrations of 4-OH ASDN and two repeat tubes of each control will be run following the repetition of 4-OH ASDN. Three repetitions will be prepared for each concentration of 4-OH ASDN.

For each repeat tube (full enzyme activity, background activity, positive and negative controls and each 4-OH ASDN concentration), the Excel spreadsheet will include total observed (uncorrected) disintegrations per minutes (dpms) per tube and total aromatase activity per tube. The dpm and aromatase activity values will be corrected for background dpms, as measured by average of background activity control tubes. The aromatase activity will be calculated as the corrected dpm, normalized by the specific activity of the [^{3}H] ASDN, the mg of protein of the microsomes, and the incubation time. The average (corrected) dpms and aromatase activity across the four background activity control repeat tubes must necessarily be equal to 0 within each replicate.

For each tube percent of control will be determined by dividing the background corrected aromatase activity for that tube by average background corrected aromatase for activity for the four full enzyme activity tubes and multiplying by 100. Nominally, one might expect for an inhibitor the percent of control activity values to vary between approximately 0% near the high inhibition concentrations and approximately 100% near the low inhibition concentrations. However, due to experimental variation individual observed percent control values will sometimes extend below 0% or above 100%.

Concentration response trend curves will be fitted to the percent of control activity values within each of the repeat tubes at each 4-OH ASDN concentration. Concentration will be expressed on the log scale. In agreement with past convention, logarithms will be common logarithms (i.e. base 10). Let X denote the logarithm of the concentration of 4-OH ASDN (e.g. if concentration = 10^{-5} then X = -5). Let

Y = percent of control activity in the inhibitor tube
X = logarithm (base 10) of the concentration

DAVG = average dpms across the repeat tubes with the same 4-OH ASDN concentration
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 will be fitted to relate percent of control activity to logarithm of concentration within each replicate:

$$Y = 100/[1 + 10^{(\mu - X)\beta}] + \varepsilon$$

where ε is the variation among repetitions, distributed with mean 0 and variance proportional to DAVG (based on Poisson distribution theory for radiation counts). The variance is approximated by Y.

The response curve will be fitted by weighted least squares nonlinear regression analysis with weights equal to 1/Y. Observed individual percent activity values above 100% will be set to 99.5%. Observed individual percent activity values below 0% will be set to 0.5%. Model fits will be carried out using Prism software (Version 3 or higher).

Concentration response models will be fitted for each replicate test. Based on the results of the fit within each replicate the extent of aromatase inhibition will be summarized as IC_{50} (10^{μ}) and slope (β). The estimated IC_{50} for 4-OH ASDN will be a (weighted) geometric mean across the replicates. The estimated overall standard error will be based on the standard errors within each replicate and the replicate-to-replicate variability. The average value and standard error of $\log_{10}IC_{50}$ or β and the replicate-to-replicate component of variation will be calculated based on a one-way random effects analysis of variance model fit. For each replicate the estimated $\log_{10}IC_{50}$ (μ), the within replicate standard error of μ, the IC_{50}, the slope (β), the within replicate standard error of β, and the "Status" of each response curve will be displayed in a table. The "Status" of each response curve will be indicated as:

- Complete. Data points ranging from essentially 0% to 100 % of control.
- Incomplete – Interpolate. Data points to at least 50% inhibition.
- Incomplete – Extrapolate. Data points all above 50% inhibition.
- No inhibition. No data below 80% of control.

6.2.2 Graphical and Analysis of Variance Comparisons Among Concentration Response Curve Fits

For each replicate, the individual percent of control values will be plotted versus logarithm of the 4-OH ASDN concentration. The fitted concentration response curve will be superimposed on the plot. Individual plots will be prepared for each replicate.

Additional plots will be prepared to compare the percent of control activity values across replicates. For each replicate, the average percent of control values will be plotted versus logarithm of 4-OH ASDN concentration on the same plot. Plotting symbols will distinguish among replicates. The fitted concentration response curves for each replicate will be superimposed on the plots. On a separate plot the average percent of control values for each replicate will be plotted versus logarithm of 4-OH ASDN concentration.

The average concentration response curve across replicates will be superimposed on the same plot.

For each replicate, β and μ will be treated as a random variable with mean (β_{avg}, μ_{avg}). X and Y ($0< Y <100$) denote logarithm of concentration and percent of control, as defined above. The average response curve will be

$$Y_{avg} = 100/[1 + 10^{\beta avg(\mu avg - X)}].$$

Slope (β) and $\log_{10}IC_{50}$ (μ) will also be compared across replicates based on one-way random effects analysis of variance, treating the replicates as random effects. For each of β and μ, plots will be prepared that display the parameters within each replicate with associated 95% confidence intervals based on the within replicate standard error and average across replicates with associated 95% confidence interval incorporating replicate-to replicate variation.

6.2.3 Graphical and Analysis of Variance Comparisons of Full Enzyme Activity Control, Background Activity Control, and Positive and Negative Control Percent of Control Across Replicates

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

To assess whether this is the case the control responses will be adjusted for background dpms, divided by the average of the (background adjusted) full enzyme activity control values, and expressed as percent of control. The average of the four background activity controls within a replicate must necessarily be 0 percent and the average of the four full enzyme activity controls within a replicate must necessarily be 100 percent. The full enzyme activity controls percent of control, the background activity controls percent of control, and the negative and positive controls percent of control values will be plotted across replicate with plotting symbol distinguishing between beginning and end, and with reference line 0% (background activity control) or 100% (full enzyme activity control) respectively. These plots will display the extent of consistency across replicates with respect to average value and variability and will provide comparisons of beginning versus end of each replicate.

Two-factor analysis of variance models will be fitted, separately for the full enzyme activity control, the background activity control, and the positive and negative control tubes. The factors in the analysis of variance will be:

- Portion (beginning or end) 1 df
- Replicate 1 df
- Portion by replicate interaction 1 df.

The residual error variation corresponds to repetition within replicate, and portion (with 4 degrees of freedom). The response will be percent of control. Since for the background

activity and full enzyme activity controls the average of the repetitions within replicate are constrained to be 0 and 100 respectively, by the way in which "percent of control" is defined, the variation associated with the replicate is necessarily constrained to be 0.

If the daily replicates are in control the portion main effect and the portion by replicate interaction should be nonsignificant. If the portion by replicate interaction is significant, the nature of the effect will be assessed by comparing the portion effect within each replicate to the portion effect averaged across replicates.

6.2.4 Microsomal Characterization Within and Between Laboratories

Battelle will supply microsomes to RTI, WIL and In Vitro laboratories and In Vitro will supply microsomes to RTI, Battelle and WIL laboratories. Each laboratory will determine protein concentration and aromatase activity of each microsomal preparation, as discussed in the protocol. Each laboratory will compare the protein concentrations and the aromatase activity between the two microsomes sources by two-sample t-test, using the within laboratory microsomes preparation replicate determination variation as an error criterion.

The results of the determinations of protein concentration and aromatase activity from each microsomal preparation will be sent to the Data Coordination Center where an interlaboratory comparison will be carried out.

A two-way analysis of variance model will be fitted to the protein concentration and aromatase activity responses. The factors in the model will be:

- Laboratory 3 df
- Microsomes preparation 1 df
- Laboratory x Microsomes preparation 3 df
- Within laboratory –preparation variation 8 df.

The significance of the microsomes preparation main effect will be based on the laboratory x microsomes preparation interaction. The significance of the laboratory x microsomes preparation interaction will be based on comparisons with the within laboratory-preparation variation. The within laboratory-preparation variation will be based on three replicate determinations within each laboratory, pooled across laboratories. If either is significant, estimates and confidence intervals of microsomes preparation effect will be prepared, either averaged across laboratories or separately within laboratories, as appropriate.

6.2.5 Statistical Software

Concentration response curves will be fitted to the data using the non-linear regression analysis features in the Prism statistical analysis package, Version 3 or higher. Supplemental statistical analyses and displays such as summary tables, graphical displays, analysis of variance, and multiple comparisons will be carried out using Prism,

the SAS statistical analysis system, Version 8 or higher, or other general purpose statistical packages (e.g. SPSS), as convenient.

6.2.6 Interlaboratory Statistical Analysis

The lead laboratory and each of the participating laboratories will carry out "intra-laboratory" statistical analyses based on their test data, according to this common statistical analysis plan, developed by the Data Coordination Center (Battelle). The Data Coordination Center will carry out the "inter-laboratory" statistical analysis. It will combine summary values developed in each of the intra-laboratory analyses to assess relationships among the laboratory results, the extent of laboratory-to-laboratory variation, and overall consensus estimates among the laboratories.

7.0 RETENTION OF RECORDS

All records that remain the responsibility of the testing laboratory will be retained in the archives for the life of the contract.

8.0 QUALITY CONTROL/QUALITY ASSURANCE PROCEDURES

Quality control (QC) and quality assurance (QA) procedures will follow those outlined in the Quality Assurance Project Plan (QAPP) that was prepared for this study. The study will be conducted in compliance with the Federal Register, 40 CFR Part 160, Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) Good Laboratory Practices Standards.

9.0 REPORTS

Interim data summaries, draft and final reports will be submitted as described in Section 9.5 of the QAPP.

The data to be reported in the interim data summaries will include (but is not limited to) the following information: assay date and run number, technician code, chemical code and log chemical concentration, background corrected aromatase activity (for each control and 4-OH ASDN repetition), percent of control activity, IC_{50}, slope and graphs of activity versus log of 4-OH ASDN concentration.

In addition, draft and final reports will contain tables and graphs, as appropriate, containing the results of the intra-laboratory statistical analyses described in Section 6 of this document.

10.0 STUDY RECORDS TO BE MAINTAINED

- All records that document the conduct of the laboratory experiments and results obtained, as well as the equipment and chemicals used
- Protocol and any Amendments

- List of any Protocol Deviations
- List of Standard Operating Procedures
- Quality Assurance Project Plan (QAPP) and any Amendments
- List of any QAPP Deviations

PROTOCOL AMENDMENT NUMBER 1

STUDY NUMBER: **G608316**

STUDY TITLE: **Aromatase Assay Validation: Preparation and Characterization of Human Placental Microsomes (WA 4-16, Task 6)**

PART TO BE CHANGED: Section 2.4.2, page 9, entire Section

> On the day of use, microsomes will be thawed quickly in a 37 ± 1°C water bath and then will be immediately transferred to an ice bath. The microsomes will be rehomogenized using a Potter-Elvejhem homogenizer (about 5-10 passes) or vortexed to mix prior to use. The microsomes will be diluted in buffer (serial dilutions may be necessary) to an approximate protein concentration of 0.025 mg/mL. The addition of 1 mL of the microsomes dilution will result in a final approximate protein concentration of 0.0125 mg/mL in the assay tubes. All microsomes samples will be kept on ice until they are placed in the water bath just prior to their addition to the aromatase assay. It is recommended that microsomes not be left on ice for longer than approximately 1 h before proceeding with the assay or microsomal enzyme activity may be decreased.
>
> Under no conditions should thawed or diluted microsomes be refrozen for later use in the assay.

CHANGE TO:

1. Thaw and rehomogenize the microsomes as per normal procedures.
2. Prepare an appropriate dilution (ca. 0.025 mg/mL) in buffer for use in the aromatase assay and its associated protein assay. For a microsome stock that contains ca. 8 mg/mL protein, a 1:320 dilution is recommended. Stock microsomes that contain ca. 20 mg/mL protein should be diluted 1:800.
3. Prepare a separate dilution (using the same or similar dilution factors as used in step 2- or as necessary to fall near the middle of the 5-250 µg/mL protein standard range) in buffer for use in the protein assay.
4. Be sure to prepare completely independent protein standards and QC for both protein assays. Run all assays (aromatase plus two protein assays) required for a given tube of microsomes on the same day.

REASON FOR CHANGE:

> Revision done on the request by Sponsor.
> This change is made to allow that all required experiments (four protein assays and two aromatase assays) can be conducted using a total of two tubes of microsomes.

PART TO BE CHANGED: Section 3.0, page 10, entire Section

> The protein concentration in the microsomes will be determined each day of microsome use in the aromatase assay (and at other times as appropriate) by using a DC Protein Assay kit purchased from BioRad (Hercules, CA).

A 6-point standard curve will be prepared; target range will be from 0.11 to 1.0 mg protein/mL. The protein standards will be made from bovine serum albumin (BSA). QC standards (0.125, 0.5 and 1.0 mg protein/mL), obtained from Pierce (Rockford, IL) will be run in duplicate with each assay. To a 25 µL aliquot of microsomes solution (1:50 dilution of microsomes may be required) or standard, 125 µL of BioRad DC Protein Kit Reagent A will be added and mixed. Next, 1 mL of BioRad DC Protein Kit Reagent B will be added to each standard or microsomes solution and the samples will be gently mixed. The samples will be allowed to sit at room temperature for at least 15 min to allow color development. (The absorbances are stable for about 1 hour.) Each sample (unknown and standards) will be transferred to disposable polystyrene cuvettes and the absorbance (750 nm) will be measured using a spectrophotometer. The protein concentration of the microsomal sample will be determined.

CHANGE TO:

Low Protein Concentration Assay

Standard curve range: 5-250 µg/mL

Protein Assay Kit: BioRad DC Protein Assay Kit

Prepare standards.

1. Prepare a 2.5 mg/mL solution of bovine serum albumin (BSA) in buffer (preferably using the same buffer as the unknown will be in) by dissolving 25 mg BSA in 10 mL buffer.
2. Prepare a 1:10 dilution of the above solution – yields a 250 µg/mL solution.
3. Prepare a 1:5 dilution of the 250 µg/mL solution – yields a 50 µg/mL solution.

Prepare QC samples.

1. For the 100 µg/mL QC: combine 3.3 mL of the 125 µg/mL standard from the Pierce set with 825 µL buffer.
2. For the 10 µg/mL QC: combine 400 µL of the 100 µg/mL solution with 3.6 mL buffer.
3. Store QC samples refrigerated.

Prepare Standard Curve.

[Protein] µg/mL	µL Buffer	µL 250 µg/mL BSA	µL 50 µg/mL BSA
250	0	200	-
125	100	100	-
50	0	-	200
25	100	-	100
10	160	-	40
5	180	-	20
0	200	-	0

Assay procedure.

1. Pipet 200 µL unknown or QC sample into each tube. (Standard are prepared in tubes as described above).
2. Add 100 µL BioRad DC Reagent A to each tube. Swirl.
3. Add 800 µL BioRad DC Reagent B to each tube. Vortex to mix.
4. Let stand at least 15 min, but less than 1 h for color to develop.
5. Read absorbance of each sample at 750 nm.

Standard and Unknowns are generally run in triplicate. QCs will be run in duplicate.

REASON FOR CHANGE:

Revision done on the request by Sponsor.
The change is made in order to improve the accuracy of the protein determination.

EFFECTIVE DATE: May 25, 2005

APPROVED BY:

Bozena D. Lusiak, Study Director — Date: 05-25-05

Sponsor Representative — Date: 5-25-05

PROTOCOL AMENDMENT NUMBER 2

STUDY NUMBER: **G608316**

STUDY TITLE: **Aromatase Assay Validation: Preparation and Characterization of Human Placental Microsomes (WA 4-16, Task 6)**

PART TO BE CHANGED: Section 2.4.1.3 Placental Microsomes Preparation, page 8, second paragraph, following sentence:

Buffer A will be added to an approximate 2:1 (w: v) ratio and the mixture will be homogenized using a homogenizer.

CHANGE TO:

Buffer A will be added to an approximate 2:1 (v: w) ratio and the mixture will be homogenized using a homogenizer.

REASON FOR CHANGE: Clerical error.

EFFECTIVE DATE: February 9, 2006

APPROVED BY:

Bozena D. Lusiak, Study Director — 02-09-06 Date

Sponsor Representative — 2-9-06 Date

Appendix B

1.0 TITLE AND APPROVAL

Quality Assurance Project Plan (QAPP)
For Work Assignment 4-16
Placental Aromatase Validation Study

Task 6: Prepare/Analyze Microsomes and Conduct Positive Control Study At Two Participating Laboratories; Analyze Microsomes at Each Laboratory

for

EPA CONTRACT NUMBER 68-W-01-023

March, 2005

SIGNATURE PAGE

Quality Assurance Project Plan for WA 4-16, Task 6
Placental Aromatase Validation Study
EPA CONTRACT NUMBER 68-W-01-023

Concurrences and Approvals

Terri L. Pollock, B.A.
EDSP Quality Assurance Manager
Battelle
Columbus, OH

Terri L Pollock 3-21-05
Signature Date

David P. Houchens, Ph.D.
EDSP Program Manager
Battelle
Columbus, OH

JBJ for Dave Houchens 3-21-05
Signature Date

Jerry D. Johnson, Ph.D., DABT
EDSP Work Assignment Leader
Battelle
Columbus, OH

Jerry D. Johnson 3-21-05
Signature Date

Gary Timm, M.S., M.A.
EPA Work Assignment Manager
U.S. EPA
Washington, D.C.

3/21/05
Signature Date

J. Thomas McClintock, Ph.D.
EPA Quality Assurance Manager
U.S. EPA
Washington, DC

3/21/05
Signature Date

Linda J. Phillips, Ph.D.
EPA Project Officer
U.S. EPA
Washington, DC

Linda J Phillips 3/21/05
Signature Date

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3.0 DISTRIBUTION LIST

Debra A. Drissel, B.S.
Quality Assurance Unit Manager
RTI International
3040 Cornwallis Road
Research Triangle Park, NC 27709-2194
919-541-6587

James M. Mathews, Ph.D., DABT
Study Director
RTI International
3040 Cornwallis Road
Research Triangle Park, NC 27709-2194
919-541-7461

Terri Pollock, B.A.
Quality Assurance Program Manager
Battelle
505 King Ave
Columbus, OH 43201
614-424-5883

David P. Houchens, PhD
Program Manager
Battelle
505 King Avenue
Columbus, OH 43201
614-424-3564

Jerry D. Johnson, Ph.D., DABT
Work Assignment Leader
Battelle
505 King Avenue
Columbus, OH 43201
614-424-4499

J. Thomas McClintock, Ph.D.
Quality Assurance Manager
U.S. Environmental Protection Agency
Room 4121-A
1201 Constitution Ave., NW
Washington, DC 20004
202-564-8488

Gary Timm, M.S., M.A.
EPA Work Assignment Manager
U.S. Environmental Protection Agency
EPA East Building
Room 4106-L, Mail Code 7201M
1201 Constitution Ave., NW
Washington, DC 20004
202-564-8474

Linda J. Phillips, Ph.D.
EPA Project Officer
U.S. Environmental Protection Agency
EPA East Building
Room 4106-G, Mail Code 7203M
1201 Constitution Ave., NW
Washington, DC 20004
202-564-1264

Christopher J. Bowman, Ph.D.
Staff Toxicologist, Developmental and
Reproductive Toxicology
WIL Research Laboratories, LLC
1407 George Road
Ashland, OH 44805-9281
(419) 289-8700

Neil S. Jensen, Ph.D.
Director, Technology Development
1450 South Rolling Road
In Vitro Technologies, Inc.
Baltimore, MD 21227
(410) 455-1242

Bozena D. Lusiak, Ph.D.
Principal Research Scientist
PK/TK and ADMET
Battelle
505 King Avenue
Columbus, OH 43201
(614) 424-6310

4.0 PROJECT ORGANIZATION

The U.S. Environmental Protection Agency (EPA) is implementing the Endocrine Disruptor Screening Program (EDSP). To support this program, the EPA has contracted with Battelle to provide comprehensive toxicological and ecotoxicological testing services, including chemical, analytical, statistical, and quality assurance (QA)/quality control (QC) support, to assist EPA in developing, standardizing, and validating a suite of *in vitro*, mammalian, and ecotoxicological screens and tests for identifying and characterizing endocrine effects through exposure to pesticides, industrial chemicals, and environmental contaminants. The studies conducted will be used to develop, standardize and validate methods, prepare appropriate guidance documents for peer review of the methods, and develop technical guidance and test guidelines in support of the Office of Prevention, Pesticides and Toxic Substances regulatory programs. The validation studies will be conducted under the EDSP Quality Management Plan (QMP), study protocols, applicable Quality Assurance Project Plans (QAPPs), relevant program and facility Standard Operating Procedures (SOPs), guidance documents, and Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) Good Laboratory Practice Standards (GLPs).

One of the assays recommended for validation and consideration for inclusion in the screening program is the aromatase assay. A Detailed Review Paper (DRP) was prepared for the U.S. EPA in 2002 to review the scientific basis of the aromatase assay and examine assays reported in the literature used to measure the effect of chemical substances on aromatase.

Prevalidation studies on the aromatase assay (Work Assignment [WA] 2-24) were conducted to optimize the microsomal aromatase assay protocol for human placental microsomes, demonstrate the utility of the microsomal assay to detect known aromatase inhibitors, and compare the performance of a recombinant assay system and the placental microsomal assays. Concerns with this initial work involving high variability in some runs and partial inhibition curves were addressed in a supplemental prevalidation study (WA 4-10).

The objectives of this work assignment are to use the now optimized assay: (1) to obtain intra- and interlaboratory assay variability estimates by conducting experiments at multiple laboratories, (2) to conduct microsome preparation and analysis experiments at multiple laboratories, and (3) to test up to 10 reference chemicals with different modes of action in order to evaluate assay relevance.

This work assignment is composed of multiple studies that will be conducted by the lead laboratory (Research Triangle Institute International [RTI], Research Triangle Park, NC) and three participating laboratories (Battelle, Columbus, OH; In Vitro Technologies, Baltimore, MD; WIL Research Laboratories, LLC, Ashland, OH). This QAPP will address the work to be conducted in Task 6 of the work assignment.

A summary of the work assignment organization is shown in Figure 4-1.

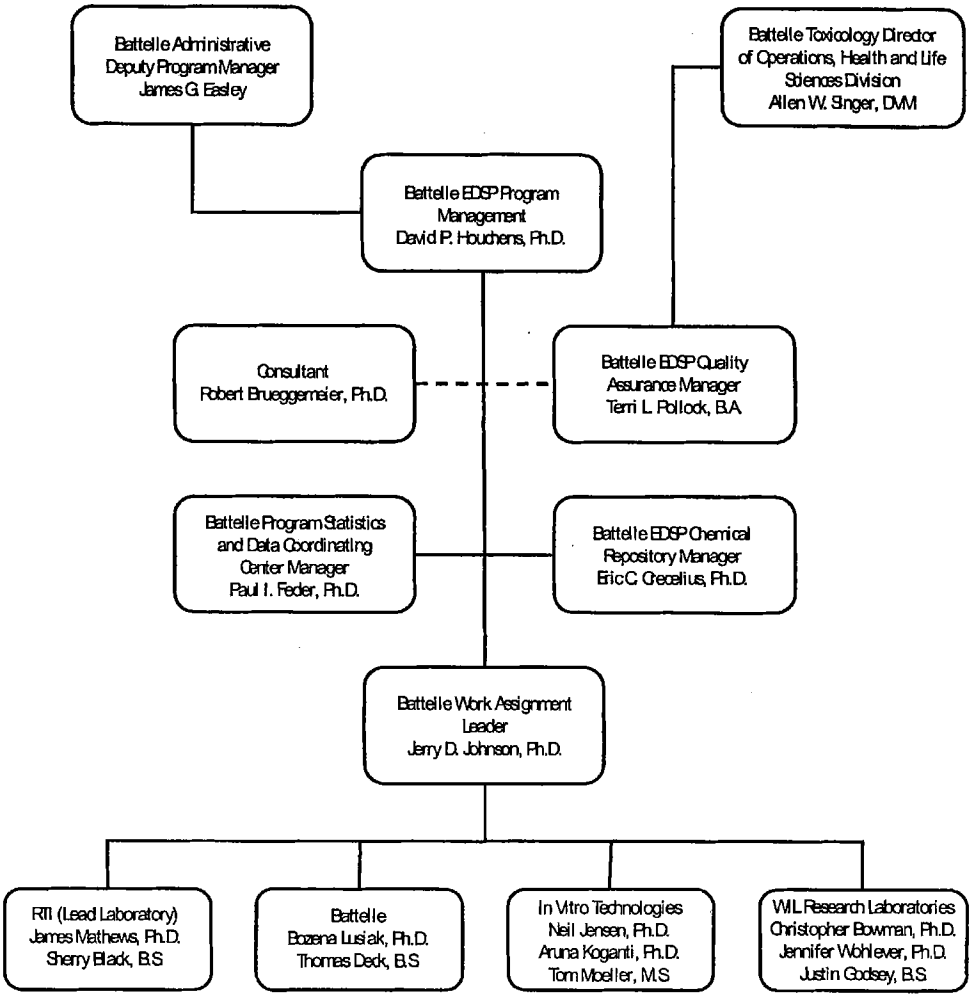

Figure 4-1. WA 4-16 Project Organization Overview

Portions of this work assignment will be managed at RTI, Battelle, WIL, and In Vitro. At each of these laboratories, there will be a person responsible for preparing the protocol, assigning appropriate staff to complete specified tasks within the protocol, and monitoring the progress of both technical and fiscal milestones as outlined in the technical work plan. A Study Director from each laboratory will report on the progress of the work assignment to Drs. David Houchens and Jerry D. Johnson at Battelle through a series of planned conference calls and through the use of written monthly reports.

General scientific direction and supervision of the work performed under this work assignment will be provided by Dr. Jerry D. Johnson, Battelle, and Dr. James Mathews, RTI International. Dr. Johnson will serve as the Work Assignment Leader (WAL) for the participating laboratories and Dr. Mathews for the lead laboratory (RTI).

Each laboratory will have a Study Director in charge of overseeing the daily operation and conduct of the study. The individual laboratory teams will execute the necessary tasks required in the study protocols and ensure the data are collected and handled appropriately. All of these tasks will be clearly defined in the study protocol.

The QAU representative for each laboratory will administer the QAPP for the EDSP facility QA team members. The specific responsibilities will include:

- Interact with the Study Director to ensure that QA and QC procedures are understood by WA personnel.
- Conduct technical systems audits (TSAs) and audits of data quality (ADQs) to evaluate the implementation of the program WAs with respect to the EDSP QMP, the WA QAPPs and/or GLP protocol, and applicable program and facility SOPs.
- Prepare and track reports of deficiencies and submit them to both line and program management.
- Consult with the Study Director and, as necessary, the EDSP Battelle QA Manager and Program Manager on actions required to correct deficiencies noted during the conduct of the WA.
- Ensure that all data produced as part of the EDSP WAs are maintained in a secure, environmentally-protected archive.
- Ensure, during the conduct of TSAs, that all staff participating on the EDSP are adequately trained.
- Maintain complete facility-specific QA records related to the program.

- Submit copies of resolved audits to the EDSP Battelle QA Manager.

- Submit a QA Statement to the EDSP Battelle QA Manager and Program Manager with each written deliverable that describes the audit and review activities completed and any outstanding issues that could affect data quality or interpretation of the results discussed in the report.

- Maintain effective communication with the EDSP QA Manager.

- Act as the facility's EDSP SOP Custodian for all SOPs received from the SOP Administrator.

As EDSP Manager, Dr. David Houchens will have ultimate responsibility for quality, timeliness, and budget adherence for all activities on the contract. He also will serve as the principal interface with the EPA's Project Officer on all contract-level administrative and technical issues. Because of the high level of subcontracting and purchases required by the program, such as test laboratory subcontracts and purchases of chemical supplies, Dr. Houchens will be assisted by an Administrative Deputy Manager, Mr. James Easley. Mr. Easley will manage the procurement of all subcontracts, consultants, and purchased materials and services, and will facilitate schedule and cost control. He has played a similar role on ten other large, multi-year, level-of-effort task-order contracts for EPA. Thus, he will be able to assure that all purchases are compliant with government regulations and that EPA is provided timely, accurate accounting of these substantial costs in our monthly progress reports.

Ms. Terri Pollock, the EDSP QA Manager at Battelle, will direct a team of QA specialists to monitor the technical activities on the chemical repository program, and provide oversight to all associated QA functions. Ms. Pollock will be responsible for reporting her findings and any quality concerns to Dr. Houchens. Ms. Pollock will report, for the purposes of this program, to Dr. Allen W. Singer, Director of Operations in the Toxicology Product Line in Battelle's Health and Life Sciences Division. This reporting relationship will assure that the QA function is independent of the technical activities on the program.

5.0 PROBLEM DEFINITION/BACKGROUND

5.1 Problem Definition

Prevalidation studies on the placental aromatase assay (WA 2-24) were conducted to optimize the microsomal aromatase assay protocol for human placenta, demonstrate the utility of the microsomal assay to detect known aromatase inhibitors, and compare the performance of a recombinant assay system and the placental microsomal assays. Concerns with this initial work

involving high variability in some runs and partial inhibition curves were addressed in a supplemental prevalidation study (WA 4-10).

With the prevalidation studies successfully completed, this work assignment directs Battelle to conduct the interlaboratory studies to determine the performance of several laboratories in conducting the assay and should complete the validation of the placental aromatase assay. A companion work assignment (WA 4-17) has been issued for the conduct of the recombinant aromatase assay.

The work assignment is comprised of 9 tasks of which five tasks involve experimentation. Task 3 is a training task. The work in Tasks 4 through 7 is described in this QAPP. Table 5-1 summarizes the validation tasks and the laboratory(ies) involved for each experimental task.

Table 5-1. Validation Study Plan Experiments

Task Number	Description of Experimental Task	Experimental Task Assignment
1	Not applicable (develop work plan, study plan, and identify/select participating laboratories)	Not an experimental task
2	Not applicable (develop QAPP and protocols)	Not an experimental task
3	Training Participating Laboratories in the Conduct of the Assay	Lead Laboratory + 3 Participating Laboratories
4	Conduct Positive Control Studies in the Participating Laboratories	3 Participating Laboratories
5	Conduct Multiple Chemical Studies with Centrally Prepared Microsomes (RTI/Participating Laboratories)	Lead Laboratory + 3 Participating Laboratories
6	Two Labs: Procure Placenta/Prepare & Analyze Microsomes/Determine Protein Concentration and Aromatase Activity/Determine 4-OH ASDN Inhibition Response/Distribute Microsomes to Labs All Labs: Using Microsomes Rec'd, Determine the Protein Concentration and Aromatase Activity	Lead Laboratory + 3 Participating Laboratories
7	Conduct Multiple Chemical Studies with Microsomes Prepared in Participating Laboratories (RTI/Participating Laboratories)	Lead Laboratory + 3 Participating Laboratories
8	Prepare Study Reports (RTI/Participating Laboratories)	Not an experimental task
9	Prepare Presentation for EDMVAC*	Not an experimental task

*EDMVAC = Endocrine Disruptor Method Validation Committee

5.2 Background

The Food Quality Protection Act of 1996 was enacted by Congress to authorize the EPA to implement a screening program on pesticides and other chemicals found in food or water sources for endocrine effects in humans. Thus, the U.S. EPA is implementing an EDSP. In this program, comprehensive toxicological and ecotoxicological screens and tests are being developed for identifying and characterizing the endocrine effects of various environmental contaminants, industrial chemicals, and pesticides. The program's aim is to develop a two-tiered approach, e.g., a combination of *in vitro* and *in vivo* mammalian and ecotoxicological screens (Tier 1) and a set of *in vivo* tests (Tier 2) for identifying and characterizing endocrine effects of pesticides, industrial chemicals, and environmental contaminants. Validation of the individual screens and tests is required, and the EDMVAC will provide advice and counsel on the validation assays.

Estrogens are sex steroid hormones that are necessary for female reproduction and affect the development of secondary sex characteristics of females. Estrogens are biosynthesized from cholesterol by a series of enzymatic steps, with the last step involving the conversion of androgens into estrogens by the enzyme aromatase. Estrogen biosynthesis occurs primarily in the ovary in mature, premenopausal women. During pregnancy, the placenta is the main source of estrogen biosynthesis and pathways for production change. Small amounts of these hormones are also synthesized by the testes in the male and by the adrenal cortex, the hypothalamus, and the anterior pituitary in both sexes. The major source of estrogens in both postmenopausal women and men occurs in extraglandular sites, particularly in adipose tissue. One potential endocrine target for environmental chemicals is the enzyme aromatase, which catalyzes the biosynthesis of estrogens. An aromatase assay is proposed as one of the Tier 1 Screening Battery Alternate Methods. A detailed literature review on aromatase was performed and encompassed (1) searching the literature databases, (2) contacting individuals to obtain information on unpublished research, and (3) evaluating the literature and personal communications.

Aromatase is a cytochrome P450 enzyme complex responsible for estrogen biosynthesis and converts androgens, such as testosterone and androstenedione, into the estrogens estradiol and estrone. Aromatase is present in the ovary, placenta, uterus, testis, brain, and extraglandular adipose tissues. Two proteins, cytochrome P450arom and NADPH-cytochrome P450 reductase, are necessary for enzymatic activity, and the enzyme complex is localized in the smooth endoplasmic reticulum. The aromatase gene, designated CYP19, encodes the cytochrome P450arom and consists of 10 exons, with the exact size of the gene exceeding 70 kilobases. Aromatase is found in breast tissue, and the importance of intratumoral aromatase and local estrogen production is being unraveled. Effective aromatase inhibitors have been developed as therapeutic agents for estrogen-dependent breast cancer to reduce the growth stimulatory effects of estrogens in breast cancer. Investigations on the development of aromatase inhibitors began in the 1970's and have expanded greatly in the past three decades.

An *in vitro* aromatase assay could easily be utilized as an alternative screening method in the Tier 1 Screening Battery to assess the potential effects of various environmental toxicants on aromatase activity. Both *in vitro* subcellular (microsomal) assays and cell-based assays are available for measuring aromatase activity. The *in vitro* subcellular assay, using human placental microsomes, is commonly used to evaluate the ability of pharmaceuticals and environmental chemicals to inhibit aromatase activity. In addition, human JEG-3 and JAR choriocarcinoma cell culture lines, originally isolated from cytotrophoblasts of malignant placental tissues, have been used as *in vitro* systems for measuring the effects of compounds on aromatase activity. These cell lines are also utilized for investigations on the effects of agents in placental toxicology.

Numerous flavonoids and related phytoestrogen derivatives have been extensively evaluated for their ability to inhibit aromatase activity for two primary reasons: (1) these natural plant products can serve as possible leads for the development of new nonsteroidal aromatase inhibitors; and (2) humans and other animals are exposed to these agents through the diet. In general, the flavonoids and related analogs demonstrate aromatase inhibition with IC_{50} values in the micromolar range; however, these compounds lack both the potency and specificity of aromatase inhibitors developed for breast cancer therapy. Several pesticides have also demonstrated inhibition of aromatase activity in the human placental microsomal assay system, with IC_{50} values for aromatase inhibition ranging from 0.04 mM to greater than 50 mM.

The human placental microsomal aromatase assay was recommended as the *in vitro* aromatase screening assay to be included in the Tier 1 Screening Battery. This assay will detect environmental toxicants that possess the ability to inhibit aromatase activity. Prevalidation studies on recombinant aromatase (WA 2-24) were conducted to optimize the microsomal aromatase assay protocol for human placenta, demonstrate the utility of the microsomal assay to detect known aromatase inhibitors, and compare the performance of a recombinant assay system and the placental microsomal assays. Concerns with this initial work involving high variability in some runs and partial inhibition curves were addressed in a supplemental prevalidation study (WA 4-10). The objective of the current work assignment is to use the now optimized assay to obtain intra- and interlaboratory assay variability estimates to complete the validation of the human placental microsome aromatase assay.

6.0 PROJECT/TASK DESCRIPTION

Only Task 6 is under the control of this QAPP. However, this QAPP also addresses the other three experimental tasks in this work assignment and will be reissued prior to the start of each new task together with a finalized task-specific template protocol included as an attachment. The Task 6 template protocol is attached to the present QAPP. The task numbering scheme for the original work assignment is employed in this document for ease of cross-referencing.

Task 4: Conduct Positive Control Studies in the Participating Laboratories

This task was completed by staff at Battelle, WIL and In Vitro. RTI staff did not conduct any experiments on this task but were involved in the review of the data produced by the other laboratories. RTI provided human placental microsomes to the other laboratories for use in this task. Battelle/RTI provided a boilerplate protocol for this task to the participating laboratories which they used to prepare their laboratory-specific protocols. These protocols contained all necessary technical detail for the conduct of this task. Briefly, the task required that each laboratory conduct three independent replicates of a Positive Control Study. In this Study, 4-OH androstenedione (4-OH ASDN, a known aromatase inhibitor) was tested in the aromatase assay at 6 concentrations to construct a dose/response curve from which an IC_{50} was calculated. Control runs also were included in the assay set to measure full aromatase activity (without any inhibitor added) and background activity (without NADPH co-factor). Battelle's Chemical Repository (CR) supplied 4-OH ASDN to each laboratory as a stock solution and conducted all necessary pre-assay chemistry activities for 4-OH ASDN.

Each laboratory presented their results in a separate spreadsheet for each of the three replicates and the results were compared both within and between laboratories.

The results of this experiment required technical review and approval prior to proceeding to Task 5.

Task 5: Conduct Studies with Centrally Prepared Microsomes

This task was completed by staff at RTI, Battelle, WIL and In Vitro. RTI provided human placental microsomes to the other laboratories for use in this task. Battelle/RTI provided a boilerplate protocol for this task to the participating laboratories, which they used to prepare their laboratory-specific protocols. These protocols contained all necessary technical detail for the conduct of this task. Briefly, the task required that each laboratory conduct three independent replicate studies on each of four test chemicals. All three replicates for a given chemical were conducted by the same technician within a laboratory. Control runs were also included in each assay set to measure full aromatase activity (without any inhibitor added) and background activity (without NADPH co-factor). In addition, positive control samples (containing a known aromatase inhibitor) and negative control samples (containing a known aromatase non-inhibitor) were included in each assay set. Battelle's CR supplied the test and control chemicals to each laboratory as individual stock solutions and conducted all necessary pre-assay chemistry activities for the test and control chemicals.

Each laboratory presented their results in a separate spreadsheet for each of the three replicates and the results were compared both within and between laboratories.

The results of this experiment required technical review and approval prior to proceeding to Task 7.

Task 6: Prepare Microsomes in Two Participating Laboratories

There will be two activities in this task. The first, to be conducted by Battelle and In Vitro, will require those laboratories to obtain a human placenta, prepare microsomes and then to analyze their microsome preparations for protein content and (uninhibited) aromatase activity. In addition, those laboratories will conduct two independent replicates of the Positive Control Study (as used in Task 4) using their microsomal preparations. RTI/Battelle will supply a template protocol that includes all technical detail required for the conduct of these experiments. Battelle's CR will supply 4-OH ASDN to each laboratory as a stock solution. The laboratories will submit the results of these studies to Battelle and the data will be reviewed by Battelle and RTI prior to submission to EPA. After EPA approves the results, the second portion of the task can be initiated.

For the second activity in this task, Battelle and In Vitro will each ship portions of their placental microsome preparations to the other three participating laboratories. All laboratories will measure the protein content and (uninhibited) aromatase activity of the microsomal preparations received from both laboratories.

Each laboratory will present their results in a separate spreadsheet for each replicate and the results will be compared both within and between laboratories.

Task 7: Conduct Studies with Microsomes Prepared in Participating Laboratories

Battelle and In Vitro will conduct the studies in this task with microsomes prepared in their laboratory in Task 6. RTI and WIL will receive microsomes from Battelle and In Vitro, respectively, for use on this task.

RTI/Battelle will supply a template protocol describing all technical details for this task to the participating laboratories from which they will prepare their laboratory-specific protocols. Each laboratory will conduct three independent replicate studies with each of 10 chemicals. All three replicates for a given chemical will be conducted by the same technician within a laboratory. Control runs will also be included in each assay set to measure full aromatase activity (without any inhibitor added) and background activity (without NADPH co-factor). In addition, positive control samples (containing a known aromatase inhibitor) and negative control samples (containing a known aromatase non-inhibitor) will be included in each assay set. Battelle's CR will supply the test and control chemicals to each laboratory as individual stock solutions and will conduct all necessary pre-assay chemistry activities for the test and control chemicals.

7.0 QUALITY OBJECTIVES AND CRITERIA

There are several critical components to the aromatase assay. Criteria for acceptance of each of these components are described below.

7.1 INCUBATION TEMPERATURE

The water bath for incubation of aromatase assay tubes will be held at 37 ± 1°C.

7.2 PLACENTA ACCEPTANCE CRITERIA

Human placentas will be obtained from a 21 to 40 year old nonsmoker, with a full term delivery. The tissue will be placed on ice within 30 min of delivery in order to preserve aromatase activity.

7.3 MINIMUM AROMATASE ACTIVITY IN NEW PLACENTAL PREPS

The minimum acceptable aromatase activity in human placenta microsomes will be set at 0.03 nmol product/mg protein/min. If the aromatase activity for any human placenta microsomal preparation is below the minimum acceptable level, then this preparation will not be used in further studies. In this case, new microsomal preparations will be made from additional placenta(s). If it becomes necessary to combine microsomes from two (or more) placentas in order to have enough placental protein for the conduct of the studies, the lots will be thawed, combined in a single vessel and rehomogenized using a Potter Elvejhem homogenizer. The combined, homogenized preparation will be divided into assay-appropriate volumes, flash frozen in liquid nitrogen and stored at -70 to -80°C.

7.4 MINIMUM PROTEIN YIELD FROM NEW PLACENTAL PREPS

It is essential that, for each microsomal preparation, enough protein be on hand for all of the planned studies. The microsomal preparations will also demonstrate acceptable aromatase activity.

It is anticipated that ca 200 to 250 mg of protein from each microsomal preparation will be necessary to run all of the proposed human placental aromatase studies. Therefore, if less than that amount is available on-hand, additional placental microsomes will be prepared until sufficient protein is obtained. If microsomes from more than one placenta are to be used, they will be combined and rehomogenized to make a single pooled sample.

7.5 Data Quality Indicators

7.5.1 Precision

The activities of replicate tubes will be within the mean activity ± 15%. Each control activity for each assay/laboratory will be within the overall mean ± 15% activity for that control type for that laboratory.

Variance between laboratories and within laboratories will be assessed for an appropriate level of precision as part of this WA. It is anticipated that full aromatase control activity between and within laboratories will be statistically equivalent at the $p > 0.1$ level. Any modifications to this criterion will be discussed with the sponsor and added to the QAPP by amendment.

IC_{50} and slope values calculated for each inhibitor will be statistically equivalent at the $p>0.1$ level both between and within laboratories. If data from an assay are statistical outliers, the assay will be repeated.

7.5.2 Bias

The control samples that are run with each assay will be used to control for bias. If the control samples for any assay do not meet the precision criteria described above, the assay will be rerun. Assays will be conducted blind at the technician level for test chemical identity.

7.5.3 Accuracy

Accuracy of the liquid scintillation spectrometry (LSS) data (from which is derived the aromatase activity) will be assessed by analysis of a sealed standard of known radioactive content. If the radioactivity in the sealed standard is more than 5% different from the known value, the data will not be used. Samples will be recounted on another LSS or on the same LSS after any problems with the instrument are corrected.

8.0 SPECIAL TRAINING/CERTIFICATION

All personnel involved in handling radiolabeled materials will have completed a Radiation Safety Training course. Training documentation will be maintained in the individual training files. Each laboratory will be licensed to receive radiolabeled materials.

All personnel involved in handling human placental and human placental microsomes will have appropriate training in the handling and disposition of biohazards. Training documentation will be maintained in the individual training files.

Staff from the participating laboratories have been trained on the performance of the aromatase assay at RTI International as part of Task 3 of this Work Assignment. Personnel participating in this training conducted the aromatase assay including full aromatase control and background control samples and a series of samples containing varying amounts of a known aromatase inhibitor (4-OH ASDN). The resultant data was evaluated by Battelle and RTI International and then submitted to EPA for review.

9.0 DOCUMENTS AND RECORDS

9.1 Retention of Specimens and Records

Archiving procedures will be specified in the individual protocols.

9.2 Quality Assurance Project Plan

This QAPP will be distributed to project participants initially, and whenever revised. Previous versions will be marked as "obsolete" when newer versions are distributed, or collected and destroyed so that there is no confusion regarding the version in effect. The right-justified document control header example shown here

Version 1
Month, Year
Page 1 of 1

will be used to ensure that revision numbers and dates are obvious to document users. The QAPP will be reviewed annually and a determination made to either modify the document based on new or modified project requirements, or leave as is.

Controlled copies of the QAPP will be maintained, tracked, and managed by the laboratories' QAU through the use of a master distribution list.

9.3 Data Forms

All data forms will include a title identifying the type of data to be recorded, a unique study code or protocol number, and the initials and date of the data recorder(s) to authenticate the records.

Corrections to data entries will be made by drawing a single line through the error, recording the correct entry, initials, date, and error code that explains the reason for the correction.

9.4 Microsome Storage Conditions

Microsomes will be stored at -70 to -80°C and the freezer temperature records will be maintained.

9.5 Reports

9.5.1 Interim Data Summary, and Draft and Final Reports

An interim data summary from each laboratory will be submitted to the EPA after completion of each task. These data summaries will not be audited by Quality Assurance but will be checked for accuracy by technical staff. This procedure is necessary to provide a rapid turn around of the data so that approval to proceed can be given by EPA.

Each laboratory will prepare an individual report for each task to be based on a template provided by Battelle and will submit these reports to Battelle. The purpose of these reports is to provide a complete description about how the experiments were performed, present the results that were obtained (including tables and graphs), and state the conclusions that were made for each applicable WA task. RTI/Battelle will prepare a report for each task that summarizes all work on the particular task and incorporates the reports from the participating laboratories as Appendices for submission to EPA. After EPA comments have been received on each task report and, if applicable, incorporated into a new version of the draft task report, then it will be issued as a final report.

Each final task report will include:

- Abstract
- Objectives
- Materials and Methods
- Results
- Discussion
- Conclusions
- References
- Summary data with statistical analyses
- Appendices which will include final reports with compliance statements for each participating laboratory
- Protocol, any amendments, or any deviations from the protocol
- QAPP, any amendments, or any deviations from the QAPP.

RTI/Battelle will prepare a final Work Assignment report that summarizes the results of the entire Work Assignment. This report will consist of a statement of the objectives of the work

assignment, a summary of the results and a statement of conclusions for the Work Assignment. The individual task reports will be referenced within this final report.

9.5.2 QA Assessment Reports

QA assessment reports will be maintained as confidential files in the QAU.

9.5.3 Status Reports

Status/progress reports will be submitted to the EPA Project Officer by Battelle on a monthly basis as stipulated in the contract.

10.0 SAMPLING PROCESS DESIGN (EXPERIMENTAL DESIGN)

The details of the experimental design for the task subject to this QAPP will be contained in a GLP compliant protocol. A template protocol for this task is attached as an Appendix to this document.

11.0 SAMPLING METHODS

The entire aqueous portion of the incubation mixtures remaining after extraction with methylene chloride (CH_2Cl_2) will be placed in appropriate containers. The samples will be mixed well prior to the removal of aliquots for liquid scintillation counting (LSC). If there is insufficient time for preparing LSC samples on the day the assay is run, the samples will be refrigerated overnight. Samples remaining after preparation of LSC aliquots will be frozen and stored at about -20°C. These samples will be thawed, mixed and realiquoted, if necessary, due to problems with LSC samples.

Each test and standard chemical will be supplied to the participating laboratories by Battelle as a stock solution at the highest concentration necessary for use in the assay. These solutions will be well-mixed prior to the preparation of dilutions of these stock solutions by the individual participating laboratories.

12.0 SAMPLE HANDLING AND CUSTODY

12.1 Test and Reference Chemical Solutions

The test and standard chemical stock solutions will be transferred to the Laboratories' Material Handling Facility with a study specific transfer of material form. The samples will be processed according to the SOPs for packing, shipment and documentation of shipment and receipt.

12.2 Sample Collection Documentation

All samples (or sample sets) will be labeled with enough information to allow for unequivocal identification of each sample along with suitable storage conditions in accordance with applicable regulations.

13.0 ANALYTICAL METHODS

Analytical methods are described in the study protocol (Appendix). Failures of analytical systems are addressed in the relevant SOPs.

14.0 QUALITY CONTROL

14.1 Methods

Control samples will be run with each assay. These include: (1) full aromatase enzyme activity controls, (2) background controls, (3) positive controls and (4) negative controls. Acceptance criteria and corrective actions where acceptance criteria are not met are described in Section 7. Replicates will be used as a means to monitor variability of the assay. Replicates will be assessed for variance and those that are outside the acceptable range (mean ± 15%) will be flagged as statistical outliers.

14.2 Data Collection

Data collection documentation will be as described in applicable SOPs or protocols.

Assay data, including weights and/or volumes of chemicals, solvents or other materials used to prepare necessary solutions or samples, will be recorded manually on data sheets. Protein assay absorbance data will also be recorded manually on data sheets. All data sheets will include a title identifying the type of data to be recorded, the unique study code or protocol number, and the initials and date of the data recorder(s) to authenticate the records.

Scintillation counter data will be automatically saved to a data file that will automatically be assigned a unique filename. The data will be annotated to identify samples with the sequential vial number. Procedures for converting CPM data to DPM data will be documented.

Relevant data from the data sheets and scintillation counter output (as DPM) will be typed into a validated MS Excel spreadsheet for calculation of (1) substrate specific activity (2) protein content and/or (3) aromatase activity. All transcribed data will be verified (100% QC) before they are reported and this QC check will be documented on the spreadsheet printouts by technician initials and date.

Aromatase activity data will be entered automatically (through linked validated spreadsheets) or manually into Prism data files for calculation of IC_{50}. Data will be entered automatically (through linked validated spreadsheets) or manually into spreadsheets for import into SAS data files for statistical analysis. All manually entered data will undergo a 100% QC check.

15.0 INSTRUMENT/EQUIPMENT TESTING, INSPECTION, AND MAINTENANCE

The following types of equipment will be required for this WA: temperature controlled shaking water bath, pH meter, analytical balances, centrifuges (low and high speed and ultracentrifuges), pipettors, scintillation counters, spectrophotometer, and high performance liquid chromatography (HPLC) equipment (injector, pumps, detectors [radiochemical and ultraviolet {UV}], data collection system). The equipment will be tested, inspected and maintained according to schedules contained in the relevant SOPs.

16.0 INSTRUMENT/EQUIPMENT CALIBRATION AND FREQUENCY

Balances used to obtain weight measurements, as well as the check weights that are used to verify a balance's calibration status, will be calibrated and maintained according to the schedule specified in relevant SOPs. Balances that do not meet the criteria specified in the SOP will not be used for this work assignment.

Scintillation Counters will be calibrated using procedures described in the relevant SOPs. Calibration of pH meters will occur as specified in relevant SOPs. The water bath, pipettes, spectrophotometer, and HPLC equipment will be calibrated using the procedures and schedule in applicable SOPs. Any equipment or instrument that does not meet acceptance criteria as described in the relevant SOP will not be used for this work assignment.

17.0 INSPECTION/ACCEPTANCE OF SUPPLIES AND CONSUMABLES

Upon receipt, purchased items will be inspected for conformance to quality requirements prior to use. All use of the product will be prior to the expiration dates, if applicable. Chemicals will be received and stored in accordance with applicable SOPs.

18.0 NON-DIRECT MEASUREMENTS

No collection of any samples or sample data will be obtained from non-direct measures such as computer data bases or programs.

19.0 DATA MANAGEMENT

19.1 Data Management Overview

Data will be maintained in notebooks and/or files according to applicable facility SOPs. The records will be kept in the appropriate rooms until there is a signed final report at which time they will be inventoried and placed in the facility archives according to applicable facility SOPs, unless the sponsor requests that they be transferred to another archive location.

19.2 Data Transfer

Information will be sent to the Data Coordination Center in electronic format as specified in SOP EDSP.D-003-01. Specifically all raw data, all tables, graphs summarizing results of statistical analyses as presented in study reports, statistical analysis data files, statistical analysis programs, and all study documents will be sent to the EDSP Data Coordination Center in electronic format.

20.0 ASSESSMENTS AND RESPONSE ACTIONS

EDSP QA team members will perform assessments on WA activities and operations affecting data quality and the raw data and final report. They will report any findings to the Study Director and management to ensure that the requirements in relevant SOPs, study protocols and WA QAPP, the QMP, and the FIFRA GLPs are met. The assessments for this study will include TSAs and ADQs. Performance Evaluations will not apply to this QAPP.

20.1 Technical Systems Audits

A TSA is a process by which the quality of a study is assessed through evaluating a study activity's conformance with the protocols, applicable facility or program SOPs, QAPP, QMP, and GLPs. The acceptance criteria will be that WA activities and operations must meet the requirements of these planning documents and the GLPs or be explained and evaluated in a deviation report. Deviations from the GLPs, QAPP, protocol, or SOPs will be properly documented and assessed by management and the Study Director as to their impact on the study.

20.2 Type, Scheduling, and Performance of Technical Systems Audits

The following paragraphs provide an example of how the laboratories will perform technical system audits.

Prior to the experimental start, the facility QA Team Member will convey a list of inspections targeted for the study to the Study Director. Whenever possible, TSAs will be done at the commencement of the WA critical phase to ensure WA integrity based on compliance with

the protocol, QAPP, SOPs, and GLPs. Critical phases targeted for TSAs will include, but are not limited to:

- Protocol review
- Placental collection and microsome preparation
- Aromatase assay sample preparation and analysis.

During the TSA, EDSP QA team members will record observations to be used later in preparing the audit report. EDSP QA team members will observe the procedure, data recording, and any equipment maintenance and calibration procedures and/or documentation, noting whether or not the activities adhered to the study protocols and QAPP, applicable SOPs, QMP, and the GLPs. Any findings will be communicated to the technical personnel at the completion of the procedure unless an error could compromise the study (e.g., misdiluting the stock solution). EDSP QA team members will immediately notify the Study Director by telephone and/or e-mail of any adverse findings that could impact the conduct of the study. This direct communication will also be documented in the audit report.

20.3 Audits of Data Quality

An ADQ is a process by which the accuracy of data calculations and reporting will be assessed to ensure that the reported results are of high quality and accurately reflect the raw data and accurately describe the materials used in the study. The acceptance criteria for the ADQ will be that data collection, analysis, and reporting must meet the requirements of the applicable facility and program SOPs, the WA protocols and QAPP, QMP, and the FIFRA GLPs, or be explained and evaluated in a deviation report, as previously described.

20.4 Scheduling and Performance of Audits of Data Quality

Direct and frequent communication between the WA Leader/Study Director, laboratory supervisor, and the QA Manager will provide for sufficient time to perform an ADQ so that the submission date of the draft final report meets that specified in the study protocol. The scheduling process will also allow for a reasonable amount of time for corrections and subsequent verification of the corrections by QA.

EDSP QA team members will audit the study records at a frequency adequate to ensure that approved protocol requirements are met. The frequency required is specified by the type of data in the QMP, Section 2.4.1. Findings will be reported and corrective actions undertaken as described earlier. EDSP QA team members will review the final report using the audited data and corrected tables. The report text will be reviewed to ensure that every statement is supported by the data and any discussions or conclusions drawn from the study are supported by the data. Findings will then be reported and corrective actions undertaken as described earlier.

20.5 Audit Report Format

The following paragraphs provide an example of how the laboratories will format an audit report.

The audit report will consist of a cover page for study information and additional page(s) with the audit findings. All pages will have header information containing the study protocol number, audit report date, and audit type. The audit report date will be the date on which the EDSP QA team member signs the audit report and sends it to the Study Director and management.

The cover page will contain the study protocol title, number, and code; Sponsor; Study Director; audit type; audit date(s); EDSP QA team member; distribution list; the dated signature of the auditor; the date that the Study Director received the audit report; and the dated signatures of the Study Director and management. The distribution list will include additional names for individuals who have findings pertaining to their area of responsibility (e.g., the ARF Manager would address a finding pertaining to the ARF) and is used to ensure that the report is sent to all who need to respond. Subsequent page(s) will contain the audit finding(s), any recommended remedial actions, and space for the Study Director to respond to the findings and document remedial actions taken or to be taken.

20.6 Response Actions and Resolution of Issues

The Study Director will respond to the TSA report within a specified number of working days of receipt of the report as required by the laboratory's SOPs. There will be no deadline for the Study Director's response to an ADQ report except for the time constraint deriving from the submission date of the final WA report. The Study Director will forward the audit report to management for review. Management will add comments as necessary, sign and date the report and return it to the EDSP QA team member. The EDSP QA team member will assess the responses and verify the corrective actions. If a disagreement between the Study Director and EDSP QA team member arises over a finding, it will be discussed among the other EDSP QA team members. The EDSP QA team member will then present the majority opinion to the Study Director for further consideration. If the disagreement remains, the issue will be reported to the Study Director's management. The action decided on by management will be documented in the QA files.

During an assessment, if the auditor determines that adverse health effects could result or WA objectives of acceptable quality cannot be achieved, the auditor will follow the Stop Work Procedure specified in the EDSP QMP (Section 3.3).

20.7 Independent Assessments

The EDSP Battelle QA Manager (QAM), or designee, will conduct an independent TSA and ADQ during the conduct of this work assignment. Typically one independent audit will be conducted during the work assignment. If major deficiencies are uncovered, additional independent audits will be scheduled. The conduct and reporting of the audits will be consistent with the procedures described in the EDSP QMP (Section 3.3).

In addition, the EDSP EPA QAM, or designee, will have the option of conducting external TSAs/ADQs.

21.0 REPORTS TO MANAGEMENT

The QA Manager will send periodic reports to the Study Director and management, which detail significant regulatory, protocol, and SOP issues. Also, the participating laboratories will report to the EDSP Program Manager and WAL.

22.0 DATA REVIEW, VERIFICATION, AND VALIDATION

The data produced under this work assignment will be reviewed by the technical personnel for the validation process and by EDSP QA team members for the verification process (see section 23). The criteria used for validation will depend on the type of data. For dose solution sample data, information regarding the condition of the containers and whether or not samples were compromised will be recorded in the sample chain-of-custody records. Compromised samples will not be analyzed. The criteria for validating data are those found in Section 7 (Quality Objectives and Criteria).

23.0 VERIFICATION AND VALIDATION METHODS

23.1 Chain of Custody for Data

Study data, records, and specimens will be maintained in a secure and designated location, e.g., in the respective laboratory offices until study completion. Chain-of-custody procedures will be implemented according to facility SOPs. Chain-of-custody information, including the date, study record(s) removed or returned, and the name of the person removing or returning the data will be documented. At study completion, the Study Director will follow the procedures specified in the facility SOP for archiving study materials.

23.2 Data Validation

Data validation is a process by which the WA Leader/Study Director and/or other technical personnel evaluate the data for conformance to the stated requirements for methodology

and quality. These personnel will be responsible for reviewing the data, evaluating any technical deviations or non-conformances, and then determining the degree to which the data meet the quality criteria stated in Section 7.

23.3 Data Verification

Data verification will constitute part of the ADQ process performed by EDSP QA team members and described earlier. Verification will ensure that (1) the data are of high quality and were collected according to the planning documents' requirements, and (2) the reported results accurately reflect the raw data. Each data type will be evaluated against its collection and reduction requirements specified in the planning documents. Errors discovered during the data evaluation will be corrected. The reported conclusions drawn from the data will be verified by EDSP QA team members during the report audit to confirm that they are true and accurate. The procedure for resolving issues of data verification has been detailed in prior sections of this document.

24.0 RECONCILIATION AND USER REQUIREMENTS

Proposed methods for data analysis, including a test for statistical outliers, will be specified in the Study Plan and/or protocols.

25.0 REFERENCES

The following references were used to prepare the QAPP. Not all references are cited in the text.

Battelle (2003). Endocrine Disruptor Screening Program Quality Management Plan, Version 2. May 12, 2003.

Battelle (2004). Technical Work Plan on Microsomal Aromatase Validation Study, EPA Contract Number 68-W-01-023, Work Assignment 4-16. September 8, 2004.

FQPA (1996). Food Quality Protection Act of 1996, U.S. Public Law 104-170, 21 U.S.C. 46a(p), Section 408(p), 110 STAT.1489. August 3, 1996.

APPENDIX A

TEMPLATE PROTOCOL FOR TASK 6

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EPA Contract No.: 68-W-01-023
EPA Work Assignment No.: 4-16
Task Number: 6

TITLE: Prepare/Analyze Microsomes and Conduct Positive Control Study at Two Participating Laboratories; Analyze Microsomes at Each Laboratory

SPONSOR: Battelle, 505 King Avenue, Columbus, OH 43201

TESTING FACILITY:

PROPOSED EXPERIMENTAL START DATE:

PROPOSED EXPERIMENTAL END DATE:

AMENDMENTS:

Number	Date	Section(s)	Page(s)
1			
2			
3			

Approved By:

James M. Matthews, Ph.D. DABT — Date
RTI Study Director

Jerry D. Johnson, Ph.D., DABT — Date
Work Assignment Leader (WAL), Battelle

Gary Timm, M.S., M.A. — Date
EPA Work Assignment Manager

David P. Houchens, Ph.D. — Date
EDSP Program Manager, Battelle

Linda J. Phillips, Ph.D. — Date
EPA Project Officer

Reviewed By:

Kim Collier, B.A. — Date
RTI, Quality Assurance Specialist

Terri L. Pollock, B.A. — Date
EDSP Quality Assurance Manager, Battelle

J. Thomas McClintock, Ph.D. — Date
EPA Quality Assurance Manager

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1.0 OBJECTIVES

Task 6: Prepare/Analyze Microsomes and Conduct Positive Control Study at Two Participating Laboratories; Analyze Microsomes at Each Laboratory

The objectives of this protocol are to describe procedures for the preparation of human placental microsomes, the analysis of microsomal preparation for protein content and uninhibited aromatase activity, and the conduct of a study with the known aromatase inhibitor 4-hydroxy-androstenedione (4-OH ASDN) to demonstrate the responsiveness of the assay to an aromatase inhibitor. This task is to be conducted in two stages as described below.

1.1 Stage 1 - Placental Procurement/Microsomal Preparation and Characterization/ Positive Control Study

In Stage 1, only two of the four laboratories will perform these activities – procurement of the placenta, preparation and characterization of microsomes, and conduct of a positive control study with the microsomes.

Battelle's Endocrine Disruptor Screening Program (EDSP) Office selected two laboratories, Battelle and In Vitro, and assigned the activities of Stage 1 to them. These two laboratories were selected because of their proximity to and previous working experience with nearby teaching hospitals and large population areas. The third laboratory, WIL, is going to investigate the feasibility of obtaining a human placenta in case one of the other two laboratories is unable to obtain a placenta.

In addition to procuring a placenta and preparing microsomes, these two laboratories will determine the protein concentration and aromatase activity (uninhibited) of the microsomes that they prepared. In addition, they will run two independent replicates of a study to determine the response of the microsomal aromatase to 6 concentrations of 4-OH ASDN using their own microsomal preparations. These activities (from placental procurement to completion of the positive control assay) are described in detail in other sections in this protocol.

The data from these studies will be sent to Battelle's EDSP Program Office and, together with staff at RTI, the data will be reviewed prior to submission to the Environmental Protection Agency (EPA). Approval from the EPA Work Assignment Manager (WAM) will be obtained before the labs can proceed to Stage 2.

1.2 Stage 2 - Distribution of Microsomes and Conduct of Aromatase Activity Studies

In Stage 2, the two labs that procured/prepared and characterized the microsomes in the first stage will distribute their microsomes to the lead lab (RTI) and other participating laboratories, i.e., Battelle will distribute microsomes to In Vitro, RTI, and WIL, whereas In Vitro will distribute microsomes to Battelle, RTI, and WIL. In this way, each laboratory will use

microsomes prepared by both laboratories in their tests. Battelle and In Vitro will include with the shipped microsomes the protein concentration and aromatase activity determinations. Upon receipt of the microsomes, each laboratory will determine for themselves the protein concentration and aromatase activity (uninhibited). From these experiments, comparisons between microsome preparations will be carried out within laboratories and comparisons among laboratories will be carried out within microsome preparations. The preparation and analysis effects will be independently estimated.

1.3 Justification for Test System

The test system for this study is human placental microsomes. This test system was selected because it provides a biological source of the aromatase enzyme and since the assay is being evaluated for its potential to serve as a screening assay, the use of human tissue enhances its predictive potential.

1.4 Test Method

This *in vitro* test method involves combining microsomes, substrate, appropriate co-factors and test substances in a common reaction vessel. The effect of the test substances on microsomal enzyme activity is evaluated by measuring the amount of the product of the enzyme-catalyzed substrate oxidation that is formed.

There is no applicable route of administration in the sense of a dose administration route for this *in vitro* test.

2.0 MATERIALS RECEIPT AND/OR PREPARATION

A sufficient supply of chemical reagents, radiolabeled and non-radiolabeled androstenedione, and microsomal preparation from the human placenta will be obtained prior to initiation of the first set of experiments to ensure that sufficient quantities are available to conduct the studies.

2.1 Substrate

2.1.1 Substrate Name/Supplier

The substrate for the aromatase assay is androstenedione (ASDN). Non-radiolabeled and radiolabeled ASDN will be used. The non-radiolabeled ASDN and the radiolabeled androstenedione ([1β-^{3}H]-ASDN, [^{3}H]ASDN) will be provided to the laboratories by Battelle's Chemical Repository (CR). The CR will forward all applicable information regarding supplier, lot numbers and reported/measured purity for the substrate to the laboratories and this information will be included in study reports. The radiochemical purity of the [^{3}H]ASDN was assessed by the lead laboratory in a previous task and was found to be 97%.

2.1.2 Preparation of Substrate Solution for use in Aromatase Assay

Since the specific activity of the stock [^{3}H]ASDN is too high for use directly in the assay, a solution containing a mixture of nonradiolabeled and radiolabeled [^{3}H]ASDN is prepared such that the final concentration of ASDN in the assay is 100 nM and the amount of tritium added to each incubation is about 0.1 µCi. This substrate solution should have a concentration of 2 µM with a radiochemical content of about 1 µCi/mL.

The following illustrates the preparation of a substrate solution using a stock of [^{3}H]ASDN with a specific activity of 25.3 Ci/mmol and a concentration of 1 mCi/mL. Prepare a 1:100 dilution (10 µCi/mL) of the radiolabeled stock in buffer. Prepare a 1 mg/mL solution of ASDN in ethanol and then prepare dilutions in buffer to a final concentration of 1 µg/mL. Combine 4.5 mL of the 1 µg/mL solution of ASDN, 800 µL of the [^{3}H]ASDN dilution and 2.7 mL buffer to make 8 mL of substrate solution (enough for 80 tubes). Record the weight of each component added to the substrate solution. After mixing the solution well, weigh aliquots (ca. 20 µL) and combine with scintillation cocktail for radiochemical content analysis. The addition of 100 µL of the substrate solution to each 2 mL assay volume yields a final [^{3}H]ASDN concentration of 100 nM with 0.1 µCi/tube.

2.2 Test Substances

4-Hydroxyandrostenedione (4-OH ASDN) is a known aromatase inhibitor. Other known or potential inhibitors may be tested.

2.2.1 4-Hydroxyandrostenedione (4-OH ASDN)

CAS No.: 566-48-3

Molecular Formula/Weight: $C_{19}H_{26}O_3$; 302.4 g/mol

Supplier: Sigma

Lot No: tbd

Purity: tbd

Storage Conditions: 2-8°C (for bulk chemical and solutions)

2.2.2 Test Substance Formulation and Analysis

Test substance stock solutions will be prepared and analyzed by the CR and distributed to the laboratories. 4-OH ASDN will be formulated in ethanol. The total volume of test substance formulation used in each assay should be no more than 1% of the total assay volume (i.e., 20 µL in a 2 mL assay) in order to minimize the potential of the solvent to inhibit the enzyme. Dilutions of the stock solution will be prepared in ethanol on the day of use such that the target concentration of inhibitor can be achieved by the addition of 20 µL of the dilution to a 2 mL assay volume.

2.3 Control Substances

The known aromatase inhibitor, 4-hydroxyandrostendione (4-OH ASDN), is used as the test substance and positive control substance for this task. A known aromatase non-inhibitor, lindane, will be used as the negative control substance. Table 1 contains identity and property information for these substances.

Table 1. Control Substances

Test Substance	CAS Number	Molecular Formula	Molecular Weight (g/mol)	Target Concentration in Assay (M)	Basis for Selection
4-OH ASDN	566-48-3	$C_{19}H_{26}O_3$	302.4	5E-8	Known aromatase inhibitor
Lindane	58-89-9	$C_6H_6Cl_6$	290.8	1E-6	Affects StAR and cholesterol metabolism; no aromatase activity

2.3.1 Control Substance Formulation and Analysis

Control substance stock solutions will be prepared and analyzed by the CR and distributed to the laboratories. Control substances will be formulated in ethanol or DMSO. The total volume of control substance formulation used in each assay should be no more than 1% of the total assay volume (i.e., 20 μL in a 2 mL assay) in order to minimize the potential of the solvent to inhibit the enzyme. Fresh dilutions of the stock solution will be prepared in the same solvent as the stock solution on the day of use. Dilutions will be prepared such that the target concentration of control substance (Table 3) can be achieved by the addition of 20 µL of the dilution to a 2 mL assay volume. Information on storage conditions for control substance stock solutions will be provided by the CR.

2.4 Human Placental Microsomes

2.4.1 Preparation

Appropriate precautions must be taken in the handling of the human placenta, which should be considered potentially infectious. These precautions should be extended to the handling of the human placental microsomes as well.

2.4.1.1 Source of the Placentas. Human placenta will be obtained from a local hospital. The exact source of the placenta will be documented in the study records. The human placenta will be from a non-smoking, 21-40 year old mother with a full term delivery. Within 30 minutes of the delivery of the placenta by the mother, it will be placed in a tissue bag, sealed, and packed in wet ice in an insulated shipping container. The placenta tissue bag will be labeled with date and time of delivery. Laboratory personnel will be on-call and will be responsible for transporting the placenta to their laboratory for processing into microsomes, as described below. Efforts will be made to minimize the time from delivery to the initiation of microsome preparation. Ideally, microsome preparation should begin within 2 hours of obtaining the placenta.

2.4.1.2 Microsome Preparation Buffers.

Buffer A: 0.25 M Sucrose, 0.04 M nicotinamide, 0.05 M sodium phosphate (pH 7.0). First prepare the 0.05M sodium phosphate buffer, pH 7.0: Dissolve 6.90 ± 0.55 g of sodium phosphate monobasic (JT Baker, cat # 4011-01, 137.99 g/mol; or equivalent) in 1 L distilled, deionized water to prepare 0.05 M NaH_2PO_4. Dissolve 7.10 ± 0.57 g of sodium phosphate dibasic (JT Baker, cat # 4062-01, 141.96 /mol; or equivalent) in 1 L distilled, deionized water to prepare 0.05 M Na_2HPO_4. Combine these solutions to a final pH of 7.0. The buffer may be stored for up to one month in the refrigerator (2-8 °C).

To complete preparation of Buffer A, dissolve 85.58 ± 1.36 g sucrose (JT Baker, cat # 4097-04, 342.3 g/mol; or equivalent) and 4.88 ± 0.48 g nicotinamide (Sigma, cat # N3376, 122.1 g/mol) in 1L 0.05M sodium phosphate buffer, pH 7.0. The buffer may be stored for up to one month in the refrigerator (2-8 °C).

Buffer B: 0.1 M sodium phosphate (pH 7.4). Dissolve 13.80 ± 0.55 g sodium phosphate monobasic (JT Baker, cat # 4011-01, 137.99 g/mol; or equivalent) in 1 L distilled, deionized water to prepare 0.1 M NaH_2PO_4. Dissolve 14.20 ± 0.56 g sodium phosphate dibasic (JT Baker, cat # 4062-01, 141.96 g/mol; or equivalent) in 1 L distilled, deionized water to prepare 0.1 M Na_2HPO_4. Combine these solutions to a final pH of 7.4. The assay buffer may be stored for up to one month in the refrigerator (2-8 °C).

Buffer C: 0.1 M sodium phosphate (pH 7.4) with 0.25 M sucrose, 20% glycerol and 0.05 mM dithiothreitol. Dissolve 17.12 ± 0.27 g sucrose and 1.54 ± 0.12 mg dithiothreitol (Sigma, cat # D5545, 154.3 g/mol) in about 100 mL 0.1 M sodium phosphate buffer, pH 7.4 (prepared as described above). Dilute to 160 mL with additional 0.1 M sodium phosphate buffer, pH 7.4. Add glycerol (Sigma, cat # G7893, 92 g/mol) to a total solution volume of 200 mL.

2.4.1.3 Placental Microsome Preparation. *Caution*: Microsomes can be denatured by detergents. Therefore, it is important to ensure that all glassware, etc. that is used in the preparation or usage of microsomes is free of detergent residue. New disposable test tubes, bottles, vials, pipets and pipet tips may be used directly in the assay. Durable labware that may have been exposed to detergents should be rinsed with water and/or buffer prior to use in the assay.

Human placentas are discoid in shape and have a fetal surface (with umbilicus attached) and a maternal surface. Each of these surfaces is covered with a fibrous, vascularized membrane. To ensure the preservation of aromatase activity, the tissue will be kept well-chilled on ice and work will commence quickly. The placenta will be placed on a tray that is set over/in a pan of ice to aid in keeping the tissue chilled during dissection operations. While keeping the placenta chilled on ice, the membrane and fibrous material will be dissected, removed and discarded. The spongy tissue will be cut into small portions and placed on ice in pre-chilled (refrigerated) Buffer A. Batches of the tissue will be sequentially removed to a beaker and

minced with scissors. Buffer A will be added to an approximate 2:1 w:v ratio and the mixture will be homogenized using a Polytron homogenizer. Some fibrous material may be resistant to homogenization and this tissue will be removed from the homogenate or allowed to remain with the knowledge that it will be removed in the centrifugation step to follow. The homogenate will be transferred to centrifuge tubes (recommended approximately 40-mL capacity, appropriate to use at forces of 10,000 *g*) and kept on ice until all of the tissue is processed or until the capacity of the centrifuge rotor is reached. Tissue homogenization will continue in batches as described until all tissue is processed. The tissue homogenate will be centrifuged (in batches, as necessary, dependent on rotor capacity and the number of tubes to be processed) at a setting of 10,000 *g* for 30 minutes inan appropriate centrifuge (such as an IEC B-22M) at 4 °C. The supernatant will be removed by pipetting and transferred to ultracentrifuge tubes (recommended approximate capacity is 26-mL) and will be centrifuged at a setting of 35,000 rpm (or another speed as necessary to produce approximately 100,000 g) in an appropriate ultracentrifuge (such as a Beckman L5-50B Ultracentrifuge) for one hour at about 4°C to obtain the crude microsomal pellet. The supernatant will be decanted and the microsomal pellet will be dislodged from the wall of the tube by gentle swirling with a few mL of Buffer B. Care will be taken to not dislodge the clear pellet that is often visible under the microsomal pellet. The microsomal pellet (along with the buffer) will be poured into a (suggested 15-mL size) Potter-Elvejhem homogenizer and resuspended in Buffer B. The suspension will be transferred to ultracentrifuge tubes. The suspensions of multiple pellets may be combined in a single ultracentrifuge tube. The samples will be centrifuged at a setting of 35,000 rpm (ca. 100,000 *g*, Beckman L5-50B) for one hour to wash the microsomes. This washing procedure (supernatant decanting, pellet resuspension and centrifugation) will be repeated one additional time. Then the supernatant will be decanted and the twice-washed microsomal pellet will be dislodged from the wall of the tube by gentle swirling in a few mL of Buffer C. All microsomal pellets will be combined into a single lot and resuspended in Buffer C using a Potter Elvejhem homogenizer. An appropriate final volume of suspended microsomes will range from 20-30 mL, dependent on the amount of protein that is isolated from the placenta. The concentration of microsomes in the final suspension will be at least 15 mg/mL, which will be measured at this point using the protein assay. The microsomes will be aliquoted (ca. 200 µL/tube) into labeled tubes (NUNC cryotubes), flash frozen in liquid nitrogen, and stored at approximately -70 to -80°C until removed for use.

2.4.2 Use of Microsomes

On the day of use, microsomes will be thawed quickly in a 37 ± 1°C water bath and immediately transferred to an ice bath. The microsomes will be rehomogenized using a Potter-Elvejhem homogenizer (about 5-10 passes) or vortexed to mix prior to use. The microsomes will be diluted in buffer (serial dilutions may be necessary) to an approximate protein concentration of 0.025 mg/mL. The addition of 1 mL of that microsome dilution will result in a final approximate protein concentration of 0.0125 mg/mL in the assay tubes. All microsome samples must be kept on ice until they are placed in the water bath just prior to their addition to the aromatase assay. It is recommended that microsomes not be left on ice for longer than approximately 1 h before proceeding with the assay or microsomal enzyme activity may be decreased.

Under no conditions should thawed or diluted microsomes be refrozen for later use in the assay.

2.5 Other Assay Components

2.5.1 Buffer

The assay buffer is 0.1 M sodium phosphate buffer, pH 7.4. It is prepared as described in Section 2.4.1.2 above for Buffer B. The assay buffer may be stored for up to one month in the refrigerator (2-8°C).

2.5.2 Propylene Glycol

Propylene glycol (JT Baker, cat # 9402-01, 76.1 g/mol) is added to the assay directly as described below.

2.5.3 NADPH

NADPH (β-nicotinamide adenine dinucleotide phosphate, reduced form, tetrasodium salt, Sigma, cat # 1630, 833.4 g/mol) is the required co-factor for CYP19. The final concentration in the assay is 0.3 mM. Typically, a 6 mM stock solution is prepared in assay buffer and then 100 µL of the stock is added to the 2 mL assay volume. NADPH must be prepared fresh each day and is kept on ice.

3.0 PROTEIN ASSAY

The protein concentration of each microsome preparation prepared in this task will be measured by all participating laboratories. The protein concentration of the microsome preparation will be determined on each day of use of the microsomes in the aromatase assay and at other times as appropriate. A 6-point standard curve will be prepared, ranging from 0.13 to 1.5 mg protein/mL using bovine serum albumin (BSA). Protein will be determined by using a DC Protein Assay kit purchased from Bio-Rad (Hercules, CA). QC standards (0.125, 0.5 and 1 mg/mL BSA), obtained from Pierce (Woburn, MA) will be run in duplicate with each assay. To a 25 µL aliquot of unknown or standard, 125 µL of BioRad DC Protein Kit Reagent A will be added and mixed. Next, 1 mL of BioRad DC Protein Kit Reagent B will be added to each standard or unknown and the samples will be vortex mixed. The samples will be allowed to sit at room temperature for at least 15 min to allow for color development. The absorbances are stable for about 1 h. Each sample (unknown and standards) will be transferred to disposable polystyrene cuvettes and the absorbance (@ 750 nm) will be measured using a spectrophotometer. The protein concentration of the microsomal sample will be determined by extrapolation of the absorbance value using the curve developed using the protein standards.

4.0 AROMATASE ASSAY METHOD

This procedure will be to measure the aromatase activity in the microsomal preparations. Four types of control samples will be included for each replicate. These include:

- full enzyme (aromatase) activity controls (substrate, NADPH, propylene glycol, buffer, vehicle [used for preparation of test substance solutions] and microsomes)
- background activity controls (all components that are in the full aromatase activity controls, except NADPH)
- positive control (all components that are in the full aromatase activity controls, except vehicle, and with the addition of 4-OH ASDN at a single concentration)
- negative control (all components that are in the full aromatase activity controls, except vehicle, and with the addition of lindane at a single concentration).

Four test tubes of each type of control will be included with each replicate and treated the same as the other samples. The controls sets will be split so that two tubes (of each control type) will be run at the beginning and two at the end of each set.

The assays will be performed in 13x100 mm test tubes maintained at 37 ± 1°C in a shaking water bath. Each test tube will be uniquely identified by applying a label or writing directly on the test tube. Propylene glycol (100 µL), [^{3}H]ASDN, NADPH, and buffer (0.1 M sodium phosphate buffer, pH 7.4) will be combined in the test tubes (total volume 1 mL). The final concentrations for the assay components are presented in Table 2. The tubes and the microsomal suspension will be placed at 37 ± 1°C in the water bath for five minutes prior to initiation of the assay by the addition of 1 mL of the diluted microsomal suspension. The total assay volume will be 2 mL, and the tubes will be incubated for 15 min. The incubations will be stopped by the addition of methylene chloride (2 mL); the tubes will be vortex-mixed for ca. 5 s and placed on ice. The tubes will be vortex-mixed an additional 20-25 s. The tubes will be centrifuged using a Beckman GS-6R centrifuge with GH-3.8 rotor for 10 minutes at a setting of 1000 rpm. The methylene chloride layer will be removed and discarded; the aqueous layers are extracted again with methylene chloride (2 mL). This extraction procedure will be performed one additional time, each time discarding the methylene chloride layer. The aqueous layers will be transferred to vials and duplicate aliquots (0.5 mL) will be transferred to 20-mL liquid scintillation counting vials. Liquid scintillation cocktail (Ultima Gold, Packard, 10 mL) will be added to each counting vial and shaken to mix the solution. The radiochemical content of each aliquot will be determined as described below.

Table 2. Optimized Aromatase Assay Conditions

Assay factor (units)	Human Placental [1]
Microsomal Protein (mg/mL)[a]	0.0125
NADPH (mM)[a]	0.3
[^{3}H]ASDN (nM)[a]	100
Incubation Time (min)	15

[a] Final concentrations

Analysis of the samples will be performed using Liquid Scintillation Spectrometry (LSS). Radiolabel found in the aqueous fractions represents ^3H_2O formed.

5.0 DETERMINATION OF THE RESPONSE OF AROMATASE ACTIVITY TO 4-OH ASDN

Only the laboratories that procured the placenta and prepared the microsomes will perform the experiments described in this section, which is similar to the Positive Control experiment conducted in WA 4-16, Task 4. Two independent replicates will be performed per laboratory. Each concentration of 4-OH ASDN will be run in triplicate tubes per replicate (Table 3). The four types of control samples described in Section 4.0 will be included in each replicate. Each control type will be run in quadruplicate with the controls sets split so that two tubes (of each control type) will be run at the beginning and two at the end of each replicate set.

The assay will be conducted as described in Section 4.0 with the following modification. 4-OH ASDN solution (or vehicle) will be added to the mixture of propylene glycol, substrate, NADPH and buffer in a volume not to exceed 20 µL prior to preincubation of that mixture. The volume of buffer used will be adjusted so the total incubation volume remains at 2 mL.

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Table 3. Study Design – Aromatase Response to 4-OH ASDN

Sample type	Repetitions (test tubes)	Description	Control or Test chemical concentration (M final)
Full Enzyme Activity Control	4	Complete assay[a] with inhibitor vehicle control	N/A
Background Activity Control	4	Complete assay with inhibitor vehicle control omitting NADPH	N/A
Positive Control	4	Complete assay with positive control chemical (4-OH ASDN) added	5×10^{-8}
Negative Control	4	Complete assay with negative control chemical (lindane) added	1×10^{-6}
4-OH ASDN Concentration 1	3	Complete assay with 4-OH ASDN added	1×10^{-6}
4-OH ASDN Concentration 2	3	Complete assay with 4-OH ASDN added	1×10^{-7}
4-OH ASDN Concentration 3	3	Complete assay with 4-OH ASDN added	5×10^{-8}
4-OH ASDN Concentration 4	3	Complete assay with 4-OH ASDN added	2.5×10^{-8}
4-OH ASDN Concentration 5	3	Complete assay with 4-OH ASDN added	1×10^{-8}
4-OH ASDN Concentration 6	3	Complete assay with 4-OH ASDN added	1×10^{-9}

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

6.0 DATA ANALYSIS

The data analysis described in the following subsections addresses all of the experiments of this task. The laboratories will only be responsible for performing the data analysis that corresponds to the experiments that they are assigned to conduct.

6.1 Aromatase Activity and Percent of Control Calculations

Relevant data will be entered into the latest version of the spreadsheet Aromatase_Master_Versionx.y.xls (where x and y denote version number designation) for calculation of aromatase activity and percent of control. The version of the spreadsheet used will be included in the reports. A working document detailing the use of this spreadsheet has been issued in a previous task on this work assignment.

6.2 Statistical Analyses

6.2.1 Concentration Response Fits for the Reference Chemicals

For the 4-OH ASDN, two independent replicates of the concentration response curve fit will be carried out.

For each replicate two repeat tubes of the full enzyme activity controls, the background activity controls and the positive and negative controls will be run prior to the repetitions of the graded concentrations of 4-OH ASDN and two repeat tubes of each control will be run following the repetition of 4-OH ASDN. Three repetitions will be prepared for each concentration of 4-OH ASDN.

For each repeat tube (full enzyme activity controls, background activity controls, positive, and negative controls and each 4-OH ASDN concentration) the Excel database spreadsheet will include total observed (uncorrected) disintegrations per minute (DPMs) per tube and total aromatase activity per tube. The DPM and aromatase activity values will be corrected for the background DPMs, as measured by the average of the background activity control tubes. The aromatase activity will be calculated as the corrected DPM, normalized by the specific activity of the [^{3}H]ASDN, the mg of protein of the aromatase, and the incubation time. The average (corrected) DPMs and aromatase activity across the four background activity control repeat tubes must necessarily be equal to 0 within each replicate.

For each tube percent of control will be determined by dividing the background corrected aromatase activity for that tube by the average background corrected aromatase activity for the four full enzyme activity control tubes and multiplying by 100. Nominally one might expect for an inhibitor the percent of control activity values to vary between approximately 0% near the high inhibition concentrations and approximately 100% near the low inhibition concentrations. However due to experimental variation individual observed percent of control values will sometimes extend below 0% or above 100%.

Concentration response trend curves will be fitted to the percent of control activity values within each of the repeat tubes at each 4-OH ASDN concentration. Concentration is expressed on the log scale. In agreement with past convention, logarithms will be common logarithms (i.e. base 10). X will denote the logarithm of the concentration of 4-OH ASDN (e.g. if concentration = 10^{-5} then X = -5). Let

Y = percent of control activity in the inhibitor tube
X = logarithm (base 10) of the concentration
DAVG = average DPMs across the repeat tubes with the same 4-OH ASDN concentration
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 will be fitted to relate percent of control activity to logarithm of concentration within each replicate:

$$Y = 100/[1 + 10^{(\mu - X)\beta}] + \varepsilon$$

where ε is the variation among repetitions, distributed with mean 0 and variance proportional to DAVG (based on Poisson distribution theory for radiation counts). The variance will be approximated by Y.

The response curve will be fitted by weighted least squares nonlinear regression analysis with weights equal to 1/Y. Model fits will be carried out using Prism software (Version 3 or higher). Observed individual percent activity values above 100% will be set to 99.5%. Observed individual percent activity values below 0% will be set to 0.5%. Model fits will be carried out using Prism software (Version 3 or higher).

Concentration response models will be fitted for each replicate test. Based on the results of the fit within each replicate the extent of aromatase inhibition will be summarized as IC_{50} (10 μ) and slope (β). The estimated IC_{50} for 4-OH ASDN will be a (weighted) geometric mean across the replicates. The estimated overall standard error will be based on the standard errors within each replicate and the replicate-to-replicate variability. The average value and standard error of $\log_{10}IC_{50}$ or β and the replicate-to-replicate component of variation will be calculated based on a one-way random effects analysis of variance model fit. For replicate the estimated $\log_{10}IC_{50}$ (μ), the within replicate standard error of μ, the IC_{50}, the slope (β), the within replicate standard error of β, and the "Status" of each response curve will be displayed in a table. The "Status" of each response curve will be indicated as:

- Complete. Data points ranging from essentially 0 percent to 100 percent of control
- Incomplete - Interpolate. Data points to at least 50% inhibition
- Incomplete – Extrapolate. Data points all above 50% inhibition
- No Inhibition. No data below 80% of control.

6.2.2 Graphical and Analysis of Variance Comparisons Among Concentration Response Curve Fits

For each replicate the individual percent of control values will be plotted versus logarithm of the 4-OH ASDN concentration. The fitted concentration response curve will be superimposed on the plot. Individual plots will be prepared for each replicate.

Additional plots will be prepared to compare the percent of control activity values across replicates. For each replicate the average percent of control values will be plotted versus logarithm of 4-OH ASDN concentration on the same plot. Plotting symbols will distinguish among replicates. The fitted concentration response curves for each replicate will be

superimposed on the plots. On a separate plot the average percent of control values for each replicate will be plotted versus logarithm of 4-OH ASDN concentration. The average concentration response curve across replicates will be superimposed on the same plot.

For each replicate, β and μ will be treated as a random variable with mean (β_{avg}, μ_{avg}). X and Y ($0< Y <100$) will denote logarithm of concentration and percent of control, as defined above. The average response curve will be

$$Y_{avg} = 100/[1 + 10^{\beta avg(\mu avg - X)}].$$

Slope (β) and $\log_{10}IC_{50}$ (μ) will also be compared across replicates based on one-way random effects analysis of variance, treating the replicates as random effects. For each of β and μ, plots will be prepared that display the parameters within each replicate with associated 95% confidence intervals based on the within replicate standard error and the average across replicates with associated 95% confidence interval incorporating replicate-to-replicate variation.

6.2.3 Graphical and Analysis of Variance Comparisons of Full Enzyme Activity Control, Background Activity Control, and Positive and Negative Control Percent of Control Across Replicates

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

To assess whether this is the case the control responses will be adjusted for background DPMs, divided by the average of the (background adjusted) full enzyme activity control values, and expressed as percent of control. The average of the four background activity controls within a replicate must necessarily be 0 percent and the average of the four full enzyme activity controls within a replicate must necessarily be 100 percent. The full enzyme activity controls percent of control, the background activity controls percent of control, and the negative and positive controls percent of control values will be plotted across replicate, with plotting symbol distinguishing between beginning and end, and with reference line 0% (background activity controls) or 100% (full enzyme activity controls) respectively. These plots will display the extent of consistency across replicates with respect to average value and variability and will provide comparisons of beginning versus end of each replicate.

Two-factor analysis of variance models will be fitted, separately for the full enzyme activity control, the background activity control, and the positive and negative control tubes. The factors in the analysis of variance will be

- Portion (beginning or end) 1 df
- Replicate 1 df

- Portion by replicate interaction 1 df

The residual error variation corresponds to repetition within replicate and portion (with 4 degrees of freedom). The response will be percent of control. Since for the background activity and full enzyme activity controls the average of the repetitions within a reference chemical and replicate are constrained to be 0 and 100 respectively, by the way in which "percent of control" is defined, the variation associated with the replicate is necessarily constrained to be 0.

If the daily replicates are in control the portion main effect and the portion by replicate interaction should be nonsignificant. If the portion by replicate interaction is significant the nature of the effect will be assessed by comparing the portion effect within each replicate to the portion effect averaged across replicates.

6.2.4 Microsomal Characterization Within and Between Laboratories

Laboratory 2 (Battelle) will supply microsomes to laboratories 1 (RTI), 3 (WIL), and 4 (In Vitro) and laboratory 4 will supply microsomes to laboratories 1, 2, and 3. Each laboratory will determine protein concentration and aromatase activity of each microsomal preparation, as discussed in the protocol. Each test laboratory will compare the protein concentrations and the aromatase activity between the two microsome sources by two-sample t-tests, using the within laboratory-microsome preparation replicate determination variation as an error criterion.

The results of the determinations of protein concentration and aromatase activity from each microsomal preparation within each of the test laboratories will be sent to the Data Coordination Center where an inter-laboratory comparison will be carried out.

A two-way analysis of variance model will be fitted to the protein concentration and aromatase activity responses. The factors in the model will be

- Laboratory 3 df
- Microsome preparation 1 df
- Laboratory × Microsome preparation 3 df
- Within laboratory-preparation variation 8 df

The significance of the microsome preparation main effect will be based on the laboratory × microsome preparation interaction. The significance of the laboratory × microsome preparation interaction is based on comparisons with the within laboratory-preparation variation. The within laboratory-preparation variation will be based on three replicate determinations within each laboratory, pooled across laboratories. If either is significant, estimates and confidence intervals of microsome preparation effect will be prepared, either averaged across laboratories or separately within laboratories, as appropriate.

6.2.5 Statistical Software

Concentration response curves will be fitted to the data using the non-linear regression analysis features in the PRISM statistical analysis package, Version 3 or higher. Supplemental statistical analyses and displays such as summary tables, graphical displays, analysis of variance, and multiple comparisons will be carried out using PRISM, the SAS statistical analysis system, Version 8 or higher, or other general purpose statistical packages (e.g. SPSS), as convenient.

6.2.6 Interlaboratory Statistical Analysis

The lead laboratory and each of the participating laboratories will carry out "intra-laboratory" statistical analyses based on their test data, according to this common statistical analysis plan, developed by the Data Coordination Center (Battelle). The Data Coordination Center will carry out the "inter-laboratory" statistical analysis. It will combine summary values developed in each of the intra-laboratory analyses to assess relationships among the laboratory results, the extent of laboratory-to-laboratory variation, and overall consensus estimates among the laboratories. Also see section 6.2.4

7.0 RETENTION OF RECORDS

All records that remain the responsibility of the testing laboratory will be retained in the archives for the life of the contract.

8.0 QUALITY CONTROL/QUALITY ASSURANCE PROCEDURES

Quality control (QC) and quality assurance (QA) procedures will follow those outlined in the Quality Assurance Project Plan (QAPP) that was prepared for this study. The study will be conducted in compliance with the Federal Register, 40 CFR Part 160. Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) Good Laboratory Practices Standards.

9.0 REPORTS

Interim data summaries, draft and final reports will be submitted as described in Section 9.5 of the QAPP.

The data to be reported in the interim data summaries will include (but is not limited to) the following information: assay date and run number, technician code and log 4-OH ASDN concentration, background corrected aromatase activity (for each control and 4-OH ASDN repetition), percent of control activity, IC_{50}, slope and graphs of activity versus log 4-OH ASDN concentration.

In addition, draft and final reports will contain tables and graphs, as appropriate, containing the results of the intra- and inter-laboratory statistical analyses described in Section 6 of this document.

10.0 STUDY RECORDS TO BE MAINTAINED

- All records that document the conduct of the laboratory experiments and results obtained, as well as the equipment and chemicals used
- Protocol and any Amendments
- List of any Protocol Deviations
- List of Standard Operating Procedures
- QAPP and any Amendments
- List of any QAPP Deviations

Appendix C

FINAL ANALYSIS REPORT

PLACENTAL AROMATASE VALIDATION STUDY

[^{3}H]ASDN Radiochemical Purity Determination

EPA Contract Number 68-W-01-023
Work Assignment 4-16

Sponsor:

Battelle Memorial Institute
505 King Avenue
Columbus, OH 43201-2693

Performing Laboratory:
Drug Metabolism and Pharmacokinetics
RTI International
Post Office Box 12194
Research Triangle Park, NC 27709

FINAL REPORT

Title:	PLACENTAL AROMATASE VALIDATION STUDY [^{3}H]ASDN Radiochemical Purity Determination
Author:	Sherry Black
Performing Laboratory:	Drug Metabolism and Pharmacokinetics RTI International Post Office Box 12194 Research Triangle Park, NC 27709
Sponsor:	Battelle Memorial Institute 505 King Avenue Columbus, OH 43201-2693
Sponsor's Representatives:	David Houchens, Ph.D. EDSP Program Manager Battelle Jerry D. Johnson, Ph.D. Diplomate, A.B.T. Work Assignment Leader Battelle
Analysis Date:	January 5, 2005
Final Report Date	September 28, 2005

Author:

Sherry Black 9/28/05
Sherry Black Date
Research Chemist

Approved:

J Mathews 9-28-05
James Mathews, Ph. D, DABT Date
Study Director

RTI INTERNATIONAL

Quality Assurance Statement

Study Title: [^{3}H] ASDN Radiochemical Purity Determination
WA 4-16 and WA 4-17

Sponsor: Battelle Memorial Institute

Study Code: An05-928

Protocol Number: RTI-928-AN

This study was audited by the Science and Engineering – Health Sciences Quality Assurance Unit and the results of the inspections and audits were reported to the study director and management as identified below. To the best of our knowledge, the reported results accurately describe the study methods and procedures used, and the reported results accurately reflect the raw data.

Inspections and Audits	Inspection and Audit Date(s)	Date Inspection/Audit Report Sent to Study Director and Management
Data and Report Audit	March 24, 2005	March 25, 2005

K. Collier
Quality Assurance Specialist

9/28/2005
Date

Approval:

Carrie Ingalls
Quality Assurance Assistant Manager

09/28/2005
Date

Introduction

The objective of this work is to determine the radiochemical purity of the [3H]ASDN to be used in the conduct of WA 4-16 and WA 4-17. The criteria for acceptance of the material for this use is 95% radiochemical purity as determined by high performance liquid chromatography (HPLC) and liquid scintillation counting.

Materials and Methods

[^{3}H]Androstenedione ([^{3}H]ASDN) of lot number 3538496 was received from Perkin Elmer Life Science (Boston, MA).

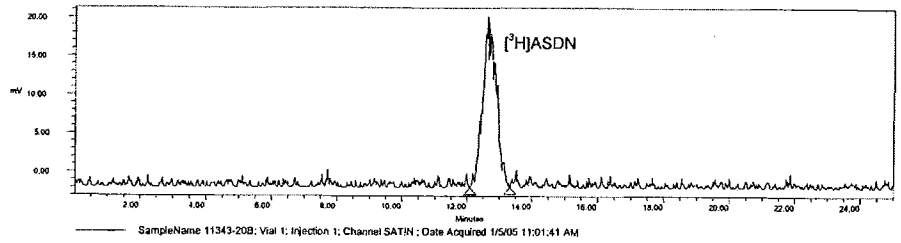
The radiochemical purity of the [^{3}H]ASDN (1:100 dilution in ethanol) was determined using high performance liquid chromatography (HPLC) and liquid scintillation counting. The HPLC system consists of a Waters 2690 Separations Module, a Waters 2487 Dual λ Absorbance Detector and a β-RAM Model 3 flow-through radioactivity detector (IN/US, Inc., Tampa, FL) with a 250 µL glass scintillant cell. Data was collected using Waters Millennium^{32} Client/Server Chromatography Data System Software, Version 4.0.

The HPLC method used a Zorbax Rx-C_{18} column (4.6 x 250 mm) with a mobile phase of 55:15:30 (v:v:v) distilled, deionized water: tetrahydrofuran: methanol and a flow rate of 1 mL/min. The eluant was monitored by ultraviolet (UV) absorbance at 240 nm and by a flow-through radiochemical detector. Eluant fractions were collected manually into vials containing ca. 10 mL Ultima Gold and assayed for radiochemical content by liquid scintillation spectrometry (LSS)

Results

The HPLC radiochromatogram of the [^{3}H]ASDN, lot number 3538496, is presented in Figure 1. The measured radiochemical purity of the [^{3}H]ASDN was 97%.

Figure 1. HPLC Radiochromatogram of [^{3}H]ASDN



Conclusion

[^{3}H]ASDN, lot number 3538496, is acceptable for use on WA 4-16 and WA 4-17.

Appendix D

Battelle
The Business of Innovation

ANALYTICAL CHEMISTRY ACTIVITIES REPORT

4-HYDROXYANDROSTENEDIONE (4-OH ASDN)

CAS No.: 566-48-3

Lot No.: 063K4069 (Sigma Aldrich)

Receipt Date: 10/22/04

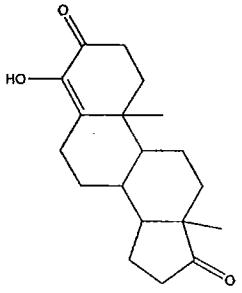
Amount Received: 3.1 g

Appearance: Solid

Vendor Purity: 99% by TLC

Storage Conditions (@ Battelle): Refrigerated (~5°C)

STRUCTURE:



Mol. Wt.: 302.41 g/mol

Mol. Formula: $C_{19}H_{26}O_3$

Prepared By:

Denise A. Contos 12/19/05

Denise A. Contos, M.S.

Approved By:

Steven W. Graves

Steven W. Graves, B.S.

Manager, Chemistry Technical Center

Battelle Study No. WA 4-16/17

QUALITY ASSURANCE STATEMENT

This study was inspected by the Quality Assurance Unit (QAU) and reports were submitted to the Study Director and Management as follows:

Phase Inspected	Inspection Date	Date Reported to Study Director/Management
Test substance receipt*	10/26/04	10/26/04
Formulation preparation	12/2/04	12/2/04
Dispensing	12/2/04	12/2/04
Formulation analysis	12/2/04	12/2/04
Audit analytical report	10/20/05	10/20/05
Audit study file	10/20/05	10/20/05

* These inspections are serving the purpose for all reference chemicals since QA was required to see only one phase inspection of a chemical.

Quality Assurance Unit — 12-14-05 Date

EXECUTIVE SUMMARY

The title compound, 4-hydroxyandrostenedione (4-OH ASDN), was analyzed in support of the Environmental Protection Agency (EPA) Placental and Recombinant Aromatase Assay Prevalidation Work, Work Assignment 4-16/17.

The solubility of 4-hydroxyandrostenedione was determined to be acceptable in 95% ethanol for preparing formulations.

A formulation analysis method was developed and validated to analyze 4-hydroxyandrostenedione in 95% ethanol at a concentration of 3.02 mg/mL (0.01M). This method was used to analyze samples from both formulation and formulation storage stability studies at 3.02 mg/mL.

The storage stability study indicated that a 3.02 mg/mL formulation, stored in sealed amber glass bottles and protected from light, was stable for 173 days at approximately 5°C.

The stock formulation prepared for shipment to the testing laboratory was analyzed and met the established acceptance criteria.

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LIST OF FIGURES

1 INTRODUCTION

The purpose of this work was to provide all necessary chemistry support activities for 4-hydroxyandrostenedione on Environmental Protection Agency (EPA) Work Assignment 4-16/17, and consisted of:

- Determining solubility in 95% ethanol.
- Developing and validating a formulation analysis method.
- Conducting a storage stability study.
- Preparing and analyzing a stock formulation.

This work was done at Battelle, 505 King Avenue, Columbus, OH 43201.

2 CHEMICAL RECEIPT AND STORAGE

One 15-mL amber glass bottle of 4-hydroxyandrostenedione, 063K4069, was received from the repository at Battelle's Marine Sciences Laboratory in Sequim, WA on October 22, 2004. The label amount indicated 3.1 grams was sent. The chemical was received and subsequently stored at approximately 5°C.

A copy of the manufacturer's Certificate of Analysis for this lot is shown in Figure 1. This states that purity was 99% based on thin layer chromatography (TLC).

3 SOLUBILITY STUDIES

A solubility study was conducted to determine the solubility of 4-hydroxyandrostenedione (4-OH ASDN) in 95% ethanol, at a concentration of at least 30.2 mg/mL. The 4-OH ASDN (0.30200 ± 0.03020 g) was weighed into a 10-mL volumetric flask, diluted to approximately 80% volume with 95% ethanol, sealed and shaken to mix. The flask was diluted to volume with 95% ethanol, sealed, shaken, sonicated for approximately 50 minutes and stirred. The 4-OH ASDN did not go into solution.

A second solubility study was conducted to determine the solubility of 4-OH ASDN in 95% ethanol, with a solubility of at least 3.02 mg/mL being required for acceptability. The 4-OH ASDN (0.03020 ± 0.00302 g) was weighed into a 10-mL volumetric flask, diluted to approximately 80% volume with 95% ethanol, sealed and shaken to mix. The flask was diluted to volume with 95% ethanol, sealed, shaken and sonicated for approximately 2 minutes. The 4-OH ASDN went into solution. This experiment showed that 95% ethanol was an acceptable solvent for the 3.02 mg/mL formulation (0.01M).

SIGMA-ALDRICH

Certificate of Analysis

Product Name	4-Androsten-4-ol-3,17-dione
Product Number	A5791
Product Brand	SIGMA
CAS Number	566-48-3
Molecular Formula	$C_{19}H_{26}O_3$
Molecular Weight	302.41

TEST	SPECIFICATION	LOT 063K4069 RESULTS
APPEARANCE		WHITE POWDER
SOLUBILITY		CLEAR COLORLESS SOLUTION AT 10 MG/ML OF METHANOL
ELEMENTAL ANALYSIS		75.45% CARBON
PROTON NMR SPECTRUM		CONSISTENT WITH STRUCTURE
PURITY BY THIN LAYER CHROMATOGRAPHY		99%
QC ACCEPTANCE DATE		JUNE 2003

Lori Schulz, Manager
Analytical Services
St. Louis, Missouri USA

Figure 1 – Certificate of Analysis

4 FORMULATION ANALYSIS METHOD PERFORMANCE EVALUATION (MPE)

This section describes the evaluation of a method developed to analyze formulations of 4-hydroxyandrostenedione in 95% ethanol at a target concentration of 3.02 mg/mL (0.01 M) for the stability study and the results and conclusions from this evaluation.

4.1 Method Development

Method development for this chemical involved the evaluation of various chromatographic columns and conditions. The selected method was one which produced acceptable retention time for the major peak, apparent resolution of significant impurities and acceptable peak shape. The detection method chosen was gas chromatography with flame ionization detection (GC/FID).

4.2 Method

The GC parameters for 4-hydroxyandrostenedione are presented in Table 1.

Table 1 – GC System

GC	Agilent 6890 (Palo Alto, CA)
Column	RTX-5 MS, 15 m × 0.25 mm (ID), 0.25 µm film thickness (Restek, Bellefonte, PA)
Carrier Gas and Flow Rate	Helium at 2 mL/minute
Oven Temperature	150°C, hold for 1 minute, increase at 15°C/minute to 320°C
Detector Type	Flame Ionization (FID)
Detector Flow Rates	Hydrogen at 30 mL/minute; Air at 380 mL/minute
Detector Temperature	320°C
Injector Temperature	250°C
Injection Volume	1 µL
Injection Mode	Split 1:10
Run Time	~12 minutes

4.3 Method Validation

Validation was accomplished using a single experiment.

Triplicate vehicle/calibration standards at the highest and lowest of four concentrations were prepared. A single standard was prepared at each intermediate concentration. The high and low concentrations were used to assess the precision of the method. The precision of the low concentration was used to calculate limits of detection (LOD) and limits of quantitation (LOQ). Triplicate vehicle blanks with and without internal standard (IS) were used to assess the specificity of the method.

4.3.1 Preparation of Standards and Blanks

4.3.1.1 Internal Standard (IS)

Fifty (50) milligrams ± 4 mg of benzophenone was added to a 25-mL volumetric flask. The content of the flask was diluted to volume with methanol, sealed, and mixed well.

4.3.1.2 Stock Standards

Two stock standards (A, B) were prepared by accurately weighing 50 ± 1 mg of 4-OH ASDN each into individual 50-mL volumetric flasks and dissolving in and diluting to volume with methanol. This produced stocks A and B with target concentrations of 1000 µg/mL each.

4.3.1.3 Vehicle/Calibration Standards

Vehicle/calibration standards were prepared as shown in Table 2. The contents of the flasks were diluted to volume with methanol, and mixed well. Triplicate vehicle/calibration standards were prepared at the low and high concentrations with single vehicle/calibration standards prepared at the two intermediate concentrations.

Table 2 – Preparation of Vehicle/Calibration Standards

Vehicle/Calibration Std	Target Final Conc (µg/mL)	Source	Source Volume (mL)	IS (mL)	95% Ethanol (mL)	Final Volume (mL)
VS1	500	A	5	1	1	10
VS2	300	B	3	1	1	10
VS3	200	A	2	1	1	10
VS4	100	B	1	1	1	10

4.3.1.4 Blanks

Triplicate blanks without IS were prepared by pipetting 1 mL of 95% ethanol into three individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with methanol, sealed, and mixed well.

Triplicate blanks with IS were prepared by pipetting 1 mL IS and 1 mL of 95% ethanol into three individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with methanol, sealed, and mixed well.

4.3.2 Analysis

A portion of each vehicle/calibration standard and blank was transferred to individual autoinjector vials and the vials were sealed. Single injections were made from each vial using the same chromatographic system and parameters determined during method development (Table 1).

4.3.3 Calculations

The integration of the 4-OH ASDN and IS peaks by the chromatography data system was evaluated to assure it was consistent in all chromatograms and manually reintegrated, if necessary. A linear regression equation weighted 1/x was calculated relating the response ratio of 4-OH ASDN divided by the IS (y) to the concentration of the vehicle/calibration standards (x). The concentration of each vehicle/calibration standard was calculated using its individual response ratio and the regression equation. These values were used to calculate the individual and average concentrations, percent relative errors (RE), standard deviation (s), and percent relative standard deviation (RSD) as appropriate for the vehicle/calibration standard at each concentration.

4.3.4 Results

Specificity is shown by representative overlaid chromatograms from low and high vehicle/calibration standards, blank with IS, and a blank from the validation data as presented in Figure 2.

The blank and blank with IS exhibited no peaks that would significantly interfere with the 4-OH ASDN or IS peaks.

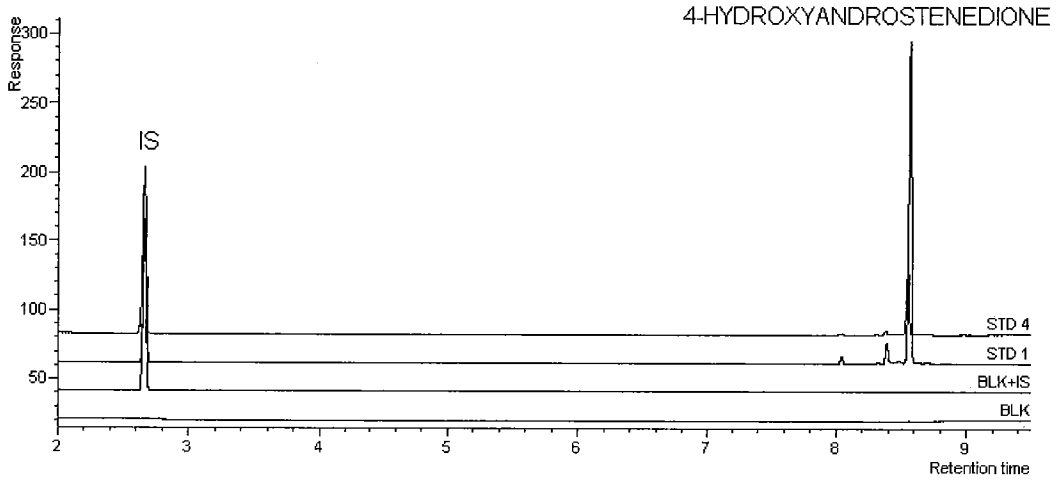

Figure 2 – Representative Overlaid Chromatograms from a Low and High Vehicle/Calibration Standard, Blank with IS, and Blank from the Validation (Shown Top to Bottom)

The regression analysis results from the validation standard curve indicate linearity and are shown in Table 3.

Table 3 – Regression Analysis Validation Results

Slope	y-Intercept	Correlation Coefficient	Standard Error
0.0038	-0.0272	0.9975	0.0565

The vehicle/calibration standard validation results are shown in Table 4.

Table 4 –Vehicle/Calibration Standard Validation Results

Nominal Std Conc (µg/mL)	Det'd Std Conc (µg/mL)	Avg Det'd Std Conc (µg/mL)	s (µg/mL)	% RSD	%RE	Avg %RE
506.4	496.8	509.6	24.2	4.7	-1.9	0.6
	494.5				-2.3	
	537.5				6.1	
298.1	289.4	NA	NA	NA	-2.9	NA
202.6	198.8	NA	NA	NA	-1.9	NA
99.38	100.7	100.4	0.4	0.4	1.3	1.0
	99.89				0.5	
	100.5				1.1	

The method validation sensitivity was 1.266 µg/mL, the LOD, which is defined as three times the standard deviation of the low vehicle/calibration standard. This is equivalent to a formulation concentration of 13 µg/mL when a formulation is diluted 1 to 10 for analysis. The LOQ was 4.219 µg/mL, defined as ten times the standard deviation of the lowest standard because there was no blank response. This is equivalent to a formulation concentration of 42 µg/mL when a formulation is diluted 1 to 10 for analysis. The estimated limit of quantitation (ELOQ), defined as the lowest standard with acceptable accuracy and precision, was 99.38 µg/mL.

4.3.5 Conclusions

The method met all acceptance criteria for precision, accuracy, linearity, sensitivity and specificity. The method was suitable for the stability study and subsequent formulation analyses for which it was used.

5 FORMULATION STABILITY STUDIES

A formulation stability study was conducted at a concentration of 3.02 mg/mL (0.01 M) in 95% ethanol for 173 days in sealed, amber glass bottles stored at approximately 5°C.

5.1 Study Design

A sample was analyzed on the day of preparation (Day 0) and Day 14. A second sample was analyzed on the day of preparation Day 0, Days 27, 54, 83 and 173. Three aliquots were analyzed from each sample at each storage time.

5.2 Formulation Method

A formulation was prepared on November 10, 2004 (Day 0) for the storage stability study at a target concentration of 3.02 mg/mL (0.01 M) in 95% ethanol by accurately weighing 75.50 ± 0.75 mg of 4-OH ASDN into a 25-mL volumetric flask. The chemical was dissolved in and diluted to approximately three quarters of the total volume with 95% ethanol. The flask was sealed, sonicated for 10 minutes and allowed to cool to room temperature. The contents of the flask was diluted to volume with 95% ethanol, sealed, and mixed well.

Approximately 6 mL of formulation was transferred into each of four, 8-mL amber glass vials which were then sealed. One vial was used for the Day 0 analysis and the other three were stored at approximately 5°C until use. After 14 days of storage, a vial was removed from the refrigerator, allowed to warm to room temperature, and triplicate aliquots were prepared and analyzed.

A second formulation was prepared on December 2, 2004 (Day 0) at a target concentration of 3.02 mg/mL (0.01 M) in 95% ethanol by accurately weighing 151.00 ± 0.50 mg into a 50-mL volumetric flask. The content of the flask was diluted to approximately 80% volume with 95% ethanol, sealed and mixed well. The contents of the flask were diluted to volume with 95% ethanol and mixed well. Approximately 18 mL were dispensed into an amber glass bottle, sealed and stored refrigerated. A formulation sample aliquot was prepared for analysis on Days 0, 27, 54, 83 and 173 for storage stability determination.

5.3 Analysis Method

Vehicle/calibration standards, blanks with and without IS were prepared as described in the validation experiment (Section 4.3.1) of this report with the exception that the standard stocks were prepared by accurately weighing 25 ± 1 mg of 4-OH ASDN into 25-mL volumetric flasks.

In triplicate, 1 mL of the formulation and 1 mL of IS were pipetted into three individual 10-mL volumetric flasks, diluted to volume with methanol, sealed and mixed well. An appropriate volume of each was transferred to an autoinjector vial and the vials were sealed and analyzed using the chromatographic system in Table 1.

5.4 Results

The results from the storage stability study are shown in Table 5 and presented in control chart format in Figure 3.

Table 5 – Formulation Storage Stability Results (3.02 mg/mL)

Preparation Date	Analysis Date	Day	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL) ± s	% of Day 0 Conc ± s
11/10/04	11/10/04	0	2.871	2.873	2.928	2.891 ± 0.032	100.0 ± 1.1
11/10/04	11/24/04	14	3.006	3.085	3.149	3.080 ± 0.072	106.5 ± 2.5
12/2/04	12/2/04	0	3.005	3.022	3.005	3.011 ± 0.010	100.0 ± 0.3
12/2/04	12/29/04	27	3.168	3.123	3.117	3.136 ± 0.028	104.2 ± 0.9
12/2/04	1/25/05	54	3.008	3.126	3.110	3.081 ± 0.064	102.3 ± 2.1
12/2/04	2/23/05	83	3.027	3.131	3.217	3.125 ± 0.095	103.8 ± 3.2
12/2/04	5/24/05	173	3.126	3.142	3.129	3.133 ± 0.008	104.1 ± 0.3

For the sample prepared November 10, 2004, the pooled RSD of the analytical method was 1.9%. This means that there would have to be a difference of more than 4.4% from the Day 0 value for the difference to be statistically significant at a 95% confidence level.

For the sample prepared December 2, 2004, the pooled RSD of the analytical method was 1.8%. This means that there would have to be a difference of more than 4.0% from the Day 0 value for the difference to be statistically significant at a 95% confidence level.

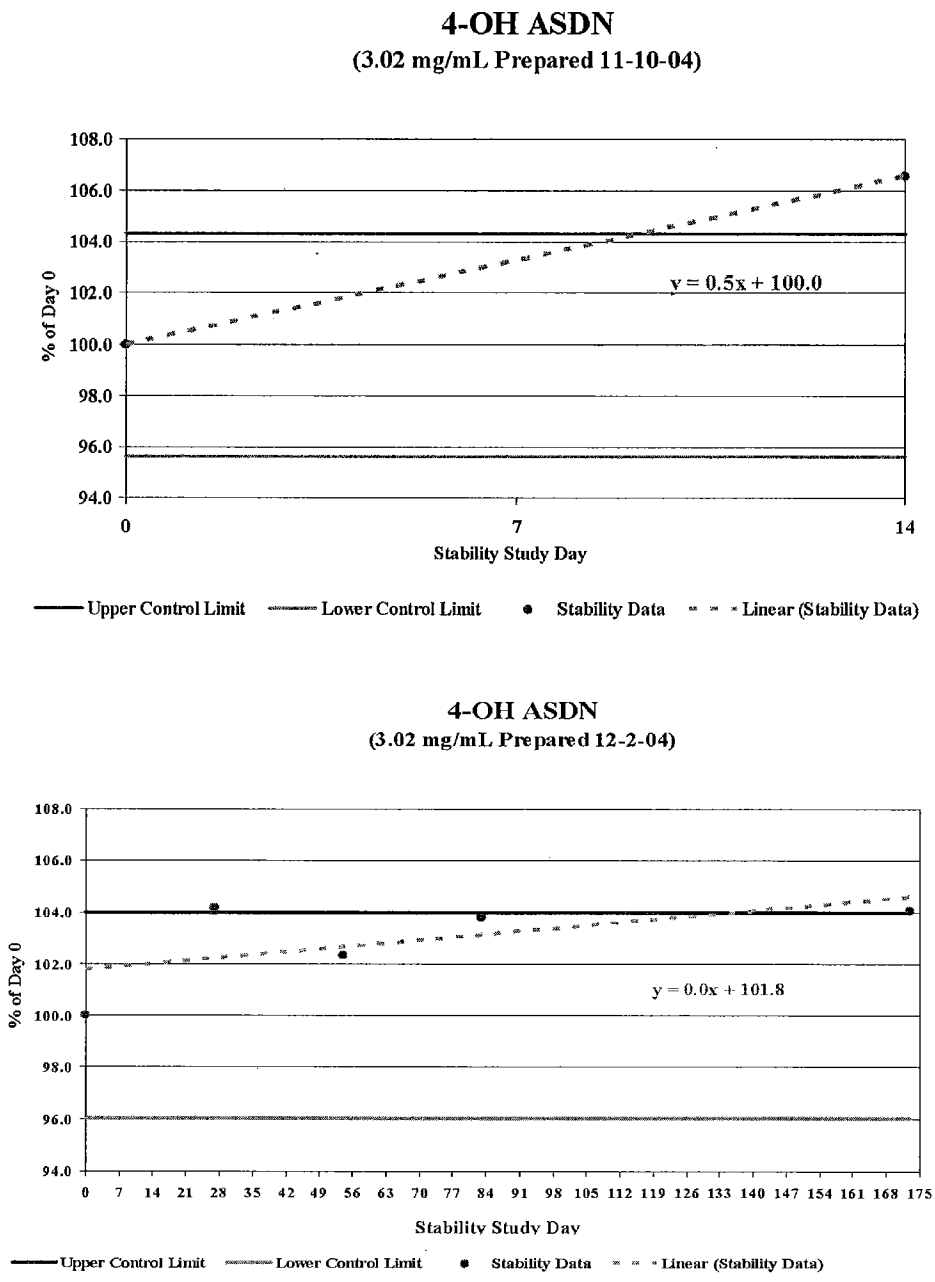

Figure 3 – Control Charts for the Storage Stability Studies

5.5 Discussion and Conclusions

The concentration of the samples stored at approximately 5°C protected from light in amber glass vials for Day 14 was above the upper significance level, but was within 6.5% of the Day 0 value (prepared November 10, 2004). Concentrations for Days 54 and 83 samples were within the upper and lower significance levels and Days 27 and 173 were just above the upper significant level. A linear trend analysis indicated there was no significant trend to changing concentration over time for the samples. These data indicate the formulation was stable when stored protected from light at approximately 5°C for 173 days.

6 FORMULATION PREPARATIONS AND ANALYSES

Formulations were prepared and analyzed on December 2, 2004, January 25, 2005, March 21, 2005, and June 27, 2005, according to SOP No. COMSPEC.II-027, "Standard Operating Procedure (SOP) for the Formulation and Analysis of 4-Hydroxyandrostenedione (4-OH ASDN) in 95% Ethanol." This section describes the method, results, and conclusions.

6.1 Preparation of Formulations

An accurate weight of 151.00 ± 0.50 mg of 4-OH ASDN was added to a 50-mL volumetric flask. The content of the flask was diluted to approximately 80% volume with 95% ethanol, sealed and mixed well. The contents of the flask were diluted to volume with 95% ethanol and mixed well. This produced a target concentration of 3.02 mg/mL (0.01 M) 4-OH ASDN in 95% ethanol.

6.2 Preparation of Standards and Blanks

Standards and blanks were prepared as described for the method validation, Section 4.3.1 of this report.

6.3 Preparation of Formulation Samples

One (1) mL of the formulation and 1-mL of IS were pipetted into three individual 10-mL volumetric flasks, diluted to volume with methanol, sealed, and mixed well.

6.4 Analysis

Autoinjector vials were filled with aliquots of each standard, blank and sample. A single injection was made from each vial using the conditions from the method validation (Table 1).

6.5 Calculations

The peaks for 4-hydroxyandrostenedione and the IS were integrated for each injection by the chromatography data system. Any peak with improper integration was manually reintegrated. A linear regression equation weighted 1/x was calculated relating the response ratio (4-hydroxyandrostenedione/IS) (y) to the concentration of the vehicle/calibration standards (x). This regression equation and the response ratios

were used to calculate the concentration in each standard and formulation sample. The percent RE for each standard was calculated by subtracting the nominal value from the determined value, dividing by the nominal value, and then multiplying by 100. The percent RE for each formulation sample was calculated by subtracting the target value from the determined value, dividing by the target value, and then multiplying by 100. The average determined concentration, standard deviation, and percent RSD were calculated for the vehicle/ calibration standards and formulation samples when applicable.

6.6 Results

Specificity is shown by the representative overlaid chromatograms of the high and low standards, blank with IS and a blank presented in Figure 4.

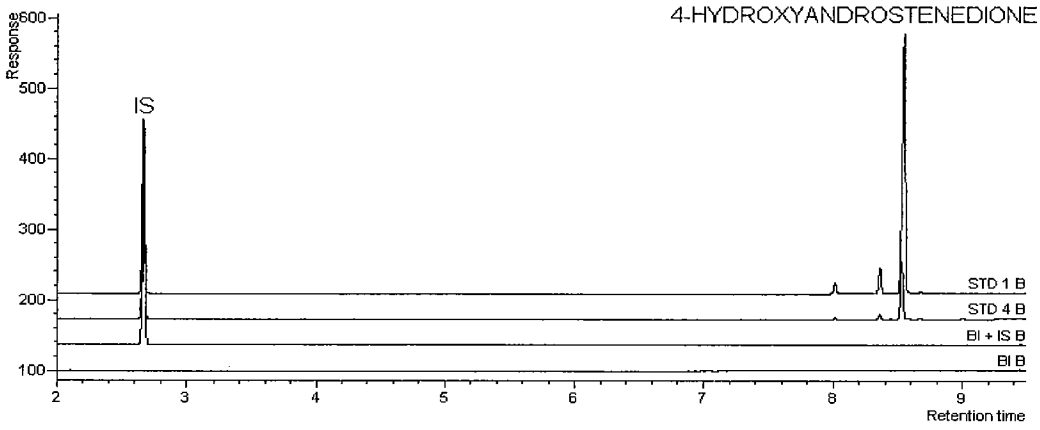

Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from Formulation Analysis

The regression analysis results of the vehicle/calibration standard curves indicated linearity and are shown in Table 6.

Table 6 – Regression Analysis Results

Slope	y-Intercept	Correlation Coefficient	Standard Error
0.0038	-0.0140	0.9999	0.0117
0.0035	-0.0037	1.000	0.0061
0.0036	-0.0251	0.9999	0.0100
0.0038	-0.0218	0.9999	0.0104

The results of the formulation analyses are shown in Table 7.

Table 7 – Formulation Analysis Results

Batch	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL)	Avg % RE	% RSD
1-ASDN	3.005	3.022	3.005	3.011	-0.3	0.3
2-ASDN	3.056	3.089	3.049	3.065	1.4	0.7
3-ASDN	3.112	3.053	3.063	3.076	1.9	1.0
4-ASDN	2.943	2.945	2.950	2.946	-2.5	0.1

The formulations met acceptance criteria (RE within 10% of target and RSD of ≤ 10%).

6.7 Conclusions

The average concentration of the stock formulations and their percent RSD were within acceptance criteria. Therefore, the formulations were suitable for use.

7 ACKNOWLEDGMENTS

Analytical support for this work was provided by Sandy Runyon, Christina Zielinski, Tudor Fernando, Kevin Carrico, and Darren Brown. The report was written by Denise Contos. Review of the data and report for completeness and accuracy was performed by Maria Evascu.

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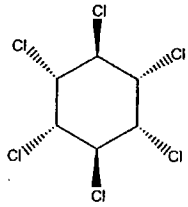
ANALYTICAL CHEMISTRY ACTIVITIES REPORT

LINDANE

CAS No.: 58-89-9
Lot No.: 14419EB (Sigma Aldrich)
Receipt Date: 1/6/05
Amount Received: 10 g
Appearance: White Solid
Vendor Purity: 99.6% by GC
Storage Conditions (@ Battelle): Room temperature (~25°C)

STRUCTURE:



Mol. Wt.: 290.83 g/mol

Mol. Formula: $C_6H_6Cl_6$

Prepared By:

Denise A. Contos, M.S.

Approved By:

Steven W. Graves, B.S.
Manager, Chemistry Technical Center

Battelle Study No. WA 4-16/17

QUALITY ASSURANCE STATEMENT

This study was inspected by the Quality Assurance Unit (QAU) and reports were submitted to the Study Director and Management as follows:

Phase Inspected	Inspection Date	Date Reported to Study Director/Management
Test substance receipt*	10/26/2004	10/26/2004
Formulation preparation*	12/2/2004	12/2/2004
Dispensing*	12/2/2004	12/2/2004
Formulation analysis*	12/2/2004	12/2/2004
Audit analytical report	12/22/2005	12/22/2005
Audit study file	12/22/2005	12/22/2005

* These inspections are serving the purpose for all reference chemicals since QA was required to see only one phase inspection of a chemical.

Kathleen E. Reed 1-6-06

Quality Assurance Unit Date

EXECUTIVE SUMMARY

The title compound, lindane, was analyzed in support of the Environmental Protection Agency (EPA) Placental and Recombinant Aromatase Assay Prevalidation Work, Work Assignment 4-16/17.

Solubility of lindane was determined to be acceptable in dimethylsulfoxide (DMSO) for preparing formulations.

A formulation analysis method was developed and validated to analyze lindane in DMSO at a concentration of 29.08 mg/mL (0.1M). This method was used to analyze samples from both formulation and formulation storage stability studies at 29.08 mg/mL.

Storage stability study indicated that a 29.08 mg/mL formulation stored in sealed amber glass bottles and protected from light was stable for 168 days at approximately 5°C.

The formulations prepared for shipment to the testing laboratory were determined and met the established acceptance criteria.

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LIST OF FIGURES

1 INTRODUCTION

The purpose of this work was to provide all necessary chemistry support activities for lindane on Environmental Protection Agency (EPA) Work Assignment 4-16/17, and consisted of:

- Determining solubility in dimethylsulfoxide (DMSO).
- Developing and validating a formulation analysis method.
- Conducting a storage stability study.
- Preparing and analyzing a stock formulation.

This work was done at Battelle, 505 King Avenue, Columbus, OH 43201.

2 CHEMICAL RECEIPT AND STORAGE

One 20-mL amber glass bottle of lindane, 14419EB, was received from the repository at Battelle's Marine Sciences Laboratory in Sequim, WA on January 6, 2005. The label amount indicated 10 grams was sent. The chemical was received and subsequently stored at room temperature.

A copy of the manufacturer's Certificate of Analysis for this lot is shown in Figure 1. This states that purity was 99.6% based on gas chromatography (GC).

SIGMA-ALDRICH

Certificate of Analysis

Product Name	Lindane
Product Number	23,339-0
Product Brand	ALDRICH
CAS Number	58-89-9
Molecular Formula	$C_6H_6Cl_6$
Molecular Weight	290.83

TEST	SPECIFICATION	LOT 14419EB RESULTS
APPEARANCE	WHITE TO OFF-WHITE POWDER	OFF WHITE POWDER
INFRARED SPECTRUM	CONFORMS TO STRUCTURE AND STANDARD.	CONFORMS TO STRUCTURE AND STANDARD
GAS LIQUID CHROMATOGRAPHY	96.5% (MINIMUM)	99.6%
QUALITY CONTROL ACCEPTANCE DATE		MAY, 2003

Ronnie J. Martin, Supervisor
Quality Control
Milwaukee, Wisconsin USA

Figure 1 – Certificate of Analysis

3 SOLUBILITY STUDIES

A solubility study was conducted to determine the solubility of lindane in 100% DMSO, at a concentration of at least 29.08 mg/mL. Lindane (0.29080 ± 0.02908 g) was weighed into a 10-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The contents were mixed until the lindane dissolved. The contents of the flask were diluted to volume with DMSO, sealed, and mixed well. The lindane went readily into solution. This experiment showed that DMSO was an acceptable solvent for the 29.08 mg/mL formulation.

4 FORMULATION ANALYSIS METHOD PERFORMANCE EVALUATION (MPE)

This section describes the evaluation of a method developed to analyze formulations of lindane in DMSO at a target concentration of 29.08 mg/mL for the stability study and the results and conclusions from this evaluation.

4.1 Method Development

Method development for this chemical involved the evaluation of various chromatographic conditions. The selected method was one which produced acceptable retention time for the major peak, apparent resolution of significant impurities and acceptable peak shape. The detection method chosen was gas chromatography with flame ionization detection (FID).

4.2 Method

The GC parameters for lindane are presented in Table 1.

Table 1 – GC System

GC	Agilent 6890 (Palo Alto, CA)
Column	RTX-5, 30 m × 0.25 mm (ID), 0.25 µm film thickness (Restek, Bellefonte, PA)
Carrier Gas and Flow Rate	Helium at ~2 mL/minute
Oven Temperature	150°C, hold for ~2 minutes, increase at 20°C/minute to 300°C; hold for 2 minutes
Detector Type	Flame Ionization (FID)
Detector Flow Rates	Hydrogen at ~30 mL/minute; Air at ~380 mL/minute
Detector Temperature	320°C
Injector Temperature	285°C
Injection Volume	1 µL
Injection Mode	Split 5:1
Run Time	~12 minutes

4.3 Method Validation

Validation was accomplished using a single experiment.

Triplicate vehicle/calibration standards at the highest and lowest of four concentrations were prepared. A single standard was prepared at each intermediate concentration. The high and low concentrations were used to assess the precision of the method. The precision of the low concentration was used to calculate limits of detection (LOD) and limits of quantitation (LOQ). Triplicate vehicle/calibration blanks with and without working internal standard (WIS) were used to assess the specificity of the method.

4.3.1 Preparation of Standards and Blanks

4.3.1.1 Internal Standard (IS)

Approximately 25 ± 1 mg of phenanthrene was added to a 25-mL volumetric flask. The contents of the flask was diluted to volume with methanol, sealed, and mixed well.

The IS was prepared by pipetting 10 mL of stock IS into a 25-mL volumetric flask. The contents of the flask was diluted to volume with methanol, sealed, and mixed well.

4.3.1.2 Stock Standards

Two stock standards were prepared by accurately weighing 50 ± 2 mg of lindane each into two individual 25-mL volumetric flasks and dissolving in and diluting to volume with methanol. This produced stocks A and B with target concentrations of 2000 µg/mL each.

4.3.1.3 Vehicle/Calibration Standards

Vehicle/calibration standards were prepared as shown in Table 2. The contents of the flasks were diluted to volume with methanol and mixed well. Triplicate vehicle/calibration standards were prepared at the low and high concentrations with single vehicle/calibration standards prepared at the two middle concentrations.

Table 2 – Preparation of Vehicle/Calibration Standards

Vehicle/Calibration Std	Target Final Conc (µg/mL)	Source	Source Volume (mL)	WIS (mL)	DMSO (mL)	Final Volume (mL)
VS1	800	A	4	1	0.1	10
VS2	600	B	3	1	0.1	10
VS3	400	A	2	1	0.1	10
VS4	200	B	1	1	0.1	10

4.3.1.4 Blanks

Triplicate blanks without IS were prepared by pipetting 0.1 mL of DMSO into three individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with methanol, sealed, and mixed well.

Triplicate blanks with IS were prepared by pipetting 1 mL IS and 0.1 mL of DMSO into three individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with methanol, sealed, and mixed well.

4.3.2 Analysis

A portion of each vehicle/calibration standard and blank was transferred to individual autoinjector vials and the vials were sealed. Single injections were made from each vial using the same chromatographic system and parameters determined during method development as shown in Table 1.

4.3.3 Calculations

The integration of the lindane and IS peaks by the chromatography data system was evaluated to assure it was consistent in all chromatograms and manually reintegrated, if necessary. A linear regression equation was calculated relating the response ratio of lindane divided by the IS (y) to the concentration of

the vehicle/calibration standards (x). The concentration of each vehicle/calibration standard was calculated using its individual response ratio and the regression equation. These values were used to calculate the individual and average concentrations, percent relative errors (RE), standard deviation (s), and percent relative standard deviation (RSD) as appropriate for the vehicle/calibration standards at each concentration.

4.3.4 Results

Specificity is shown by the representative overlaid chromatograms from a high and low vehicle/calibration standard, a blank with IS, and a blank from the validation as indicated in Figure 2. The blank and blank with IS exhibited no peaks that would significantly interfere with the lindane or IS peaks. The regression analysis results from the standard curve indicate the linearity and are shown in Table 3.

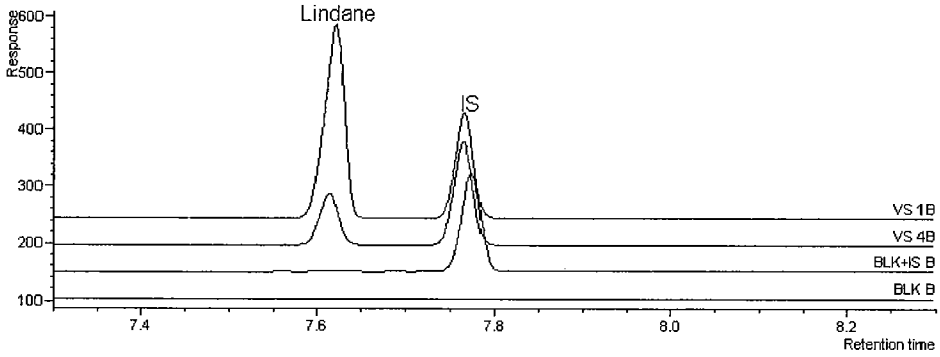


Figure 2 – Representative Overlaid Chromatograms from a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from the Validation (Shown Top to Bottom)

Table 3 – Method Validation Regression Analysis Results

Slope	y-Intercept	Correlation Coefficient	Standard Error
0.0027	-0.0351	1.000	0.0047

The precision and accuracy of the vehicle/calibration standard validation results are shown in Table 4.

Table 4 – Vehicle/Calibration Standard Validation Results

Nominal Std Conc (µg/mL)	Det'd Std Conc (µg/mL)	Avg Det'd Std Conc (µg/mL)	s (µg/mL)	%RSD	%RE	Avg %RE
776.3	777.3	776.8	1.1	0.1	0.1	0.1
	777.6				0.2	
	775.6				-0.1	
600.2	598.4	NA	NA	NA	-0.3	NA
388.2	387.0	NA	NA	NA	-0.3	NA
200.1	202.8	200.5	2.1	1.1	1.4	0.2
	200.1				0.0	
	198.6				-0.7	

The sensitivity of the method resulted in 6.4 µg/mL LOD which is defined as three times the standard deviation of the low vehicle/calibration standard. This is equivalent to a formulation concentration of 640 µg/mL when a formulation is diluted 1 to 100 for analysis. The LOQ, defined as ten times the standard deviation of the lowest standard because there was no blank response, was 21.3 µg/mL. This is equivalent to a formulation concentration of 2130 µg/mL when a formulation is diluted 1 to 100 for analysis. The estimated limit of quantitation (ELOQ), defined as the lowest standard with acceptable accuracy and precision, was 200.1 µg/mL.

4.3.5 Conclusions

The method met all acceptance criteria for specificity, linearity, precision, accuracy, and sensitivity. The method was suitable for the stability study and subsequent formulation analyses.

5 FORMULATION STABILITY STUDIES

A formulation stability study was conducted at a target concentration of 29.08 mg/mL in DMSO for 168 days (24 weeks) in sealed, amber glass bottles stored at approximately 5°C.

5.1 Study Design

A single sample was analyzed on the day of preparation (Day 0), Day 14, Weeks 4, 8 and 12. A second formulation sample was prepared and analyzed on January 24, 2005 (Day 0) and on Week 24. Three aliquots were analyzed from each sample at each storage time.

5.2 Formulation Method

A formulation was prepared on January 13, 2005, Day 0 of the storage stability study at a target concentration of 29.08 mg/mL in DMSO by accurately weighing 727 ± 7 mg of lindane into a 25-mL volumetric flask. The chemical was dissolved in and diluted to approximately three quarters of the total volume with

DMSO. The flask was sealed and manually shaken to mix the contents. The contents of the flask was diluted to volume with DMSO, sealed, and mixed well.

Approximately 6 mL of formulation was transferred into each of four, 8-mL amber glass vials which were then sealed. One vial was used for the Day 0 analysis and the other three were stored at approximately 5°C until use. After the desired storage period, a vial was removed from storage, allowed to warm to room temperature, and triplicate aliquots were prepared and analyzed.

A second formulation (Batch 1-LIN-1) was prepared on January 24, 2005 (Day 0) at a target concentration of 29.08 mg/mL in DMSO by accurately weighing 1.45400 ± 0.058 g into a 50-mL volumetric flask. The content of the flask was diluted to approximately 80% volume with DMSO, sealed and mixed well. The contents of the flask was diluted to volume with DMSO and mixed well. Approximately 9 mL were dispensed into an amber glass bottle, sealed and stored refrigerated. A formulation sample aliquot was prepared for analysis on Days 0 and 168 for storage stability determination.

5.3 Analysis Method

Vehicle/calibration standards and blanks with and without IS were prepared as described in the validation experiment (Section 4.3.1) of this report.

One (1) mL of the formulation was pipetted into three individual 10-mL volumetric flasks, diluted to volume with methanol, sealed, and mixed well. One (1) mL of the diluted formulation and 1-mL of IS were pipetted into 10-mL volumetric flasks, diluted to volume with methanol, sealed, and mixed well. An appropriate volume of each was transferred to an autoinjector vial and the vials were sealed and analyzed using the chromatographic system in Table 1.

5.4 Results

The results from the storage stability study are shown in Table 5 and presented in control chart format in Figure 3.

Table 5 – Formulation Storage Stability Results (29.08 mg/mL)

Preparation Date	Analysis Date	Day	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL) ± s	% of Day 0 Conc ± s
1/13/05	1/13/05	0	29.38	29.48	29.18	29.35 ± 0.15	100 ± 0.5
1/13/05	1/27/05	14	28.56	28.56	28.67	28.60 ± 0.06	97.4 ± 0.2
1/13/05	2/10/05	28	31.36	31.30	31.64	31.43 ± 0.18	107 ± 0.6
1/13/05	3/10/05	56	28.77	28.76	28.65	28.73 ± 0.07	97.9 ± 0.2
1/13/05	4/7/05	84	29.22	29.67	29.47	29.45 ± 0.23	100 ± 0.8
1/24/05	1/24/05	0	30.02	29.88	29.93	29.95 ± 0.07	100 ± 0.2
1/24/05	7/11/05	168	29.64	29.72	29.95	29.77 ± 0.16	99.4 ± 0.5

For the formulation sample prepared on January 13, 2005, the pooled relative standard deviation of the analytical method was 0.5%. This means that there would have to be a difference of more than 1.2% from the Day 0 value for the difference to be statistically significant at a 95% confidence level.

For the formulation sample prepared on January 24, 2005, the pooled RSD of the analytical method was 0.6%. This means that there would have to be a difference of more than 1.3% from the Day 0 value for the difference to be statistically significant at a 95% confidence level.

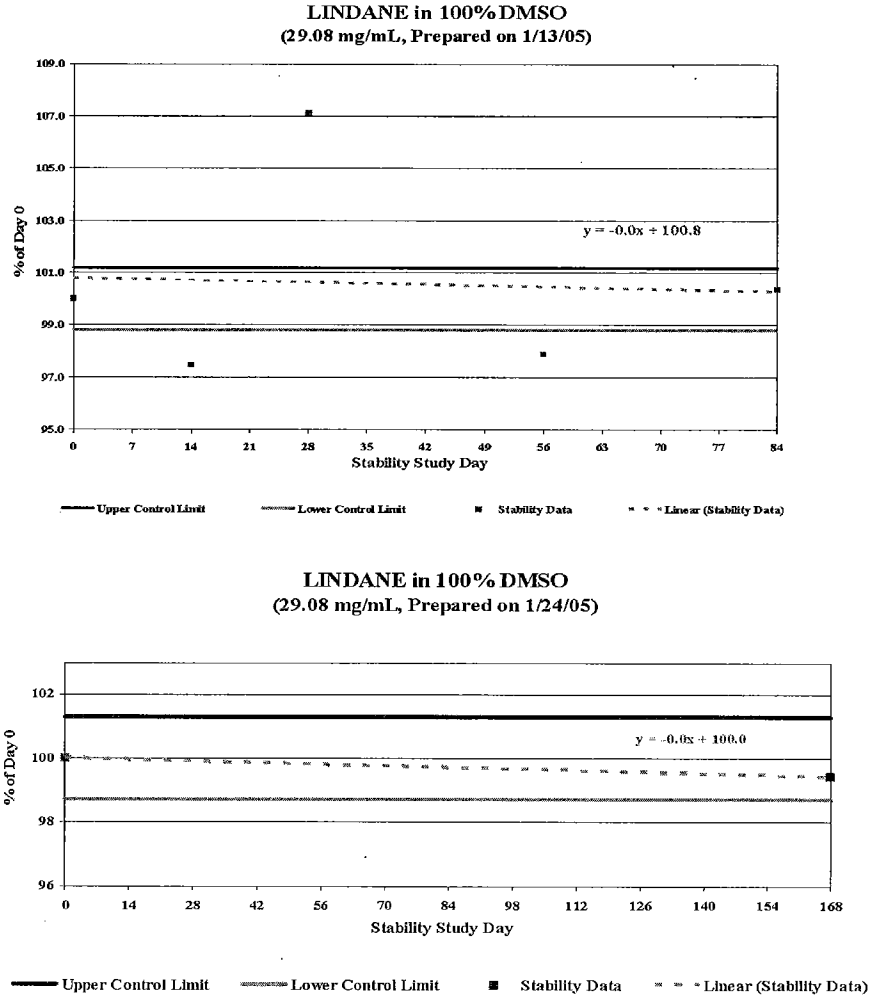


Figure 3 – Control Chart for the Storage Stability Study

5.5 Discussion and Conclusions

The Day 0 determined value for the formulation prepared on January 13, 2005 was approximately 1.0% above nominal (the calculated concentration based on the weight of the chemical). The concentrations of the samples stored at approximately 5°C protected from light in amber glass vials for Days 14 and 56 were below the lower significance level and for Day 28 it was above the upper significance level due to the tight precision of the assay. The average concentrations of the samples were within 2.6% (Day 14), 7.1% (Day 28), 2.1% (Day 56), and 0.4% (Day 84) of the Day 0 value and met acceptance criteria of ± 10%. These data indicate the formulation was stable at approximately 5°C for 84 days.

The formulation stability sample prepared on January 24, 2005 (Day 0) and analyzed on Day 0 and Day 168 (July 11, 2005) was approximately 3.0% above nominal for Day 0 (the calculated concentration based on the weight of the chemical) and for Day 168, 0.6% below the Day 0 value and met acceptance criteria of ± 10%. These data indicate the formulation was stable at approximately 5°C protected from light for 168 days.

6 FORMULATION PREPARATION AND ANALYSIS

Formulations were prepared and analyzed on January 24, 2005, March 21, 2005 and July 1, 2005, according to SOP COMSPEC.II-029, "Standard Operating Procedure (SOP) for the Formulation and Analysis of Lindane in 100% Dimethylsulfoxide (DMSO)." This section describes the method, results, and conclusions.

6.1 Preparation of Formulation

Lindane (1.45400 ± 0.058 g) was weighed into a 50-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The contents were mixed until the lindane dissolved. The contents of the flask were diluted to volume with DMSO, sealed, and mixed well.

6.2 Preparation of Standards and Blanks

Standards and blanks were prepared as described for the validation (Section 4.3.1 of this report).

6.3 Preparation of Formulation Samples

One (1) mL of the formulation was pipetted into three individual 10-mL volumetric flasks, diluted to volume with methanol, sealed, and mixed well. One (1) mL of the diluted formulation and 1-mL of IS were pipetted into individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with methanol, sealed, and mixed well.

6.4 Analysis

Autosampler vials were filled with aliquots of each standard, blank and sample. A single injection was made from each vial using the GC conditions from the validation (Table 1). Representative overlaid chromatograms of the high and low vehicle/calibration standards, blank with IS, and a blank are shown in Figure 4.

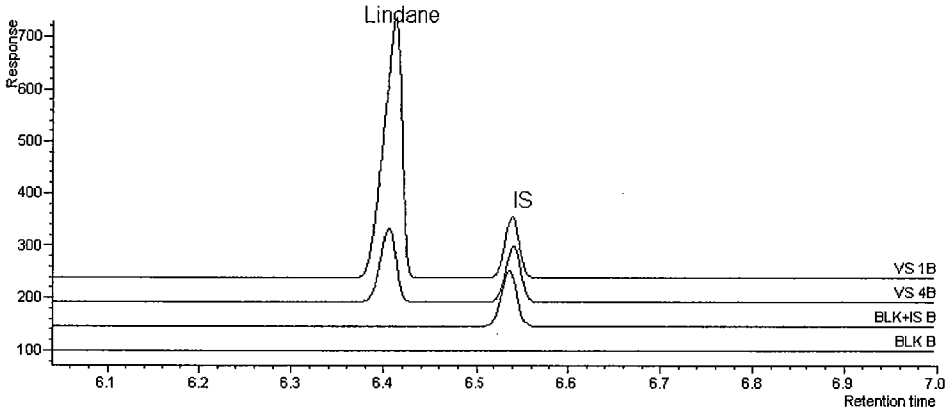


Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from a Formulation Analysis (Shown Top to Bottom)

6.5 Calculations

The peaks for lindane and the IS were integrated for each injection by the chromatography data system. Any peak with improper integration was manually reintegrated. A linear regression equation was calculated relating the response ratio (lindane/IS) to the concentration of the vehicle/calibration standards. This regression equation and the response ratios were used to calculate the concentration in each standard and formulation sample. The percent RE for each standard was calculated by subtracting the nominal value from the determined value, dividing by the nominal value, and then multiplying by 100. The percent RE for each formulation sample was calculated by subtracting the target value from the determined value, dividing by the target value, and then multiplying by 100. The average determined concentration, standard deviation, and percent RSD were calculated for the vehicle/calibration standards and formulation samples when applicable.

6.6 Results

The regression analysis results of the vehicle/calibration standard curves indicated linearity and are shown in Table 6.

Table 6 – Formulation Regression Analysis Results

Formulation Date	Slope	y-Intercept	Correlation Coefficient
1/24/05	6.8029	-0.0081	1.000
3/21/05	7.2898	-0.0197	1.000
7/1/05	6.8477	-0.1022	1.000

The results of the formulation analysis are shown in Table 7. Formulations met all acceptance criteria (RE within 10% of target and RSD of ≤ 10%).

Table 7 – Formulation Analysis Results

Formulation Date	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL)	Avg %RE	%RSD
1/24/05	30.02	29.88	29.93	29.95	3.0	0.2
3/21/05	29.23	29.67	29.20	29.37	1.0	0.9
7/1/05	29.32	29.26	29.63	29.40	1.1	0.7

6.7 Conclusions

The average concentration of the formulations and its percent RSD were within acceptance criteria. Therefore the formulation was suitable for use.

7 ACKNOWLEDGMENTS

Analytical support for this work was provided by Barb Harritos, Darren Brown, John Kelly, Christina Zielinski, Jim Hoskinson, Melinda Pauff, Tudor Fernando, and Sandy Runyon. The report was written by Denise Contos. Review of the data and report for completeness and accuracy was performed by Maria Evascu.

Appendix E

Assay Date	4/14/2005	Test Chemical ID	NA	# Concentrations tested	NA				
Technician ID	TD	Replicate #	1	Microsome type	placental	Microsome ID	6-041305		

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	1	0.8	0.6	0.4	0.2	0.11	Blank	28	20	210000238
	0.303	0.255	0.204	0.149	0.084	0.033	0.000			
	0.299	0.259	0.206	0.144	0.079	0.053	0.000			
	0.293	0.255	0.205	0.138	0.080	0.055	0.000			
Samples:	6-041305	QC1	QC2	QC3						
	0.148	0.040	0.150	0.277						
	0.152	0.039	0.158	0.294						
	0.158									

Standard concentration (mg/mL)	Volume of stock used	Final volume of Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results	
1	17.9	25	0.00100	25	0.0251	0.298	0.298	0.0240	m, b	0.087	-0.002
0.8	14.3	25	0.00080	25	0.0200	0.256	0.256	0.0203	se_m, se_b	0.004	0.001
0.6	10.7	25	0.00060	25	0.0150	0.205	0.205	0.0159	r^2, se_y	0.992	0.001
0.4	7.1	25	0.00040	25	0.0099	0.144	0.144	0.0105	F, df	517	4
0.2	3.6	25	0.00020	25	0.0050	0.061	0.061	0.0051	ss_{reg}, ss_{resid}	0.000	0.000
0.11	2	25	0.00011	25	0.0028	0.047	0.047	0.0021			

Regression results are calculated using the function LINEST

Blank	0.000	r^2=	0.992
		m=	0.087
		b=	-0.002

	A_{raw}	$A_{adj.}$	mg protein measured	µL diluted µSOMES	Vol usome prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
6-041305	0.148	0.148	0.011	25	100	5000	0.022	0.023	22.579
6-041305	0.152	0.152	0.011	25	100	5000	0.022		
6-041305	0.158	0.158	0.012	25	100	5000	0.024		
QC1	0.040	0.040	0.002	25	1	1	0.000	0.000	0.059
QC1	0.039	0.039	0.001	25	1	1	0.000		
QC1									
QC2	0.150	0.150	0.011	25	1	1	0.000	0.000	0.458
QC2	0.158	0.158	0.012	25	1	1	0.000		
QC2									
QC3	0.277	0.277	0.022	25	1	1	0.001	0.001	0.915
QC3	0.294	0.294	0.024	25	1	1	0.001		
QC3									

Assay Date 4/15/2005 | Test Chemical ID NA | # Concentrations tested NA

Technician ID TD | Replicate # 2 | Microsome type placental | Microsome ID 6-041305

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	1	0.8	0.6	0.4	0.2	0.11	Blank	28	20	210000239
	0.315	0.263	0.202	0.154	0.081	0.045	0.000			
	0.319	0.265	0.207	0.154	0.076	0.050	0.000			
	0.323	0.269	0.211	0.155	0.074	0.054	0.000			

Samples:	6-041305	QC1	QC2	QC3
	0.140	0.042	0.155	0.284
	0.146	0.042	0.165	0.299
	0.146			

Standard concentration (mg/mL)	Volume of stock used	Final volume of Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output
1	17.9	25	0.00100	25	0.0251	0.319	0.319	0.0245
0.8	14.3	25	0.00080	25	0.0200	0.266	0.266	0.0201
0.6	10.7	25	0.00060	25	0.0150	0.206	0.206	0.0152
0.4	7.1	25	0.00040	25	0.0099	0.154	0.154	0.0110
0.2	3.6	25	0.00020	25	0.0050	0.077	0.077	0.0046
0.11	2	25	0.00011	25	0.0028	0.050	0.050	0.0024

Variables	Regression results	
m, b	0.082	-0.002
se_m, se_b	0.003	0.001
r^2, se_y	0.995	0.001
F, df	842	4
ss_{reg}, ss_{resid}	0.000	0.000

Regression results are calculated using the function LINEST

Blank 0.000

r^2= 0.995
m= 0.082
b= -0.002

	A_{raw}	$A_{adj.}$	mg protein measured	µL diluted µSOMES	Vol usome prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
6-041305	0.140	0.140	0.010	25	100	5000	0.020	0.020	20.217
6-041305	0.146	0.146	0.010	25	100	5000	0.021		
6-041305	0.146	0.146	0.010	25	100	5000	0.021		
QC1	0.042	0.042	0.002	25	1	1	0.000	0.000	0.070
QC1	0.042	0.042	0.002	25	1	1	0.000		
QC1									
QC2	0.155	0.155	0.011	25	1	1	0.000	0.000	0.457
QC2	0.165	0.165	0.012	25	1	1	0.000		
QC2									
QC3	0.284	0.284	0.022	25	1	1	0.001	0.001	0.888
QC3	0.299	0.299	0.023	25	1	1	0.001		
QC3									

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0194	29878	1538517	
2	0.0198	30852	1558182	
3	0.0194	30382	1563664	
4	0.0192	30551	1591198	
5	0.0197	32416	1645482	
			Average DPM/g soln	1579409
			SD	41459
			CV	2.62
			µCi/g soln	0.711

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	11.03	10		1103.00
Dilution A			100	11.03
Dilution B			10	1.10

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	1.60253 g
Mass of dilution B used in substrate prep	0.90144 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.620449 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00805 µg/g soln.
µg/g soln.

a. µCi/g soln 0.711
b. Specific activity of [^{3}H]ASDN (µCi/mmol) 25300000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.620449 + 0.00805
= 0.628503 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.132 µCi/µg ASDN

719715 dpm/nmol

Assay Date	4/15/2005	Test Chemical ID	NA	# Concentrations tested	NA					
Technician ID	LR	Replicate #	1	Microsome type	placental	Microsome ID	6-041305	Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
								28	20	210000238

Standards:	1	0.8	0.6	0.4	0.2	0.11	Blank
	0.293	0.250	0.192	0.137	0.050	0.028	0.000
	0.268	0.235	0.203	0.119	0.056	0.034	0.000
	0.281	0.230	0.194	0.199	0.057	0.066	0.000

Samples:	6-041305	QC1	QC2	QC3
	0.134	0.041	0.162	0.296
	0.125	0.034	0.159	0.282
	0.127			

Standard concentration (mg/mL)	Volume of stock used	Final volume of Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output
1	17.9	25	0.00100	25	0.0251	0.281	0.281	0.0235
0.8	14.3	25	0.00080	25	0.0200	0.238	0.238	0.0198
0.6	10.7	25	0.00060	25	0.0150	0.196	0.196	0.0161
0.4	7.1	25	0.00040	25	0.0099	0.152	0.152	0.0122
0.2	3.6	25	0.00020	25	0.0050	0.054	0.054	0.0036
0.11	2	25	0.00011	25	0.0028	0.043	0.043	0.0026
			Blank	0.000				

Variables	Regression results	
m, b	0.088	-0.001
se_m, se_b	0.008	0.001
r^2, se_y	0.972	0.002
F, df	137	4
ss_{reg}, ss_{resid}	0.000	0.000

Regression results are calculated using the function LINEST

r^2= 0.972
m= 0.088
b= -0.001

	A_{raw}	$A_{adj.}$	mg protein measured	µL diluted µSOMES	Vol usome prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
6-041305	0.134	0.134	0.011	25	100	5000	0.021	0.020	20.270
6-041305	0.125	0.125	0.010	25	100	5000	0.020		
6-041305	0.127	0.127	0.010	25	100	5000	0.020		
QC1	0.041	0.041	0.002	25	1	1	0.000	0.000	0.084
QC1	0.034	0.034	0.002	25	1	1	0.000		
QC1									
QC2	0.162	0.162	0.013	25	1	1	0.001	0.001	0.517
QC2	0.159	0.159	0.013	25	1	1	0.001		
QC2									
QC3	0.296	0.296	0.025	25	1	1	0.001	0.001	0.970
QC3	0.282	0.282	0.024	25	1	1	0.001		
QC3									

Assay Date	4/15/2005	Test Chemical ID	NA	# Concentrations tested	NA	Microsome type	placental	Microsome ID	6-041305	Technician ID	LR	Replicate #	1

Microsome Dilution Details		
Dilution A	0.1	mL microsome Stock used
	5	mL total volume
	50	dilution factor
Dilution B	1	mL microsome Dilution A used
	18	mL total volume
	18	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	900	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	20.27
Protein Concentration (dilution added to assay, mg/mL):	0.022522

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-04
2	1.00E-05
3	2.50E-06
4	1.00E-06
5	5.00E-07
6	2.50E-07
7	1.00E-07
8	1.00E-08

Assay Date	4/15/2005	Test Chemical ID	NA	# Concentrations tested	NA	Microsome type	pooled rat	Microsome ID	6-041305	Test Initials ID	LR	Replicate #	1

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate nmol $^{3}H_2O$ formed					
Sample Type	Replicate Level	Nominal total volume (mL)	Aliq volume (mL)	Aliq.#	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate solution in necessary tube (mL)	total DPM in assay tube (dilute)	% conversion to product	Total DPM corrected for background (background Tubes)	nmol $^{3}H_2O$ formed	Volume diluted in microcentrifuge tube in assay (mL)	Final [protein] in assay (mg/mL)	Incubation time (min)	Aromatase activity (pmoles tritiated water formed/mg protein/min)
Full activity control	1	2	0.5	1	2752	5504	5617	11034	0.1	157941	6.99	10905	0.0152	1	0.011	15	0.0450
			0.5	2	2758	5530			0.1					1		15	
	2	2	0.5	1	2709	5416	5516	11032	0.1	157941	6.98	10903	0.0152	1	0.011	15	0.0450
			0.5	2	2807	5614			0.1					1		15	
	3	2	0.5	1	2686	5372	5368	10736	0.1	157941	6.80	10631	0.0148	1	0.011	15	0.0437
			0.5	2	2682	5364			0.1					1		15	
	4	2	0.5	1	2136	5440	5406	10812	0.1	157941	6.85	10713	0.0149	1	0.011	15	0.0441
			0.5	2	2696	5372			0.1					1		15	
Background control	1	2	0.5	1	21	42	46	92	0.1	157941	0.06	-8	0.0000	1	0.011	15	0.0000
			0.5	2	25	50			0.1					1		15	
	2	2	0.5	1	24	48	49	98	0.1	157941	0.06	-2	0.0000	1	0.011	15	0.0000
			0.5	2	25	50			0.1					1		15	
	3	2	0.5	1	24	48	54	108	0.1	157941	0.07	9	0.0000	1	0.011	15	0.0000
			0.5	2	30	60			0.1					1		15	
	4	2	0.5	1	23	46	50	100	0.1	157941	0.06	1	0.0000	1	0.011	15	0.0000
			0.5	2	27	54			0.1					1		15	
Positive control	1	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	2	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	3	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	4	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
Negative Control	1	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	2	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	3	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	4	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
NA	1-1	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	1-2	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	1-3	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	2-1	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	2-2	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	2-3	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	3-1	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	3-2	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	3-3	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	4-1	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	4-2	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	4-3	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	5-1	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	5-2	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	5-3	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	6-1	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	6-2	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	6-3	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	7-1	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	7-2	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	7-3	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	8-1	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	8-2	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
	8-3	2	0.5	1					0.1	157941		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	

Assay Date	4/15/2005	Test Chemical ID	NA	# Concentrations tested	NA	Microsome type	placental	Microsome ID	B-041305	Technician ID	LR	Replicate #	1

Control Type	Portion	Average	SD
Full activity	Beginning	0.0450	0.0000
Full activity	End	0.0439	0.0002
Full activity	Overall	0.0444	0.0006
Background	Beginning	0.0000	1.74491E-05
Background	End	0.0000	2.32655E-05
Background	Overall	0.0000	2.71777E-05
Positive	Beginning	#VALUE!	#VALUE!
Positive	End	#VALUE!	#VALUE!
Positive	Overall	#VALUE!	#VALUE!
Negative	Beginning	#VALUE!	#VALUE!
Negative	End	#VALUE!	#VALUE!
Negative	Overall	#VALUE!	#VALUE!

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					

Percent of control values				
Level	Log[test substance]	Replicate 1	2	3
1				
2				
3				
4				
5				
6				
7				
8				

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0196	27447	1400357	
2	0.0193	29574	1532332	
3	0.0195	29499	1512769	
4	0.0197	28977	1470914	
5	0.0197	30041	1524924	
			Average DPM/g soln	1488259
			SD	54578
			CV	3.67
			µCi/g soln	0.670

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.2	10		1020.00
Dilution A			100	10.20
Dilution B			10	1.02

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	1.6081 g
Mass of dilution B used in substrate prep	0.9054 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.574285 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00759 µg/g soln.
µg/g soln.

a. µCi/g soln	0.670
b. Specific activity of [^{3}H]ASDN (µCi/mmol)	25300000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.574285 + 0.00759
= 0.581874 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.152 µCi/µg ASDN

732525 dpm/nmol

Assay Date	4/15/2005	Test Chemical ID	NA	# Concentrations tested	NA					
Technician ID	TD	Replicate #	2	Microsome type	placental	Microsome ID	6-041305	Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
								28	20	210000238

Standards:	1	0.8	0.6	0.4	0.2	0.11	Blank
	0.301	0.267	0.216	0.158	0.089	0.041	0.000
	0.320	0.269	0.215	0.153	0.081	0.052	0.000
	0.320	0.267	0.212	0.155	0.082	0.054	0.000

Samples:	6-041305	QC1	QC2	QC3
	0.131	0.044	0.166	0.301
	0.133	0.040	0.160	0.299
	0.130			

Standard concentration (mg/mL)	Volume of stock used	Final volume of Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output
1	17.9	25	0.00100	25	0.0251	0.314	0.314	0.0241
0.8	14.3	25	0.00080	25	0.0200	0.268	0.268	0.0202
0.6	10.7	25	0.00060	25	0.0150	0.214	0.214	0.0158
0.4	7.1	25	0.00040	25	0.0099	0.155	0.155	0.0109
0.2	3.6	25	0.00020	25	0.0050	0.084	0.084	0.0049
0.11	2	25	0.00011	25	0.0028	0.049	0.049	0.0020
			Blank	0.000				

Variables	Regression results	
m, b	0.083	-0.002
se_m, se_b	0.004	0.001
r^2, se_y	0.992	0.001
F, df	476	4
ss_{reg}, ss_{resid}	0.000	0.000

Regression results are calculated using the function LINEST

r^2= 0.992
m= 0.083
b= -0.002

	A_{raw}	$A_{adj.}$	mg protein measured	µL diluted µSOMES	Vol usome prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
6-041305	0.131	0.131	0.009	25	100	5000	0.018	0.018	17.724
6-041305	0.133	0.133	0.009	25	100	5000	0.018		
6-041305	0.130	0.130	0.009	25	100	5000	0.017		
QC1	0.044	0.044	0.002	25	1	1	0.000	0.000	0.056
QC1	0.040	0.040	0.001	25	1	1	0.000		
QC1									
QC2	0.166	0.166	0.012	25	1	1	0.000	0.000	0.460
QC2	0.160	0.160	0.011	25	1	1	0.000		
QC2									
QC3	0.301	0.301	0.023	25	1	1	0.001	0.001	0.916
QC3	0.299	0.299	0.023	25	1	1	0.001		
QC3									

Assay Date	4/15/2005	Test Chemical ID	NA	# Concentrations tested	NA	Microsome type	placental	Microsome ID	6-041305	Technician ID	TD	Replicate #	2

Microsome Dilution Details

Dilution A	0.1	mL microsome Stock used
	5	mL total volume
	50	dilution factor
Dilution B	1	mL microsome Dilution A used
	18	mL total volume
	18	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	900	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	17.724
Protein Concentration (dilution added to assay, mg/mL):	0.019693

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-04
2	1.00E-05
3	2.50E-06
4	1.00E-06
5	5.00E-07
6	2.50E-07
7	1.00E-07
8	1.00E-08

Assay Date	4/15/2005	Test Chemical ID	NA	# Concentrations tested	NA	Microsome type	pooled rat	Microsome ID	5-041305	Test Item ID	TD	Replicate #	2

Sample ID		Calculate DPM in aliquots portion after extraction							Calculate % turnover			Calculate nmol ^3H_2O formed						
Sample Type	Replicate/Vessel	Nominal total volume (mL)	Aliq Volume (mL)	Aliq.#	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of reaction solution in reid assay tube (mL)	total DPM in assay tube (mL)	% conversion to product	Total DPM corrected for background (background subtracted Tubes)	nmol ^3H_2O formed	Volume diluted microsomes used in assay tube (mL)	Final protein in assay (mg/mL)	Incubation time (min)	Aromatase activity (nmoles tritiated water/mg protein/min)
Full activity control	1	2	0.5	1	3053	6106	6149	12298	0.1	148826	8.26	12160	0.0166	1	0.010	15	0.0552
			0.5	2	3096	6192			0.1					1		16	
	2	2	0.5	1	2934	5868	5827	11654	0.1	148826	7.83	11516	0.0157	1	0.010	16	0.0492
			0.5	2	2933	5866			0.1					1		15	
	3	2	0.5	1	2841	5682	5730	11460	0.1	148826	7.70	11392	0.0156	1	0.010	15	0.0523
			0.5	2	2889	5778			0.1					1		15	
	4	2	0.5	1	2708	5416	5417	10834	0.1	148826	7.28	10696	0.0145	1	0.010	15	0.0491
			0.5	2	2709	5418			0.1					1		16	
Background control	1	2	0.5	1	33	66	65	130	0.1	148826	0.09	-3	0.0000	1	0.010	15	0.0000
			0.5	3	32	64			0.1					1		16	
	2	2	0.5	1	33	66	69	139	0.1	148826	0.09	3	0.0000	1	0.010	15	0.0000
			0.5	2	36	72			0.1					1		15	
	3	2	0.5	1	35	70	75	150	0.1	148826	0.10	12	0.0000	1	0.010	15	0.0001
			0.5	2	40	80			0.1					1		15	
	4	2	0.5	1	30	60	67	134	0.1	148826	0.09	-4	0.0000	1	0.010	16	0.0000
			0.5	2	37	74			0.1					1		15	
Positive control	1	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	15	#VALUE!
			0.5	2					0.1					1		15	
	2	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	15	#VALUE!
			0.5	2					0.1					1		16	
	3	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	16	#VALUE!
			0.5	2					0.1					1		16	
	4	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	16	#VALUE!
			0.5	2					0.1					1		15	
Negative Control	1	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	15	#VALUE!
			0.5	2					0.1					1		15	
	2	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	16	#VALUE!
			0.5	2					0.1					1		16	
	3	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	16	#VALUE!
			0.5	2					0.1					1		16	
	4	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	15	#VALUE!
			0.5	2					0.1					1		16	
NA	1-1	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	16	#VALUE!
			0.5	2					0.1					1		15	
	1-2	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	15	#VALUE!
			0.5	2					0.1					1		15	
	1-3	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	15	#VALUE!
			0.5	2					0.1					1		16	
	2-1	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	15	#VALUE!
			0.5	2					0.1					1		15	
	2-2	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	16	#VALUE!
			0.5	2					0.1					1		15	
	2-3	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	15	#VALUE!
			0.5	2					0.1					1		15	
	3-1	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	15	#VALUE!
			0.5	2					0.1					1		15	
	3-2	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	15	#VALUE!
			0.5	2					0.1					1		15	
	3-3	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	15	#VALUE!
			0.5	2					0.1					1		16	
	4-1	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	15	#VALUE!
			0.5	2					0.1					1		15	
	4-2	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	16	#VALUE!
			0.5	2					0.1					1		15	
	4-3	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	15	#VALUE!
			0.5	2					0.1					1		16	
	5-1	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	15	#VALUE!
			0.5	2					0.1					1		16	
	5-2	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	16	#VALUE!
			0.5	2					0.1					1		15	
	5-3	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	15	#VALUE!
			0.5	2					0.1					1		16	
	6-1	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	16	#VALUE!
			0.5	2					0.1					1		15	
	6-2	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	15	#VALUE!
			0.5	2					0.1					1		16	
	6-3	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	16	#VALUE!
			0.5	2					0.1					1		15	
	7-1	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	15	#VALUE!
			0.5	2					0.1					1		15	
	7-2	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	16	#VALUE!
			0.5	2					0.1					1		16	
	7-3	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	15	#VALUE!
			0.5	2					0.1					1		15	
	8-1	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	16	#VALUE!
			0.5	2					0.1					1		15	
	8-2	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	15	#VALUE!
			0.5	2					0.1					1		16	
	8-3	2	0.5	1					0.1	148826		#VALUE!	#VALUE!	1	0.010	16	#VALUE!
			0.5	2					0.1					1		16	

Assay Date	4/15/2005	Test Chemical ID	NA	# Concentrations tested	NA	Microsome type	placental	Microsome ID	B-041305	Technician ID	TD	Replicate #	2

Control Type	Portion	Average	SD
Full activity	Beginning	0.0547	0.0021
Full activity	End	0.0509	0.0020
Full activity	Overall	0.0528	0.0028
Background	Beginning	0.0000	2.61422E-05
Background	End	0.0000	5.22844E-05
Background	Overall	0.0000	3.99329E-05
Positive	Beginning	#VALUE!	#VALUE!
Positive	End	#VALUE!	#VALUE!
Positive	Overall	#VALUE!	#VALUE!
Negative	Beginning	#VALUE!	#VALUE!
Negative	End	#VALUE!	#VALUE!
Negative	Overall	#VALUE!	#VALUE!

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					

Percent of control values				
	Log[test	Replicate		
Level	substance]	1	2	3
1				
2				
3				
4				
5				
6				
7				
8				

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0196	26775	1366071	
2	0.0198	27185	1372980	
3	0.0197	28722	1457970	
4	0.0196	29704	1515510	
5	0.0196	29284	1494082	
			Average DPM/g soln	1441323
			SD	68735
			CV	4.77
			µCi/g soln	0.649

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.7	10		1070.00
Dilution A			100	10.70
Dilution B			10	1.07

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.0429 g
Mass of dilution B used in substrate prep	4.5235 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.601791 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00735 µg/g soln.
µg/g soln.

a. µCi/g soln 0.649
b. Specific activity of [^{3}H]ASDN (µCi/mmol) 25300000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.601791 + 0.00735
= 0.609141 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.066 µCi/µg ASDN

677668 dpm/nmol

Assay Date	4/22/2005	Test Chemical ID	4-OHASDN	# Concentrations tested	6		
Technician ID	TD/LR	Replicate #	1	Microsome type	placental	Microsome ID	6-041305

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	1	0.8	0.6	0.4	0.2	0.11	Blank	28	20	210000238
	0.330	0.255	0.187	0.146	0.074	0.048	0.000			
	0.316	0.251	0.196	0.149	0.074	0.044	0.000			
	0.318	0.263	0.201	0.140	0.081	0.049	0.000			
Samples:	6-041305	QC1	QC2	QC3						
	0.137	0.047	0.153	0.285						
	0.138	0.045	0.172	0.303						
	0.138									

Standard concentration (mg/mL)	Volume of stock used	Final volume of Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results	
1	17.9	25	0.00100	25	0.0251	0.321	0.321	0.0251	m, b	0.082	-0.001
0.8	14.3	25	0.00080	25	0.0200	0.256	0.255	0.0198	se_m, se_b	0.002	0.000
0.6	10.7	25	0.00060	25	0.0150	0.195	0.195	0.0147	r^2, se_y	0.998	0.000
0.4	7.1	25	0.00040	25	0.0099	0.145	0.145	0.0106	F, df	2347	4
0.2	3.6	25	0.00020	25	0.0050	0.076	0.076	0.0050	ss_{reg}, ss_{resid}	0.000	0.000
0.11	2	25	0.00011	25	0.0028	0.047	0.047	0.0026			

Regression results are calculated using the function LINEST

Blank	0.000		r^2=	0.998							
			m=	0.082							
			b=	-0.001							

	A_{raw}	$A_{adj.}$	mg protein measured	µL diluted µSOMES	Vol usome prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
6-041305	0.137	0.137	0.010	25	100	5000	0.020	0.020	20.067
6-041305	0.138	0.138	0.010	25	100	5000	0.020		
6-041305	0.138	0.138	0.010	26	100	5000	0.020		
QC1	0.047	0.047	0.003	25	1	1	0.000	0.000	0.099
QC1	0.045	0.045	0.002	25	1	1	0.000		
QC1									
QC2	0.153	0.153	0.011	25	1	1	0.000	0.000	0.483
QC2	0.172	0.172	0.013	25	1	1	0.001		
QC2									
QC3	0.285	0.285	0.022	25	1	1	0.001	0.001	0.915
QC3	0.303	0.303	0.024	25	1	1	0.001		
QC3									

Assay Date	4/22/2005	Test Chemical ID	4-OHASDN	# Concentrations tested	6	Microsome type	placental	Microsome ID	6-041305	Technician ID	TD/LR	Replicate #	1

Microsome Dilution Details

Dilution A	0.1	mL microsome Stock used
	5	mL total volume
	50	dilution factor
Dilution B	2	mL microsome Dilution A used
	38	mL total volume
	19	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	950	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	20.067
Protein Concentration (dilution added to assay, mg/mL):	0.021123

Test Chemical Concentrations

Level	Final Concentration (M)
1	1.00E-06
2	1.00E-07
3	5.00E-08
4	2.50E-08
5	1.00E-08
6	1.00E-09

Assay Date 4/22/2005 | Test Chemical ID 4-OHASON | # Concentrations tested 6 | Microsome type placental | Microsome ID 6-041305 | Technician ID TD/LR | Replicate # 1

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate nmol ^3H_2O formed					
Sample type	Replicate/Level	Nominal total volume (mL)	Aliq Volume (mL)	Aliq.#	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate solution used/assay tube (mL)	total DPM in assay tube (in total)	% conversion to product	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed	Volume diluted microsomes used in assay tube (mL)	Final [protein] in assay (mg/mL)	Incubation time (min)	Aromatase activity (nmoles/mg protein/min)
Full activity control	1	2	0.5	1	3798	7596	7429	14858	0.1	144132	10.31	14711	0.0217	1	0.011	15	0.0685
			0.5	2	3631	7262			0.1					1		15	
	2	2	0.5	1	3745	7490	7457	14914	0.1	144132	10.35	14767	0.0218	1	0.011	15	0.0688
			0.5	2	3712	7424			0.1					1		15	
	3	2	0.5	1	3605	7210	7201	14402	0.1	144132	9.99	14255	0.0210	1	0.011	15	0.0664
			0.5	2	3596	7192			0.1					1		15	
	4	2	0.5	1	3508	7016	7004	14008	0.1	144132	9.72	13861	0.0205	1	0.011	15	0.0646
			0.5	2	3496	6992			0.1					1		15	
Background control	1	2	0.5	1	44	88	90	180	0.1	144132	0.12	33	0.0000	1	0.011	15	0.0002
			0.5	2	46	92			0.1					1		15	
	2	2	0.5	1	39	78	71	142	0.1	144132	0.10	-5	0.0000	1	0.011	15	0.0000
			0.5	2	32	64			0.1					1		15	
	3	2	0.5	1	34	68	63	126	0.1	144132	0.09	-21	0.0000	1	0.011	15	-0.0001
			0.5	2	29	58			0.1					1		15	
	4	2	0.5	1	37	74	70	140	0.1	144132	0.10	-7	0.0000	1	0.011	15	0.0000
			0.5	2	33	66			0.1					1		15	
Positive control	1	2	0.5	1	1743	3486	3495	6990	0.1	144132	4.85	6843	0.0101	1	0.011	15	0.0319
			0.5	2	1752	3504			0.1					1		15	
	2	2	0.5	1	1673	3346	3331	6662	0.1	144132	4.62	6515	0.0096	1	0.011	15	0.0303
			0.5	2	1658	3316			0.1					1		15	
	3	2	0.5	1	1639	3278	3300	6600	0.1	144132	4.58	6453	0.0095	1	0.011	15	0.0301
			0.5	2	1661	3322			0.1					1		15	
	4	2	0.5	1	1631	3262	3258	6516	0.1	144132	4.52	6369	0.0094	1	0.011	15	0.0297
			0.5	2	1627	3254			0.1					1		15	
Negative Control	1	2	0.5	1	3402	6804	6792	13584	0.1	144132	9.42	13437	0.0198	1	0.011	15	0.0626
			0.5	2	3390	6780			0.1					1		15	
	2	2	0.5	1	3569	7138	7095	14190	0.1	144132	9.85	14043	0.0207	1	0.011	15	0.0661
			0.5	2	3526	7052			0.1					1		15	
	3	2	0.5	1	3415	6830	6892	13784	0.1	144132	9.56	13637	0.0201	1	0.011	15	0.0636
			0.5	2	3477	6954			0.1					1		15	
	4	2	0.5	1	3539	7078	6968	13936	0.1	144132	9.67	13789	0.0203	1	0.011	15	0.0642
			0.5	2	3429	6858			0.1					1		15	
4-OHASON	1-1	2	0.5	1	217	434	419	838	0.1	144132	0.58	691	0.0010	1	0.011	15	0.0032
			0.5	2	202	404			0.1					1		15	
	1-2	2	0.5	1	233	466	457	914	0.1	144132	0.63	767	0.0011	1	0.011	15	0.0036
			0.5	2	224	448			0.1					1		15	
	1-3	2	0.5	1	211	422	415	830	0.1	144132	0.58	683	0.0010	1	0.011	15	0.0032
			0.5	2	204	408			0.1					1		15	
	2-1	2	0.5	1	1129	2258	2266	4532	0.1	144132	3.14	4385	0.0065	1	0.011	15	0.0204
			0.5	2	1137	2274			0.1					1		15	
	2-2	2	0.5	1	1112	2224	2233	4466	0.1	144132	3.10	4319	0.0064	1	0.011	15	0.0201
			0.5	2	1121	2242			0.1					1		15	
	2-3	2	0.5	1	1145	2290	2284	4568	0.1	144132	3.17	4421	0.0065	1	0.011	15	0.0206
			0.5	2	1139	2278			0.1					1		15	
	3-1	2	0.5	1	1645	3290	3284	6568	0.1	144132	4.56	6421	0.0095	1	0.011	15	0.0299
			0.5	2	1639	3278			0.1					1		15	
	3-2	2	0.5	1	1697	3394	3395	6790	0.1	144132	4.71	6643	0.0098	1	0.011	15	0.0309
			0.5	2	1698	3396			0.1					1		15	
	3-3	2	0.5	1	1659	3318	3321	6642	0.1	144132	4.61	6495	0.0096	1	0.011	15	0.0302
			0.5	2	1662	3324			0.1					1		15	
	4-1	2	0.5	1	2314	4628	4580	9160	0.1	144132	6.36	9013	0.0133	1	0.011	15	0.0420
			0.5	2	2266	4532			0.1					1		15	
	4-2	2	0.5	1	2356	4712	4594	9188	0.1	144132	6.37	9041	0.0133	1	0.011	15	0.0421
			0.5	2	2238	4476			0.1					1		15	
	4-3	2	0.5	1	2280	4560	4593	9186	0.1	144132	6.37	9039	0.0133	1	0.011	15	0.0421
			0.5	2	2313	4626			0.1					1		15	
	5-1	2	0.5	1	2744	5488	5632	11264	0.1	144132	7.82	11117	0.0164	1	0.011	15	0.0518
			0.5	2	2888	5776			0.1					1		15	
	5-2	2	0.5	1	2733	5466	5507	11014	0.1	144132	7.64	10867	0.0160	1	0.011	15	0.0506
			0.5	2	2774	5548			0.1					1		15	
	5-3	2	0.5	1	2746	5492	5513	11026	0.1	144132	7.65	10879	0.0161	1	0.011	15	0.0507
			0.5	2	2767	5534			0.1					1		15	
	6-1	2	0.5	1	3313	6626	6750	13500	0.1	144132	9.37	13353	0.0197	1	0.011	15	0.0622
			0.5	2	3437	6874			0.1					1		15	
	6-2	2	0.5	1	3328	6656	6662	13324	0.1	144132	9.24	13177	0.0194	1	0.011	15	0.0614
			0.5	2	3334	6668			0.1					1		15	
	6-3	2	0.5	1	3439	6878	6830	13660	0.1	144132	9.48	13513	0.0199	1	0.011	15	0.0629
			0.5	2	3391	6782			0.1					1		15	
									0.1					1		15	
									0.1					1		15	
									0.1					1		15	
									0.1					1		15	
									0.1					1		15	
									0.1					1		15	
									0.1					1		15	
									0.1					1		15	
									0.1					1		15	
									0.1					1		15	
									0.1					1		15	
									0.1					1		15	

Assay Date	4/22/2005	Test Chemical ID	4-OHASDN	# Concentrations tested		Microsome 6 type	placental	Microsome ID	6-041305	Technician ID	TD/LR	Replicate #	1

Control Type	Portion	Average	SD
Full activity	Beginning	0.0686	0.0002
Full activity	End	0.0655	0.0013
Full activity	Overall	0.0671	0.0020
Background	Beginning	0.0001	0.000125142
Background	End	-0.0001	4.61048E-05
Background	Overall	0.0000	0.00010769
Positive	Beginning	0.0311	0.0011
Positive	End	0.0299	0.0003
Positive	Overall	0.0305	0.0010
Negative	Beginning	0.0640	0.0020
Negative	End	0.0639	0.0005
Negative	Overall	0.0639	0.0012

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
4-OHASDN	1	1	1.00E-06	-6.00	0.0032
4-OHASDN	1	2	1.00E-06	-6.00	0.0036
4-OHASDN	1	3	1.00E-06	-6.00	0.0032
4-OHASDN	2	1	1.00E-07	-7.00	0.0204
4-OHASDN	2	2	1.00E-07	-7.00	0.0201
4-OHASDN	2	3	1.00E-07	-7.00	0.0206
4-OHASDN	3	1	5.00E-08	-7.30	0.0299
4-OHASDN	3	2	5.00E-08	-7.30	0.0309
4-OHASDN	3	3	5.00E-08	-7.30	0.0302
4-OHASDN	4	1	2.50E-08	-7.60	0.0420
4-OHASDN	4	2	2.50E-08	-7.60	0.0421
4-OHASDN	4	3	2.50E-08	-7.60	0.0421
4-OHASDN	5	1	1.00E-08	-8.00	0.0518
4-OHASDN	5	2	1.00E-08	-8.00	0.0506
4-OHASDN	5	3	1.00E-08	-8.00	0.0507
4-OHASDN	6	1	1.00E-09	-9.00	0.0622
4-OHASDN	6	2	1.00E-09	-9.00	0.0614
4-OHASDN	6	3	1.00E-09	-9.00	0.0629

Percent of control values				
		Replicate		
Level	Log[test substance]	1	2	3
1	-6.00	4.80	5.33	4.74
2	-7.00	30.45	30.00	30.70
3	-7.30	44.59	46.14	45.11
4	-7.60	62.60	62.79	62.78
5	-8.00	77.21	75.47	75.56
6	-9.00	92.74	91.52	93.85

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0195	24250	1243590	
2	0.0193	27665	1433420	
3	0.0195	28773	1475538	
4	0.0195	29059	1490205	
5	0.0195	29304	1502769	
			Average DPM/g soln	1429104
			SD	106947
			CV	7.48
			µCi/g soln	0.644

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.48	10		1048.00
Dilution A			100	10.48
Dilution B			10	1.05

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.034 g
Mass of dilution B used in substrate prep	4.5201 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.589627 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00729 µg/g soln.
µg/g soln.

a. µCi/g soln 0.644
b. Specific activity of [^{3}H]ASDN (µCi/mmol) 25300000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.589627 + 0.00729
= 0.596914 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.078 µCi/µg ASDN

685685 dpm/nmol

Assay Date	4/25/2005	Test Chemical ID	4-OHASDN	# Concentrations tested	6				
Technician ID	TD/LR	Replicate #	2	Microsome type	placental	Microsome ID	S-041305		

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	1	0.8	0.6	0.4	0.2	0.11	Blank	28	20	210000238
	0.308	0.263	0.226	0.167	0.069	0.055	0.000			
	0.324	0.255	0.223	0.162	0.085	0.055	0.000			
	0.320	0.266	0.216	0.163	0.085	0.035	0.000			
Samples:	S-041305	QC1	QC2	QC3						
	0.139	0.045	0.162	0.295						
	0.138	0.046	0.173	0.309						
	0.135									

Standard concentration (mg/mL)	Volume of stock used	Final volume of Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results	
1	17.9	25	0.00100	25	0.0251	0.317	0.317	0.0241	m, b	0.082	-0.002
0.8	14.3	25	0.00080	25	0.0200	0.261	0.261	0.0195	se_m, se_b	0.005	0.001
0.6	10.7	25	0.00060	25	0.0150	0.221	0.221	0.0162	r^2, se_y	0.984	0.001
0.4	7.1	25	0.00040	25	0.0099	0.164	0.164	0.0115	F, df	241	4
0.2	3.6	25	0.00020	25	0.0050	0.080	0.080	0.0046	ss_{reg}, ss_{resid}	0.000	0.000
0.11	2	25	0.00011	25	0.0028	0.048	0.048	0.0020			

Regression results are calculated using the function LINEST

Blank 0.000

r^2= 0.984
m= 0.082
b= -0.002

	A_{raw}	$A_{adj.}$	mg protein measured	µL diluted µSOMES	Vol usome prep. (µL)	Final vol Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
S-041305	0.139	0.139	0.009	25	100	5000	0.019	0.019	18.607
S-041305	0.138	0.138	0.009	25	100	5000	0.019		
S-041305	0.135	0.135	0.009	25	100	5000	0.018		
QC1	0.045	0.045	0.002	25	1	1	0.000	0.000	0.070
QC1	0.046	0.046	0.002	25	1	1	0.000		
QC1									
QC2	0.162	0.162	0.011	25	1	1	0.000	0.000	0.471
QC2	0.173	0.173	0.012	25	1	1	0.000		
QC2									
QC3	0.295	0.295	0.022	25	1	1	0.001	0.001	0.913
QC3	0.309	0.309	0.023	25	1	1	0.001		
QC3									

Assay Date	4/25/2005	Test Chemical ID	4-OHASDN	# Concentrations tested	6	Microsome type	placental	Microsome ID	6-041305	Technician ID	TD/LR	Replicate #	2

Microsome Dilution Details

Dilution A	0.1	mL microsome Stock used
	5	mL total volume
	50	dilution factor
Dilution B	2	mL microsome Dilution A used
	38	mL total volume
	19	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	950	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	18.607
Protein Concentration (dilution added to assay, mg/mL):	0.019586

Test Chemical Concentrations

Level	Final Concentration (M)
1	1.00E-06
2	1.00E-07
3	5.00E-08
4	2.50E-08
5	1.00E-08
6	1.00E-09

Assay Date	4/26/2005	Test Chemical ID	4-OHASDN	# Concentrations tested	6	Microsome Type	pooled	Microsome ID	6-041305	Tech Initials ID	TD/LR	Replicate #	2

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate nmol $^{3}H_2O$ formed						
Sample Type	Replicate Level	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of incubate solution used in assay tube (mL)	total DPM in assay tube (dpm)	% conversion to product	Total DPM corrected for background (background = TDHO)	nmol $^{3}H_2O$ formed	Volume diluted microsomes used in assay tube (mL)	Final protein in assay (mg/mL)	Incubation time (min)	Aromatase activity (pmoles/mg/min)
Fortifiactity control	1	2	0.5	1	2889	5778	5765	11530	0.1	142910	8.07	11392	0.0166	1	0.010	15	0.0665
			0.5	2	2879	5752			0.1					1		15	
	2	2	0.5	1	2786	5572	5515	11030	0.1	142910	7.72	10892	0.0159	1	0.010	15	0.0641
			0.5	2	2729	5458			0.1					1		15	
	3	2	0.5	1	2411	4822	4808	9616	0.1	142910	6.73	9478	0.0138	1	0.010	15	0.0478
			0.5	2	2397	4794			0.1					1		15	
	4	2	0.5	1	2642	5284	5310	10620	0.1	142910	7.43	10482	0.0153	1	0.010	15	0.0529
			0.5	2	2658	5336			0.1					1		15	
Background control	1	2	0.5	1	41	82	72	144	0.1	142910	0.10	6	0.0000	1	0.010	15	0.0000
			0.5	2	31	62			0.1					1		15	
	2	2	0.5	1	33	66	61	122	0.1	142910	0.09	-17	0.0000	1	0.010	15	-0.0001
			0.5	2	28	56			0.1					1		15	
	3	2	0.5	1	31	74	70	140	0.1	142910	0.10	2	0.0000	1	0.010	15	0.0000
			0.5	2	33	66			0.1					1		15	
	4	2	0.5	1	31	62	74	148	0.1	142910	0.10	10	0.0000	1	0.010	15	0.0000
			0.5	2	43	86			0.1					1		15	
Positive control	1	2	0.5	1	1560	3174	3135	6212	0.1	142910	4.39	6124	0.0089	1	0.010	15	0.0374
			0.5	2	1569	3138			0.1					1		15	
	2	2	0.5	1	1515	3030	3021	6042	0.1	142910	4.23	5904	0.0086	1	0.010	15	0.0363
			0.5	2	1506	3012			0.1					1		15	
	3	2	0.5	1	1452	2964	2940	5880	0.1	142910	4.11	5742	0.0084	1	0.010	15	0.0355
			0.5	2	1458	2916			0.1					1		15	
	4	2	0.5	1	1490	2986	3007	6014	0.1	142910	4.21	5876	0.0086	1	0.010	15	0.0362
			0.5	2	1514	3028			0.1					1		15	
Negative Control	1	2	0.5	1	2979	5958	5892	11784	0.1	142910	8.25	11646	0.0170	1	0.010	15	0.0575
			0.5	2	2913	5826			0.1					1		15	
	2	2	0.5	1	2952	5904	5928	11856	0.1	142910	8.30	11718	0.0171	1	0.010	15	0.0582
			0.5	2	2976	5952			0.1					1		15	
	3	2	0.5	1	2771	5542	5578	11156	0.1	142910	7.81	11018	0.0161	1	0.010	15	0.0547
			0.5	2	2808	5614			0.1					1		15	
	4	2	0.5	1	2791	5582	5534	11068	0.1	142910	7.74	10930	0.0159	1	0.010	15	0.0543
			0.5	2	2783	5566			0.1					1		15	
4-OHASDN	1-1	2	0.5	1	201	402	402	804	0.1	142910	0.56	666	0.0010	1	0.010	15	0.0033
			0.5	2	201	402			0.1					1		15	
	1-2	2	0.5	1	198	396	419	838	0.1	142910	0.59	790	0.0010	1	0.010	15	0.0035
			0.5	2	221	442			0.1					1		15	
	1-3	2	0.5	1	219	438	435	870	0.1	142910	0.61	732	0.0011	1	0.010	15	0.0036
			0.5	2	216	432			0.1					1		15	
	2-1	2	0.5	1	1026	2052	2062	4124	0.1	142910	2.89	3986	0.0058	1	0.010	15	0.0195
			0.5	2	1037	2074			0.1					1		15	
	2-2	2	0.5	1	1085	2170	2170	4340	0.1	142910	3.04	4202	0.0061	1	0.010	16	0.0209
			0.5	2	1085	2170			0.1					1		15	
	2-3	2	0.5	1	1047	2094	2144	4288	0.1	142910	3.00	4160	0.0061	1	0.010	15	0.0206
			0.5	2	1097	2194			0.1					1		15	
	3-1	2	0.5	1	1442	2884	2911	5822	0.1	142910	4.07	5684	0.0083	1	0.010	15	0.0282
			0.5	2	1469	2938			0.1					1		15	
	3-2	2	0.5	1	1459	2918	2942	5884	0.1	142910	4.12	5746	0.0084	1	0.010	15	0.0285
			0.5	2	1483	2966			0.1					1		15	
	3-3	2	0.5	1	1521	3042	3065	6130	0.1	142910	4.29	5992	0.0087	1	0.010	15	0.0297
			0.5	2	1544	3088			0.1					1		15	
	4-1	2	0.5	1	1956	3912	3970	7940	0.1	142910	5.56	7802	0.0114	1	0.010	16	0.0387
			0.5	2	1984	3968			0.1					1		15	
	4-2	2	0.5	1	1891	3782	3798	7596	0.1	142910	5.32	7458	0.0109	1	0.010	15	0.0370
			0.5	2	1907	3814			0.1					1		16	
	4-3	2	0.5	1	1964	3928	3949	7898	0.1	142910	5.53	7760	0.0113	1	0.010	15	0.0385
			0.5	2	1985	3970			0.1					1		15	
	5-1	2	0.5	1	2261	4522	4537	9074	0.1	142910	6.35	8936	0.0130	1	0.010	15	0.0444
			0.5	2	2286	4572			0.1					1		16	
	5-2	2	0.5	1	2213	4426	4419	8838	0.1	142910	6.18	8700	0.0127	1	0.010	15	0.0432
			0.5	2	2205	4410			0.1					1		15	
	5-3	2	0.5	1	2311	4622	4549	9098	0.1	142910	6.37	8960	0.0131	1	0.010	15	0.0445
			0.5	2	2235	4470			0.1					1		15	
	6-1	2	0.5	1	2501	5002	5002	10004	0.1	142910	7.00	9866	0.0144	1	0.010	15	0.0490
			0.5	2	2501	5002			0.1					1		16	
	6-2	2	0.5	1	2639	5278	5257	10514	0.1	142910	7.36	10376	0.0151	1	0.010	15	0.0515
			0.5	2	2602	5204			0.1					1		15	
	6-3	2	0.5	1	2670	5340	5432	10864	0.1	142910	7.60	10726	0.0156	1	0.010	16	0.0532
			0.5	2	2762	5524			0.1					1		16	
									0.1					1		15	
									0.1					1		16	
									0.1					1		15	
									0.1					1		16	
									0.1					1		15	
									0.1					1		16	
									0.1					1		15	
									0.1					1		15	
									0.1					1		15	
									0.1					1		15	
									0.1					1		15	
									0.1					1		15	
									0.1					1		15	
									0.1					1		15	
									0.1					1		15	

Assay Date	4/25/2005	Test Chemical ID	4-OHASDN	# Concentrations tested		Microsome 6 type	placental	Microsome ID	6-041305	Technician ID	TD/LR	Replicate #	2

Control Type	Portion	Average	SD
Full activity	Beginning	0.0553	0.0018
Full activity	End	0.0495	0.0035
Full activity	Overall	0.0524	0.0040
Background	Beginning	0.0000	7.72216E-05
Background	End	0.0000	2.80806E-05
Background	Overall	0.0000	5.69599E-05
Positive	Beginning	0.0299	0.0008
Positive	End	0.0288	0.0005
Positive	Overall	0.0294	0.0008
Negative	Beginning	0.0580	0.0003
Negative	End	0.0545	0.0003
Negative	Overall	0.0562	0.0020

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
4-OHASDN	1	1	1.00E-06	-6.00	0.0033
4-OHASDN	1	2	1.00E-06	-6.00	0.0035
4-OHASDN	1	3	1.00E-06	-6.00	0.0036
4-OHASDN	2	1	1.00E-07	-7.00	0.0198
4-OHASDN	2	2	1.00E-07	-7.00	0.0209
4-OHASDN	2	3	1.00E-07	-7.00	0.0206
4-OHASDN	3	1	5.00E-08	-7.30	0.0262
4-OHASDN	3	2	5.00E-08	-7.30	0.0285
4-OHASDN	3	3	5.00E-08	-7.30	0.0297
4-OHASDN	4	1	2.50E-08	-7.60	0.0367
4-OHASDN	4	2	2.50E-08	-7.60	0.0370
4-OHASDN	4	3	2.50E-08	-7.60	0.0385
4-OHASDN	5	1	1.00E-08	-8.00	0.0444
4-OHASDN	5	2	1.00E-08	-8.00	0.0432
4-OHASDN	5	3	1.00E-08	-8.00	0.0445
4-OHASDN	6	1	1.00E-09	-9.00	0.0490
4-OHASDN	6	2	1.00E-09	-9.00	0.0515
4-OHASDN	6	3	1.00E-09	-9.00	0.0532

Percent of control values				
		Replicate		
Level	Log[test substance]	1	2	3
1	-6.00	6.30	6.62	6.93
2	-7.00	37.74	39.79	39.29
3	-7.30	53.82	54.41	56.74
4	-7.60	73.87	70.62	73.48
5	-8.00	84.61	82.38	84.84
6	-9.00	93.42	98.25	101.56

Assay Date	6/16/2005	Test Chemical ID	NA	# Concentrations tested	0						
Technician ID	TD	Replicate #	1	Microsome type	placental	Microsome ID	Lot # BAA	Protein stock (mg BSA)	0.25	Total volume of stock (mL)	1

Protein stock ID: 082K0776

Standards:	250	125	50	25	10	5	0
	0.563	0.347	0.147	0.080	0.033	0.015	0.000
	0.563	0.342	0.152	0.084	0.033	0.015	0.000
	0.564	0.338	0.155	0.092	0.032	0.012	0.000

Samples:	Microsomes	QC1	QC2
	0.128	0.034	0.259
	0.130	0.032	0.254
	0.124		

Standard concentration (mg/mL)	Volume of stock used	Final volume of Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output
250	200	200	0.00025	200	0.0500	0.563	0.563	0.0410
125	100	200	0.00013	200	0.0250	0.342	0.342	0.0247
50	40	200	0.00005	200	0.0100	0.151	0.151	0.0106
25	20	200	0.00003	200	0.0050	0.082	0.082	0.0055
10	8	200	0.00001	200	0.0020	0.033	0.033	0.0018
5	4	200	0.00001	200	0.0010	0.014	0.014	0.0004

Blank 0.000

r^2= 0.997
m= 0.074
b= -0.001

Variables		Regression results	
m, b	0.074	-0.001
se_m, se_b	0.002	0.000
r^2, se_y	0.997	0.001
F, df	1142	3
ss_{reg}, ss_{resid}	0.000	0.000

Regression results are calculated using the function LINEST

	A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	Vol usome prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
Microsomes	0.128	0.128	0.009	200	1	200	0.009	0.009	8.800
Microsomes	0.130	0.130	0.009	200	1	200	0.009		
Microsomes	0.124	0.124	0.009	200	1	200	0.009		
QC1	0.034	0.034	0.002	200	1	1	0.000	0.000	0.009
QC1	0.032	0.032	0.002	200	1	1	0.000		
QC1									
QC2	0.259	0.259	0.019	200	1	1	0.000	0.000	0.092
QC2	0.254	0.254	0.018	200	1	1	0.000		
QC2									

Assay Date	6/16/2005	Test Chemical ID	NA	# Concentrations tested		0				
Technician ID	TD	Replicate #	2	Microsome type	placental	Microsome ID	Lot # BAA	Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
								0.25	1	082K0776

Standards:	250	125	50	25	10	5	0
	0.560	0.334	0.148	0.079	0.027	0.013	0.000
	0.556	0.329	0.148	0.078	0.029	0.013	0.000
	0.555	0.329	0.148	0.077	0.030	0.011	0.000

Samples:	Microsomes	QC1	QC2
	0.124	0.031	0.257
	0.129	0.031	0.258
	0.129		

Standard concentration (mg/mL)	Volume of stock used	Final volume of Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output
250	200	200	0.00025	200	0.0500	0.557	0.557	0.0417
125	100	200	0.00013	200	0.0250	0.331	0.331	0.0245
50	40	200	0.00005	200	0.0100	0.148	0.148	0.0107
25	20	200	0.00003	200	0.0050	0.078	0.078	0.0055
10	8	200	0.00001	200	0.0020	0.029	0.029	0.0017
5	4	200	0.00001	200	0.0010	0.013	0.013	0.0005

Blank 0.000

Variables	Regression results	
m, b	0.076	0.000
se_m, se_b	0.003	0.000
r^2, se_y	0.997	0.001
F, df	915	3
ss_{reg}, ss_{erro}	0.000	0.000

r^2= 0.997
m= 0.076
b= 0.000

Regression results are calculated using the function LINEST

	A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	Vol usome prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
Microsomes	0.124	0.124	0.009	200	1	200	0.009	0.009	9.181
Microsomes	0.129	0.129	0.009	200	1	200	0.009		
Microsomes	0.129	0.129	0.009	200	1	200	0.009		
QC1	0.031	0.031	0.002	200	1	1	0.000	0.000	0.010
QC1	0.031	0.031	0.002	200	1	1	0.000		
QC1									
QC2	0.257	0.257	0.019	200	1	1	0.000	0.000	0.095
QC2	0.258	0.259	0.019	200	1	1	0.000		
QC2									

Assay Date 6/17/2005 | Test Chemical ID NA | # Concentrations tested 0

Technician ID TD | Replicate # 1 | Microsome type placental | Microsome ID | Lot # BAA

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	250	125	50	25	10	5	0	0.25	1	082K0776
	0.529	0.320	0.144	0.074	0.031	0.018	0.008			
	0.535	0.317	0.145	0.076	0.033	0.018	0.008			
	0.533	0.316	0.141	0.077	0.033	0.017	0.009			

Samples:	Microsomes	QC1	QC2
	0.146	0.031	0.228
	0.144	0.030	0.229
	0.145		

Standard concentration (mg/mL)	Volume of stock used	Final volume of Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{sd}	Curve Output	Variables	Regression results	
250	200	200	0.00025	200	0.0500	0.532	0.532	0.0419	m, b	0.080	-0.001
125	100	200	0.00013	200	0.0250	0.318	0.318	0.0247	se_m, se_b	0.002	0.000
50	40	200	0.00005	200	0.0100	0.143	0.143	0.0107	r^2, se_y	0.998	0.001
25	20	200	0.00003	200	0.0050	0.076	0.076	0.0053	F, df	1305	3
10	8	200	0.00001	200	0.0020	0.032	0.032	0.0018	ss_{reg}, ss_{resid}	0.000	0.000
5	4	200	0.00001	200	0.0010	0.017	0.017	0.0006			

Blank 0.000

r^2= 0.998
m= 0.080
b= -0.001

Regression results are calculated using the function LINEST

	A_{raw}	A_{sd}	mg protein measured	µL diluted µSOMES	Vol usome prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
Microsomes	0.146	0.146	0.011	200	68	10000	0.008	0.008	7.987
Microsomes	0.144	0.144	0.011	200	68	10000	0.006		
Microsomes	0.146	0.146	0.011	200	68	10000	0.008		
QC1	0.031	0.031	0.002	200	1	1	0.000	0.000	0.008
QC1	0.030	0.030	0.002	200	1	1	0.000		
QC1									
QC2	0.228	0.228	0.018	200	1	1	0.000	0.000	0.088
QC2	0.229	0.229	0.018	200	1	1	0.000		
QC2									

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0195	28237	1448051	
2	0.0194	29120	1501031	
3	0.0194	29273	1508918	
4	0.0197	30391	1542690	
5	0.0196	30358	1548878	
			Average DPM/g soln	1509914
			SD	40309
			CV	2.67
			µCi/g soln	0.680

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.3	10		1030.00
Dilution A			100	10.30
Dilution B			10	1.03

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.0498 g
Mass of dilution B used in substrate prep	4.529 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.579501 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00770 µg/g soln.
µg/g soln.

a. µCi/g soln	0.680
b. Specific activity of [^{3}H]ASDN (µCi/mmol)	25300000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.579501 + 0.00770
= 0.587201 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.158 µCi/µg ASDN

736442 dpm/nmol

Assay Date	6/17/2005	Test Chemical ID	NA	# Concentrations tested	NA	Microsome type	placental	Microsome ID	lot# BAA	Technician ID	TD	Replicate #	1

Microsome Dilution Details

Dilution A	0.068	mL microsome Stock used
	10	mL total volume
	147.0588	dilution factor
Dilution B	6.68	mL microsome Dilution A used
	20	mL total volume
	2.994012	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	440.2959	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	7.987
Protein Concentration (dilution added to assay, mg/mL):	0.01814

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-04
2	1.00E-05
3	2.50E-06
4	1.00E-06
5	5.00E-07
6	2.50E-07
7	1.00E-07
8	1.00E-08

Assay Date	5/17/2005	Test Chemical ID NA	# Concentration tested NA	Microsome type	plasma fat	Microsome ID	lot# BAA	Tech Initials ID	TD	Replicate #	1

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % tritium			Calculate nmol ^3H_2O formed						
Sample Type	Replicate/Level	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave. DPM/mL	Total DPM	Volume of aqueous portion for radioassay tube (mL)	total DPM in assay tube (dpm)	% conversion to product	Total DPM corrected for background (Background = Tritium)	nmol ^3H_2O formed	Volume diluted in microcentrifuge used in assay tube (mL)	Final (protein) in assay (mg/mL)	Incubation time (min)	Aromatase activity (pmol estrogen formed/mg protein/min)
Radioactivity control	1	2	0.5	1	1514	3028	2997	5994	0.1	150991	3.97	6840	0.0079	1	0.009	15	0.0291
				2	1483	2966			0.1					1		15	
	2	2	0.5	1	1457	2914	2919	5838	0.1	150991	3.87	6684	0.0077	1	0.009	15	0.0284
			0.5	2	1462	2924			0.1					1		15	
	3	2	0.5	1	1503	3006	2985	5970	0.1	150991	3.95	6816	0.0079	1	0.009	15	0.0290
			0.5	2	1492	2984			0.1					1		15	
	4	2	0.5	1	1456	2912	2959	5918	0.1	150991	3.92	6764	0.0078	1	0.009	15	0.0288
			0.5	2	1507	3014			0.1					1		15	
Background control	1	2	0.5	1	55	110	101	202	0.1	150991	0.13	48	0.0001	1	0.009	15	0.0002
			0.5	2	46	92			0.1					1		15	
	2	2	0.5	1	33	66	68	136	0.1	150991	0.09	-18	0.0000	1	0.009	15	-0.0001
			0.5	2	35	70			0.1					1		15	
	3	2	0.5	1	37	74	69	138	0.1	150991	0.09	-16	0.0000	1	0.009	15	-0.0001
			0.5	2	32	64			0.1					1		15	
	4	2	0.5	1	31	62	70	140	0.1	150991	0.09	-14	0.0000	1	0.009	15	-0.0001
			0.5	2	39	78			0.1					1		15	
Positive control	1	2	0.5	1	817	1634	1658	3316	0.1	150991	2.20	3162	0.0043	1	0.009	15	0.0158
			0.5	2	841	1682			0.1					1		15	
	2	2	0.5	1	842	1684	1689	3378	0.1	150991	2.24	3224	0.0044	1	0.009	15	0.0161
			0.5	2	847	1694			0.1					1		15	
	3	2	0.5	1	848	1696	1689	3378	0.1	150991	2.24	3224	0.0044	1	0.009	15	0.0161
			0.5	2	841	1682			0.1					1		15	
	4	2	0.5	1	809	1618	1630	3260	0.1	150991	2.16	3106	0.0042	1	0.009	15	0.0155
			0.5	2	821	1642			0.1					1		15	
Negative Control	1	2	0.5	1	1554	3108	3083	6166	0.1	150991	4.08	6012	0.0082	1	0.009	15	0.0300
			0.5	2	1529	3058			0.1					1		15	
	2	2	0.5	1	1525	3050	3055	6110	0.1	150991	4.05	5956	0.0081	1	0.009	15	0.0297
			0.5	2	1530	3060			0.1					1		15	
	3	2	0.5	1	1501	3002	2988	5976	0.1	150991	3.96	5822	0.0079	1	0.009	15	0.0291
			0.5	2	1487	2974			0.1					1		15	
	4	2	0.5	1	1521	3042	3029	6058	0.1	150991	4.01	5904	0.0080	1	0.009	15	0.0295
			0.5	2	1508	3016			0.1					1		15	
NA	1-1	2	0.5	1					0.1	150991		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	1-2	2	0.5	1					0.1	150991		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	1-3	2	0.5	1					0.1	150991		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	2-1	2	0.5	1					0.1	150991		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	2-2	2	0.5	1					0.1	150991		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	2-3	2	0.5	1					0.1	150991		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	3-1	2	0.5	1					0.1	150991		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	3-2	2	0.5	1					0.1	150991		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	3-3	2	0.5	1					0.1	150991		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	4-1	2	0.5	1					0.1	150991		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	4-2	2	0.5	1					0.1	150991		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	4-3	2	0.5	1					0.1	150991		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	5-1	2	0.5	1					0.1	150991		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	5-2	2	0.5	1					0.1	150991		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	5-3	2	0.5	1					0.1	150991		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	6-1	2	0.5	1					0.1	150991		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	6-2	2	0.5	1					0.1	150991		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	6-3	2	0.5	1					0.1	150991		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	7-1	2	0.5	1					0.1	150991		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	7-2	2	0.5	1					0.1	150991		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	7-3	2	0.5	1					0.1	150991		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	8-1	2	0.5	1					0.1	150991		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	8-2	2	0.5	1					0.1	150991		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	8-3	2	0.5	1					0.1	150991		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	

Assay Date	6/17/2005	Test Chemical ID	NA	# Concentrations tested	NA	Microsome type	placental	Microsome ID lot# BAA	Technician ID TD	Replicate #	1

Control Type	Portion	Average	SD
Full activity	Beginning	0.0288	0.0006
Full activity	End	0.0289	0.0002
Full activity	Overall	0.0288	0.0003
Background	Beginning	0.0001	0.000232895
Background	End	-0.0001	7.05742E-06
Background	Overall	0.0000	0.000159899
Positive	Beginning	0.0159	0.0002
Positive	End	0.0158	0.0004
Positive	Overall	0.0159	0.0003
Negative	Beginning	0.0299	0.0002
Negative	End	0.0293	0.0003
Negative	Overall	0.0296	0.0004

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					

Percent of control values				
		Replicate		
Level	Log[test substance]	1	2	3
1				
2				
3				
4				
5				
6				
7				
8				

Assay Date	6/17/2005	Test Chemical ID	NA	# Concentrations tested	0		
Technician ID	LR	Replicate #	2	Microsome type	placental	Microsome ID	Lot # BAA

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	250	125	60	25	10	5	0	0.25	1	082K0776
	0.534	0.315	0.140	0.075	0.032	0.016	0.000			
	0.534	0.315	0.137	0.075	0.031	0.016	0.000			
	0.526	0.311	0.138	0.069	0.032	0.016	0.000			

Samples:	Microsomes	QC1	QC2
	0.149	0.033	0.237
	0.147	0.031	0.230
	0.147		

Standard concentration (pg/mL)	Volume of stock used	Final volume of Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{sd}	Curve Output	Variables	Regression results	
JL 2-20-06 BBL											
250	200	200	0.00025	200	0.0500	0.531	0.531	0.0424	m, b	0.091	-0.001
125	100	200	0.00013	200	0.0250	0.314	0.314	0.0247	se_m, se_b	0.002	0.000
50	40	200	0.00005	200	0.0100	0.138	0.138	0.0105	r^2, se_y	0.999	0.000
25	20	200	0.00003	200	0.0050	0.073	0.073	0.0052	F, df	2070	3
10	8	200	0.00001	200	0.0020	0.032	0.032	0.0019	ss_{reg}, ss_{sres}	0.000	0.000
5	4	200	0.00001	200	0.0010	0.016	0.016	0.0006			

Regression results are calculated using the function LINEST

Blank	0.000	r^2=	0.999	
		m=	0.091	
		b=	-0.001	

	A_{raw}	$A_{act.}$	mg protein measured	µL diluted µSOMES	Vol usome prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
Microsomes	0.149	0.149	0.011	200	69	10000	0.008	0.008	8.304
Microsomes	0.147	0.147	0.011	200	68	10000	0.008		
Microsomes	0.147	0.147	0.011	200	68	10000	0.008		
QC1	0.033	0.033	0.002	200	1	1	0.000	0.000	0.009
QC1	0.031	0.031	0.002	200	1	1	0.000		
QC1									
QC2	0.237	0.237	0.019	200	1	1	0.000	0.000	0.091
QC2	0.230	0.230	0.018	200	1	1	0.000		
QC2									

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0194	32211	1660361	
2	0.0190	32619	1716789	
3	0.0193	32955	1707513	
4	0.0185	31390	1696757	
5	0.0193	33480	1734715	
			Average DPM/g soln	1703227
			SD	27718
			CV	1.63
			µCi/g soln	0.767

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.9	10		1090.00
Dilution A			100	10.90
Dilution B			10	1.09

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.0391 g
Mass of dilution B used in substrate prep	4.5204 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.612909 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00869 µg/g soln.
µg/g soln.

a. µCi/g soln 0.767
b. Specific activity of [^{3}H]ASDN (µCi/mmol) 25300000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.612909 + 0.00869
= 0.621594 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.234 µCi/µg ASDN

784763 dpm/nmol

Assay Date	6/17/2005	Test Chemical ID	NA	# Concentrations tested	NA	Microsome type	placental	Microsome ID	lot# BAA	Technician ID	LR	Replicate #	2

Microsome Dilution Details

Dilution A	0.068	mL microsome Stock used
	10	mL total volume
	147.0588	dilution factor
Dilution B	6.68	mL microsome Dilution A used
	20	mL total volume
	2.994012	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	440.2959	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	8.304
Protein Concentration (dilution added to assay, mg/mL):	0.01886

Test Chemical Concentrations

Level	Final Concentration (M)
1	1.00E-04
2	1.00E-05
3	2.50E-06
4	1.00E-06
5	5.00E-07
6	2.50E-07
7	1.00E-07
8	1.00E-08

Assay Date	6/17/2005	Test Chemical ID	NA	# Concentrations tested	NA	Microsome type	placental	Microsome ID	lot# BAA	Tech/date ID	LR	Replicate #	2

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate nmol ^3H_2O formed					
Sample Type	Replicate/Level	Nominal total volume (mL)	Adj Volume (mL)	Adj.#	DPM/adj	DPM/mL	Ave DPM/mL	Total DPM	Volume of reaction portion transferred to assay tube (mL)	total DPM in assay tube (adj)	% conversion to product	Total DPM corrected for background (background TriSet)	nmol ^3H_2O formed	Volume diluted in fluorescence feed in assay tube (mL)	Final [protein] in assay (mg/mL)	Incubation time (min)	Aromatase activity (pmoles tritiated water formed/mg protein/min)
Fetal activity control	1	2	0.5	1	1537	3274	3267	6534	0.1	170323	3.84	6409	0.0082	1	0.009	15	0.0289
			0.5	2	1630	3260			0.1					1		15	
	2	2	0.5	1	1522	3044	3031	6062	0.1	170323	3.56	6937	0.0076	1	0.009	15	0.0267
			0.5	2	1518	3018			0.1					1		15	
	3	2	0.5	1	1440	2880	2865	5730	0.1	170323	3.36	6605	0.0071	1	0.009	15	0.0252
			0.5	2	1425	2850			0.1					1		15	
	4	2	0.5	1	1399	2797	2791	5582	0.1	170323	3.28	6451	0.0070	1	0.009	15	0.0246
			0.5	2	1392	2784			0.1					1		15	
Background control	1	2	0.5	1	31	62	65	130	0.1	170323	0.08	6	0.0000	1	0.009	15	0.0000
			0.5	2	34	68			0.1					1		15	
	2	2	0.5	1	24	48	49	98	0.1	170323	0.06	-27	0.0000	1	0.009	15	-0.0001
			0.5	2	25	50			0.1					1		15	
	3	2	0.5	1	30	60	71	142	0.1	170323	0.08	17	0.0000	1	0.009	15	0.0001
			0.5	2	41	82			0.1					1		15	
	4	2	0.5	1	30	60	65	130	0.1	170323	0.08	6	0.0000	1	0.009	15	0.0000
			0.5	2	35	70			0.1					1		15	
Positive control	1	2	0.5	1	839	1674	1691	3382	0.1	170323	1.99	3257	0.0042	1	0.009	15	0.0147
			0.5	2	864	1708			0.1					1		15	
	2	2	0.5	1	882	1764	1757	3514	0.1	170323	2.06	3369	0.0043	1	0.009	15	0.0153
			0.5	2	875	1750			0.1					1		15	
	3	2	0.5	1	834	1668	1673	3346	0.1	170323	1.96	3221	0.0041	1	0.009	15	0.0145
			0.5	2	839	1678			0.1					1		15	
	4	2	0.5	1	837	1674	1657	3314	0.1	170323	1.95	3189	0.0041	1	0.009	15	0.0144
			0.5	2	820	1640			0.1					1		15	
Negative Control	1	2	0.5	1	1619	3238	3129	6258	0.1	170323	3.67	6133	0.0078	1	0.009	15	0.0276
			0.5	2	1550	3101			0.1					1		15	
	2	2	0.5	1	1581	3162	3186	6372	0.1	170323	3.74	6247	0.0080	1	0.009	15	0.0281
			0.5	2	1605	3210			0.1					1		15	
	3	2	0.5	1	1596	3192	3199	6398	0.1	170323	3.76	6273	0.0080	1	0.009	15	0.0283
			0.5	2	1603	3206			0.1					1		15	
	4	2	0.5	1	1583	3166	3119	6238	0.1	170323	3.66	6113	0.0078	1	0.009	15	0.0275
			0.5	2	1536	3072			0.1					1		15	
NA	1-1	2	0.5	1					0.1	170323		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	1-2	2	0.5	1					0.1	170323		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	1-3	2	0.5	1					0.1	170323		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	2-1	2	0.5	1					0.1	170323		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	2-2	2	0.5	1					0.1	170323		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	2-3	2	0.5	1					0.1	170323		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	3-1	2	0.5	1					0.1	170323		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	3-2	2	0.5	1					0.1	170323		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	3-3	2	0.5	1					0.1	170323		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	4-1	2	0.5	1					0.1	170323		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	4-2	2	0.5	1					0.1	170323		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	4-3	2	0.5	1					0.1	170323		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	5-1	2	0.5	1					0.1	170323		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	5-2	2	0.5	1					0.1	170323		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	5-3	2	0.5	1					0.1	170323		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	6-1	2	0.5	1					0.1	170323		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	6-2	2	0.5	1					0.1	170323		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	6-3	2	0.5	1					0.1	170323		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	7-1	2	0.5	1					0.1	170323		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	7-2	2	0.5	1					0.1	170323		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	7-3	2	0.5	1					0.1	170323		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	8-1	2	0.5	1					0.1	170323		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	8-2	2	0.5	1					0.1	170323		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	
	8-3	2	0.5	1					0.1	170323		#VALUE!	#VALUE!	1	0.009	15	#VALUE!
			0.5	2					0.1					1		15	

Assay Date	6/17/2005	Test Chemical ID	NA	# Concentrations tested	NA	Microsome type	placental	Microsome ID	lot#BAA	Technician ID	LR	Replicate #	2

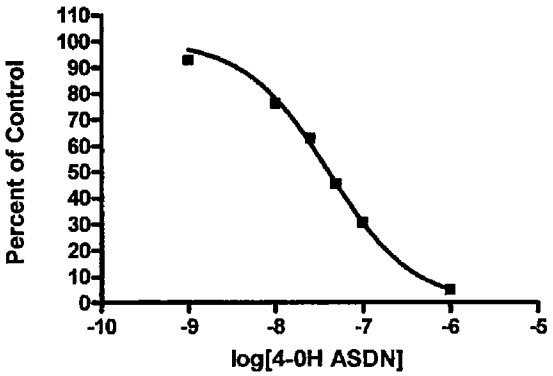
Control Type	Portion	Average	SD
Full activity	Beginning	0.0278	0.0015
Full activity	End	0.0249	0.0005
Full activity	Overall	0.0264	0.0019
Background	Beginning	0.0000	0.000101921
Background	End	0.0000	3.82202E-05
Background	Overall	0.0000	8.4987E-05
Positive	Beginning	0.0150	0.0004
Positive	End	0.0144	0.0001
Positive	Overall	0.0147	0.0004
Negative	Beginning	0.0279	0.0004
Negative	End	0.0279	0.0005
Negative	Overall	0.0279	0.0004

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					
NA					

Percent of control values				
	Log[test	Replicate		
Level	substance]	1	2	3
1				
2				
3				
4				
5				
6				
7				
8				

Appendix F

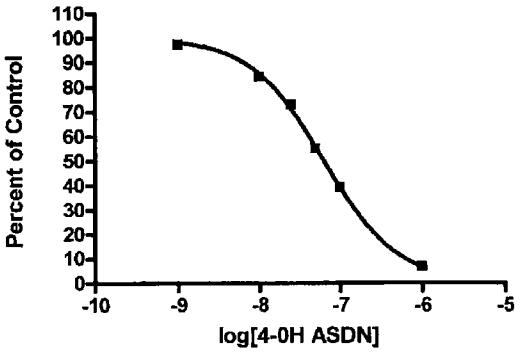
Assay Run (04-22-05)



-6.00	4.80	5.33	4.74
-7.00	30.45	30.00	30.70
-7.30	44.59	46.14	45.11
-7.60	62.60	62.79	62.78
-8.00	77.21	75.47	75.56
-9.00	92.74	91.52	93.85

Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.0
TOP	100.0
LOGEC50	-7.394
HILLSLOPE	-0.9075
EC50	4.039e-008
Std. Error	
LOGEC50	0.01109
HILLSLOPE	0.01835
95% Confidence Intervals	
LOGEC50	-7.417 to -7.370
HILLSLOPE	-0.9464 to -0.8686
EC50	3.826e-008 to 4.264e-008
Goodness of Fit	
Degrees of Freedom	16
R^2 (unweighted)	0.9949
Weighted Sum of Squares (1/Y)	1.027
Absolute Sum of Squares	77.58
Sy.x	2.202
Constraints	
BOTTOM	BOTTOM = 0.0
TOP	TOP = 100.0
Data	
Number of X values	6
Number of Y replicates	3
Total number of values	18
Number of missing values	0

Assay Run (04-25-05)



-6.00	6.30	6.62	6.93
-7.00	37.74	39.79	39.29
-7.30	53.82	54.41	56.74
-7.60	73.87	70.62	73.48
-8.00	84.61	82.38	84.84
-9.00	93.42	98.25	99.50

Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.0
TOP	100.0
LOGEC50	-7.201
HILLSLOPE	-0.9584
EC50	6.296e-008
Std. Error	
LOGEC50	0.009644
HILLSLOPE	0.01728
95% Confidence Intervals	
LOGEC50	-7.221 to -7.180
HILLSLOPE	-0.9950 to -0.9218
EC50	6.007e-008 to 6.600e-008
Goodness of Fit	
Degrees of Freedom	16
R^2 (unweighted)	0.9963
Weighted Sum of Squares (1/Y)	0.8077
Absolute Sum of Squares	59.70
Sy.x	1.932
Constraints	
BOTTOM	BOTTOM = 0.0
TOP	TOP = 100.0
Data	
Number of X values	6
Number of Y replicates	3
Total number of values	18
Number of missing values	0

Appendix G

DRAFT REPORT

PREPARATION AND CHARACTERIZATION OF HUMAN PLACENTAL MICROSOMES

INTRALABORATORY STATISTICAL ANALYSIS OF BATTELLE LABORATORIES DATA

EPA CONTRACT NUMBER 68-W-01-023
WORK ASSIGNMENT 4-16, TASK 6

January 25, 2006

Prepared for

U.S. ENVIRONMENTAL PROTECTION AGENCY
ENDOCRINE DISRUPTOR SCREENING PROGRAM
WASHINGTON, D.C.

Prepared by

BATTELLE
505 King Avenue
Columbus, Ohio 43201

Preparation and Characterization of Human Placental Microsomes
Intralaboratory Statistical Analysis of Battelle Laboratories Data

EPA CONTRACT NUMBER 68-W-01-023
WORK ASSIGNMENT 4-16, TASK 6

Ying-Liang Chou, Author	Date
Paul I. Feder, Reviewer	Date

Quality Assurance Statement

Printed: 1/24/200610:18:10AM

Study Number:G608316

This study was inspected by the Quality Assurance Unit and reports were submitted to the Study Director and Management as follows:

Phase Inspected Inspection	Date	Date Reported to Study Director / Management
Audit study file	1/24/2006	1/24/2006
Audit draft report	1/24/2006	1/24/2006

Quality Assurance Unit — Date: 1·26·06

This report discusses the methods and results of the intralaboratory statistical analysis of the Battelle Laboratories data for the placental aromatase assay, WA 4-16 Task 6 "Preparation and Characterization of Human Placental Microsomes". The microsomes that were used in the laboratory studies discussed in this report were supplied by either Battelle Laboratories (Battelle) or In Vitro Technologies, Inc. (IVT) and were analyzed by Battelle.

Summary and Conclusions

Three types of data were analyzed: 4-OH ASDN positive control inhibition, aromatase activity, and protein concentration. Battelle developed the data discussed in this report. Battelle analyzed Battelle prepared microsomes and determined 4-OH ASDN positive control inhibition curve fits, aromatase activity levels, and protein concentrations. Battelle also analyzed IVT prepared microsomes and determined aromatase activity levels and protein concentrations. For the inhibition concentration data there were two independent replicates, with microsomes prepared by Battelle. For the aromatase activity data there were four independent replicates of the full enzyme activity and background activity controls and two independent replicates of the positive and negative controls with microsomes prepared by Battelle. There were two independent replicates of the full enzyme activity, background activity, positive, and negative controls with microsomes prepared by IVT. For the protein concentration data there were six independent replicates with microsomes prepared by Battelle and four independent replicates with microsomes prepared by IVT. Statistical analyses were carried out separately for the inhibition curve fit data, the aromatase activity data, and the protein concentration data. For the inhibition curve analysis, percent of control responses for aromatase activity were used. For the aromatase activity analysis, the corrected aromatase activity values (nmol/mg protein/min) were used. Aromatase activity levels were based on four types of controls: full enzyme activity, background activity, positive, and negative controls. Statistical analyses were performed separately for these four types of controls. For the protein concentration analysis, the protein concentrations (mg/mL) were used.

For the inhibition curve data, concentration response curves were fitted within each replicate to describe the relation between 4-OH ASDN concentration and extent of inhibition. The concentration response curves were summarized by the IC_{50} (concentration corresponding to 50 percent inhibition) and the slope. Results were compared across replicates. In addition, full enzyme activity control, background activity control, positive and negative control tube responses associated with the inhibition curve tests (two replicates) were compared between the beginning and the end of each replicate to identify differences within replicates and differences across replicates. For the aromatase activity data, two-way mixed effects analysis of variance with fixed microsome preparation source effect and random replicate effect was performed. Analysis of variance tests were carried out to determine if the microsome preparation source effect was significant. For the protein concentration data, a two-sample t-test was carried out to determine if the microsome preparation source effect was significant.

The following results were obtained:

1. For the inhibition curve fits, for $\log_{10}IC_{50}$ the replicate-to-replicate variation was two orders of magnitude larger than the individual replicate within replicate variation. The within-replicate variations were close to zero. For slope, the replicate-to-replicate variation was about three times the individual replicate within-replicate variances.

2. For the controls in the inhibition curve tests, for the full enzyme activity controls and the positive controls, the averages of the two percent of controls measurements at the end were lower than the averages at the beginning for both replicates. The average difference was significant for the full enzyme activity controls and borderline significant ($p=0.055$) for the positive controls. For the background activity controls and for the negative controls, the averages of the measurements at the end were lower than the average at the beginning, but the differences were not statistically significant. In general the aromatase activity at the end of each replicate was lower than at the beginning.

3. For the aromatase activity results, significant laboratory effects were found for the full enzyme activity controls, positive controls, and negative controls. The activity levels were lower for the IVT prepared microsomes than for the Battelle prepared microsomes. (The background adjusted background activity controls are by definition constrained to have on average 0 activity within each replicate within each laboratory). Variance estimates for replicate and for repetition within replicate were small.

4. A highly significant microsome source effect was identified for the protein concentration results. The Battelle prepared microsomes had more than 2.3 times higher protein concentration than the IVT prepared microsomes.

Introduction and Background

In Task 6 of the Placental Aromatase Validation Study, Battelle and In Vitro Technologies, Inc. each prepared microsomes. They each carried out two independent replicates of a positive control inhibition study with 4-OH ASDN using their own microsomes. Battelle and IVT fitted concentration response curves to the data from each of the two replicates. Each laboratory prepared graphical displays and analysis of variance summary comparisons of the concentration response curves and for the full enzyme activity control, background activity control, positive, and negative controls associated with the inhibition curve tests, as in Task 4. There was no inter-laboratory comparison of results for the inhibition study.

Battelle supplied microsomes to IVT, RTI, and WIL laboratories and IVT supplied microsomes to Battelle, RTI, and WIL laboratories. Each laboratory determined aromatase activity and protein concentrations of each microsomal preparation, as discussed in the test protocol. Battelle and IVT compared the aromatase activity and the protein concentrations between the two microsome sources by analysis of variance and a two sample t-test, respectively.

This report discusses the methods and results of the intralaboratory statistical analyses performed on the experimental data developed by Battelle, based on the microsomes prepared by Battelle and on those prepared by IVT.

Data Used in the Analyses

Inhibition Curve Data

Aromatase activity levels were determined for six graded concentrations of the positive control inhibitor 4-OH ASDN and for the associated full enzyme activity, background activity, positive, and negative control results.

Two replicates of the positive control inhibitor study were carried out. Within each replicate three repetitions were run at each of the 4-OH ASDN log (base 10) concentrations -6, -7, -7.3, -7.6, -8, and -9. In addition, two repeat tubes of the full enzyme activity control, background activity control, positive, and negative controls were run prior to the 4-OH ASDN runs and two repeat tubes of each of the four types of controls were run following the 4-OH ASDN runs.

Statistical analyses were carried out on the "percent of control" responses. Percent of control is defined as the ratio of the background adjusted aromatase activity in the tube under consideration to the average background adjusted aromatase activity among the four full enzyme activity control tubes within the replicate, times 100. The average percent of control among the four full enzyme activity control tubes is necessarily 100 percent within each replicate. The average percent of control among the four background activity control tubes is necessarily 0 percent.

Nominally for an inhibitor the percent of aromatase activity values vary between approximately 0% near the high inhibition concentrations and approximately 100% near the low inhibition concentrations, but this may vary with the inhibitor.

The 4-OH ASDN concentration inhibition data are displayed in Table A-1. The full enzyme activity control, background activity control, positive and negative controls aromatase activity and the percent of control data associated with the inhibition curve test replicates are displayed in Table A-2.

Aromatase Activity Data

There are four types of aromatase activity data: full enzyme activity, background activity, positive, and negative controls. The microsomes were prepared by Battelle or IVT. For the full enzyme activity, background activity controls there were four independent replicates of Battelle tests with Battelle prepared microsomes. Two replicates corresponded to the inhibition curve tests and two replicates corresponded to the aromatase activity tests. For the positive and

negative controls there were two independent replicates of Battelle tests with Battelle prepared microsomes, corresponding to the inhibition curve tests. There were two independent replicates of Battelle tests with IVT produced microsomes, corresponding to the aromatase activity tests. Four repeat determinations were made in each replicate. The background corrected aromatase activity values were used as responses in the analyses. Aromatase activity values (nmol/mg protein/min) are displayed in Tables A-3 to A-6, one table for each type of control.

Protein Determination Data

Protein concentrations were determined by Battelle on microsomes prepared by Battelle and on microsomes prepared by IVT. Protein concentration determinations were made in the inhibition curve tests (Battelle microsomes only), in the aromatase activity tests (IVT and Battelle microsomes), and in the protein concentration determination tests (IVT and Battelle microsomes). Two replicate protein concentration determinations were carried out within each test type. Thus there were six replicate protein concentration determinations for the protein corresponding to the Battelle prepared microsomes and four replicate protein concentration determinations for the protein corresponding to the IVT prepared microsomes. Protein concentration determinations (mg/mL) are displayed in Table 7.

Objectives

The principal objectives of the statistical analysis are:

1. Fit concentration response models within each of the two replicates of the inhibition curve studies with Battelle microsomes to describe the trend in percent of control activity across varying inhibition concentrations of the positive control inhibitor 4-OH ASDN. Estimate the IC_{50} concentration, the slope, and associated standard errors within each replicate. Combine the results across replicates to determine the average IC_{50} concentration, the average slope, and associated standard errors across replicates.

2. Determine whether there were differences between the beginning and the end of each replicate for the full enzyme activity, background activity, positive, and negative control results within each replicate of the inhibition curve test.

3. Compare the aromatase activity values (nmol/mg protein/min) of the full enzyme activity, background activity, positive, and negative controls between the microsomes prepared by IVT and the microsomes prepared by Battelle.

4. Compare the protein concentrations (mg/mL) between the microsomes prepared by IVT and the microsomes prepared by Battelle.

Statistical Analysis Methods

Concentration Response Inhibition Curves

Within each replicate a concentration response inhibition curve was fitted to the percent of aromatase activity values at the three repetitions at each of the six graded 4-OH ASDN inhibitor concentrations.

For purposes of response curve fitting, concentration was expressed on the log scale. In agreement with past convention, common logarithms (i.e. base 10) were used. Let X denote the logarithm of the concentration of inhibitor compound (e.g. if concentration = 10^{-5} then X = -5). Let

Y ≡ (background corrected) percent of control in the inhibitor tube
X ≡ logarithm (base 10) of the concentration
DAVG ≡ average (not corrected for background) DPMs across the repeat tubes with the same inhibitor concentration
β ≡ slope of the concentration response curve (β is negative)
$\mu \equiv \log_{10}IC_{50}$ (IC_{50} is the concentration corresponding to percent of control equal to 50%)

The following two parameter concentration response curve was fitted to relate percent of aromatase activity to logarithm of concentration within each replicate

$$Y = 100/[1 + 10^{(\mu - X)\beta}] + \epsilon$$

where ϵ is the variation among repetitions, distributed with mean 0 and variance approximately proportional to DAVG (based on Poisson distribution theory for radiation counts) and also approximately proportional to the response Y.

The response curve was fitted by weighted least squares nonlinear regression analysis with weights equal to 1/Y. This weighting system gives greater weight to the lower end of the concentration response curve, where greater inhibition occurs. Observed percent of control values above 100% were set to 99.5%. Observed percent of control values below 0% were set to 0.5%. This adjustment tacitly assumes an upper bound of 100% on the concentration response curve and a lower bound of 0%.

For each replicate the estimated $\log_{10}IC_{50}$ (μ) and its associated standard error, the IC_{50} and its associated geometric standard error, the slope (β) and its associated standard error, and the "Status" of each response curve are reported. The "Status" of each response curve is indicated as "C", complete, if the concentration response curve inhibition ranges from essentially 0 percent to 100 percent of control. Otherwise it is indicated as "I", incomplete.

For each replicate the individual percent of control values were plotted versus logarithm of

inhibitor compound concentration. The fitted concentration response curve was superimposed on the same plot. These plots display the data, the fitted response curves in relation to these data, and deviations from the fits.

One-way random effects analysis of variance models with heterogeneous variances among the replicates were fitted to the parameter estimates, $\log_{10}IC_{50}$ (μ) and slope (β), from the concentration response curve fits within each replicate, using weights incorporating within replicate variances. The random effect was replicate. The within replicate variances were estimated as the squares of the standard errors for each replicate. The analysis of variance fits provide estimated weighted averages (means) across the replicates and their associated standard errors. Degrees of freedom associated with the mean effects were calculated based on Satterthwaite's approximation.

The estimated IC_{50} for the test substance was calculated as 10 to the power mean $\log_{10}IC_{50}$. The geometric standard error associated with the estimated IC_{50} was calculated as 10 to the power standard error associated with mean $\log_{10}IC_{50}$.

Slope (β) and $\log_{10}IC_{50}$ (μ) were each compared across replicates based on the one-way random effects analysis of variance model fit. For each of β and μ, plots were prepared that display the parameters within each replicate with associated 95% confidence intervals based on the within replicate standard error and the average across replicates with associated 95% confidence interval incorporating replicate-to-replicate variation.

Concentration response curves were also fitted to the averages of the three repetitions within each replicate and estimates and associated standard errors (or geometric standard error) for $\log_{10}IC_{50}$ (μ), IC_{50}, and slope (β) were displayed. The averages of the three repetitions for each of the three replicates were plotted in the same plot with plotting symbols distinguishing among replicates. The concentration response curves for each replicate, fitted to the average data, were superimposed on the same plot to compare the percent of aromatase activity values across replicates.

On a separate plot the average percent of control values for each of the replicates were plotted versus logarithm of inhibitor concentrations. The average concentration response curve across replicates was superimposed on the same plot. The average response curve was defined as

$$Y_{avg} = 100/[1 + 10^{\beta avg(\mu avg - X)}]$$

where β_{avg} and μ_{avg} were the mean values across the replicates, based on the random effects one-way analysis of variance model discussed above.

All concentration response curves were fitted to the data using the nonlinear regression analysis features in the PRISM statistical analysis package, Version 4. Supplemental statistical analyses and displays such as summary tables, graphical displays, analysis of variance, and

multiple comparisons were carried out using PRISM and the SAS statistical analysis system-Version 9.

Within each replicate, quadruplicate repetitions were made for the full enzyme activity, background activity, positive, and negative controls responses. Half the repetitions were carried out at the beginning of the replicate and half at the end. If the test conditions were consistent throughout the replicate, the control tube responses at the beginning should be equivalent to those at the end.

The control responses were expressed as percent of control. The full enzyme activity, background activity, positive, and negative control percent of control responses associated with the inhibitor concentration tests were plotted across replicates, with plotting symbol distinguishing between beginning and end, and with reference line at 0% (background activity control), at 100% (full enzyme activity control) at 50% (positive control), or at 100% (negative control). These plots indicate the extent of consistency across replicates with respect to average value and variability, and provide comparisons of beginning versus end of each replicate. Additional plots were prepared displaying the differences of the averages of the first two percent of control values (i.e. those based on the "beginning" tubes) and the averages of the last two percent of control values (i.e. those based on the "end" tubes) across replicates (end minus beginning). Each plot has a reference line of 0.

Mixed effects analysis of variance models were fitted to the full enzyme activity, background activity, positive, and negative control data. The response was percent of control. The fixed effect factor in the analysis of variance was portion (beginning or end). The random effects were replicate and portion by replicate interaction. The residual error variation was based on the variation among repetitions within replicate and portion. For the background activity and full enzyme activity controls, the average of the repetitions within a replicate are constrained to be 0 and 100 respectively, which implies that the variation associated with the replication effect is necessarily constrained to be 0.

Aromatase Activity Data

Each of the four types of aromatase activity responses (full enzyme activity, background activity, positive, and negative controls) were fitted with mixed effects analysis of variance models. The response was aromatase activity (nmol/mg protein/min) The fixed effect was microsome source (the laboratory which prepared the microsomes) and the random effect was replicate within microsome source. Analysis of variance tests were performed to determine if the microsome source effect was significant. Summary statistics (N, mean, and standard deviation) were calculated. Scatter plots were also prepared with different plotting symbols for each microsome source.

Protein Concentration Data

A two-sample t-test was performed to compare on protein concentrations between the two

microsome sources. The response was protein concentration (mg/mL). Summary statistics (N, mean, and standard deviation) were calculated by microsome source. A scatter plot was also prepared, having different plotting symbols for each microsome source.

Round Off

Some derived values in the results tables may differ from those in the computer printouts or from those obtained using hand calculations by several units in the least significant digit due to round off in intermediate numbers or in intermediate calculations.

Statistical Analysis Results

Inhibition Curve Fit Data

Concentration response curves were fitted separately to the individual repetitions within each replicate and to the averages of the repetitions at each inhibition concentration within each replicate (Table A-1). The parameters of these fitted concentration response curves are displayed in Table 1. The individual repetition data within each replicate are plotted in Figure A-1 through Figure A-2 with the corresponding fitted concentration response curves superimposed in each figure. Figure 1 displays the two concentration response curves fitted to the averages of the three repetitions within each replicate. Replicate 2 has slightly higher estimated IC_{50} and a more negative slope (Table 1).

The parameters of the mean concentration response curve, based on random effects analysis of variance model fits with replicate as a random effect are displayed in Table 1. The average concentration response curves, along with the averages of three repetitions within each replicate are plotted together in Figure 2.

The parameter estimates for each replicate and the average parameter estimates across replicates and their associated 95% confidence intervals are displayed in Table 2 and graphed in Figure 3 for $\log_{10}IC_{50}$ and Figure 4 for slope. In Figure 3, replicate 2 is seen to have a higher IC_{50} than the average. In Figure 4, the replicates 1 and 2 slopes were close to the average, with replicate 1's slope higher than the average and replicate 2's slope lower. The substantially larger widths of the confidence intervals for the overall averages in Figures 3 and 4 are due to larger replicate-to-replicate variation than within replicate variation (Table 3) and due to having just one degree of freedom with which to estimate replicate-to-replicate variation.

The results of analyses of variance for these estimates are presented in Table 3. For each replicate the squares of the standard errors associated with each parameter are given. These estimates include only within replicate variation. Across replicates, the replicate-to-replicate variation and the square of the standard error of the overall average are displayed. These estimates include both within replicate variation and replicate-to-replicate variation.

For $log_{10}IC_{50}$, the replicate-to-replicate variation was two orders of magnitude larger than the individual replicate within replicate variances. The within-replicate variances were close to zero. For slope, the replicate-to-replicate variation was about three times the individual replicate within replicate variances.

The full enzyme activity, background activity, positive, and negative control responses associated with the inhibition curve tests are displayed in Table A-2 for each replicate. These data are plotted by replicate in Figures 5 to 8, with plotting symbol distinguishing between beginning and end of the replicate. The differences between the averages at the beginning and at the end within each replicate (end minus beginning) are displayed in Figures 9 to 12.

Mixed effects analysis of variance models were fitted to the full enzyme activity, background activity, positive, and negative control data with portion as a fixed effect and with replicate and replicate by portion interaction as random effects. For the full enzyme activity controls and for the background activity controls the replicate variation is constrained to be 0 by the definitions of the background and full enzyme activity control responses. The analysis results for four types of control data are displayed in Table 4. The left panel of the table displays the results of the tests for the differences between the responses collected at the beginning and at the end of a replicate (end minus beginning). The right panel displays the estimated variance components.

For the full enzyme activity controls, the averages of the two percent of controls measurements at the end were approximately 5% and 10% lower than at the beginning for replicates 1 and 2, respectively (Figure 9). The standard error of the average of these differences was about 2.58 %. The difference between the beginning and the end, averaged across replicates, was significant (p=0.023). The estimated repetition variation was 13.32, which was substantially higher than the variation among replicates. The estimated variance for portion by replicate interaction was essentially zero (Table 4). The estimated replicate variance was zero by design.

For the background activity controls the averages of the two measurements at the end were approximately 0.15% lower and 0.1% higher than at the beginning for replicates 1 and 2, respectively (Figure 10). The standard error of the average of these differences was about 0.11 %. The difference between the beginning and the end, averaged across replicates, was not significant (p=0.711). The estimated repetition variation was 0.016 and was about five times larger than the replicate by portion interaction variance (Table 4). The estimated replicate variance was zero by design.

For the positive controls, the averages of the two measurements at the end were approximately 2% lower than at the beginning for both replicates 1 and 2 (Figure 11). The standard error of the average of these differences was about 0.77 %. The difference between the beginning and the end, averaged across replicates, was borderline significant (p=0.055). The estimated replicate variation was 1.19 and was very much smaller than the replicate-to-replicate variation. Figure 7 shows that the positive controls were about 10 percentage points higher in replicate 2 than in replicate 1. The estimated repetition variation attributed only 2 % of total

variation. There was no portion by replicate interaction variation. (Table 4).

For the negative controls, the average of the two measurements at the end was essentially the same as that at the beginning for replicate 1 and the average of the two measurements at the end was approximately 7% lower than at the beginning, for replicate 2 (Figure 12). The standard error of the average of these differences was about 3.26 %. The difference between the beginning and the end, averaged across replicates, was not statistically significant ($p=0.482$). The estimated portion by replicate interaction and repetition variations were 9.368, and 2.498 and were very much smaller than the replicate-to-replicate variation. Figure 8 shows that the negative controls were about 10 percentage points higher in replicate 2 than in replicate 1.

A possible explanation for the larger positive and negative control values in replicate 2 than in replicate 1 can be seen in Table 5, for the Battelle full enzyme activity controls associated with the inhibition curve fits. The average aromatase activity in replicate 1 is about 28 percent higher than that in replicate 2. The difference in activity is statistically significant.

Aromatase Activity

Mixed effects analysis of variance was carried out on the aromatase activity for each of the four control types (full enzyme, background activity, positive, and negative controls) pooled across the inhibition curve tests (Battelle produced microsomes) and the aromatase activity tests (Battelle and IVT produced microsomes). The fixed effect was microsome source (the laboratory that prepared the microsomes) and the random effect was replicate within laboratory. The residual variation was based on the repetition within replicate variance. Summary statistics are displayed in Table 5. Scatter plots for each of the control types are presented in Figures 13 to 16. For each of the control types, analysis of variance tests for source effect were carried out. The test was not significant for background adjusted background activity controls (since the controls within each replicate must sum to 0, by definition of background adjustment). The other three types of controls (full enzyme activity, positive, and negative controls) showed significant source effect. Table 6 shows that the variance component estimates were all close to zero for each of the four control types.

Protein Concentration Determination

Table 7 displays protein summary statistics and result of a two-sample t-test to compare microsome sources. The two-sample t-test shows that the Battelle produced microsomes had significantly higher average protein concentration than the IVT produced microsomes ($p<0.0001$). On average, the protein concentrations in the Battelle produced microsomes were more than two times higher than those in the In Vitro Technologies produced microsomes. The protein concentrations are displayed by laboratory in Figure 17.

Table 1. Parameters of the Concentration Response Curve Fits by Replicate and Averaged Across Replicates. Percent of Control Activity. Placental Aromatase Assay. Microsomes Prepared by Battelle Laboratories.

Replicate	$Log_{10}IC_{50}$ (SE)	IC_{50} (GSE)[d]	Slope (SE)	Status
Individual Values[a]				
1	-7.394 (0.011)	$4.039x10^{-8}$ (1.026)	-0.908 (0.018)	C
2	-7.201 (0.010)	$6.296x10^{-8}$ (1.022)	-0.958 (0.017)	C
Mean[c]	-7.297 (0.097)	$5.042x10^{-8}$ (1.249)	-0.933 (0.025)	--
Average Values[b]				
1	-7.394 (0.021)	$4.041x10^{-8}$ (1.049)	-0.907 (0.034)	C
2	-7.200 (0.011)	$6.304 x10^{-8}$ (1.027)	-0.958 (0.020)	C

a. Concentration response curve fitted to the data collected within each replicate, with three repetitions at each 4-OH ASDN concentration level.
b. Concentration response curve fitted to the averages of the three repetitions at each 4-OH ASDN concentration level within each replicate.
c. Weighted averages of the parameter estimates across the two replicates.
d. 10 to the power of $log_{10}IC_{50}$ and 10 to the power of its associated standard error.

Table 2. Parameter Estimates of the Concentration Response Curves and Associated 95% Confidence Intervals. Percent of Control Activity. Placental Aromatase Assay. Microsomes Prepared by Battelle Laboratories.

Parameter	Estimate (95% CI)		
	Replicate 1[a]	Replicate 2[a]	Mean [b]
$Log_{10}IC_{50}$	-7.394 (-7.418, -7.370)	-7.201 (-7.221, -7.181)	-7.297 (-8.524, -6.071)
Slope	-0.908 (-0.946, -0.869)	-0.958 (-0.995, -0.922)	-0.933 (-1.258, -0.609)

a. Parameter estimates and their associated 95% confidence intervals for each replicate, based on the concentration response curves fitted to the individual repetition values within replicates.
b. Mean and its associated 95% confidence interval, based on a one-way analysis of variance model with replicate treated as a random effect.

Table 3. Variances Associated with Estimated Parameters of Concentration Response Curves. Percent of Control Activity. Placental Aromatase Assay. Microsomes Prepared by Battelle Laboratories.

Parameter	Variance/Degree of Freedom [a,b]			
	Replicate 1	Replicate 2	Overall	
			Random Replicate (p-value)[d]	Variance of Mean[c,e]
$Log_{10}IC_{50}$	0.000123 /df=16	0.000093 /df=16	0.01852 /df=1 (p=0.241)	0.009312 /df=1
Slope	0.000337 /df=16	0.000299 /df=16	0.00098 /df=1 (p=0.297)	0.000648 /df=0.998

a. The variance estimates for each replicate were based on the concentration response curves fitted to the individual repetition results within each concentration level.
b. Variance estimates for the random replicate were estimated based on a one-way random effects analysis of variance. The variances for each replicate were fixed at their reported values.
c. Degrees of freedom for the variance of mean were estimated by $2*((1/K)* \sum(S_r^2 + S_i^2))^2/(var(S_r^2) + (2/K^2)* \sum(S_i^4/d f_i))$, where S_r^2 is random replicate variance, S_i^2 and df_i are estimated variance and degree of freedom for a given replicate, $var(S_r^2)$ is the variance associated with the estimation of S_r^2 and K is the number of replicates (Hartung and Makambi, 2001).
d. p-value is based on the Wald Z-test result.
e. Variance of mean is the square of the standard error.

Table 4. Variance Components of the Percent of Control Values for Full Enzyme Activity Control, Background Activity Control, Positive Control, and Negative Control. Position Effects and Variation Across Replicates of Portion Effects Within Replicates. Microsomes Prepared by Battelle Laboratories.

Parameter	Difference Between Beginning and End Portions (End Minus Beginning)		Variance Components [a]		
	Estimate (%) (Std. Error)	p-Value/ Degree of Freedom	Replicate	Replicate* Portion	Residual (Repetition)
Full Enzyme Activity Control	-7.867 (2.580)	0.023/ df=6	0	0.000	13.317
Background Activity Control	-0.045 (0.106)	0.711/ df=2	0	0.003	0.016
Positive Control	-1.925 (0.771)	0.055/ df=5	55.248	0	1.190
Negative Control	-3.446 (3.258)	0.482/ df=1	65.855	9.368	2.498

a. The replicate component of variation is constrained to be 0, by definitions of background and full enzyme activity control responses.

Table 5. Summary Statistics for the Aromatase Activity (nmol/mg protein/min). By Control Type, Microsome Source, and Test Type Within Source.

Control Type	Microsomes Prepared By	Data Type	Replicate	N	Mean	Standard Deviation
Full Enzyme Activity Control	Battelle	Aromatase Activity	1	4	0.0419	0.0006
			2	4	0.0528	0.0028
		Inhibition Curve Fit	1	4	0.0671	0.0020
			2	4	0.0524	0.0040
	IVT	Aromatase Activity	1	4	0.0288	0.0003
			2	4	0.0264	0.0019
Background Activity Control	Battelle	Aromatase Activity	1	4	1.0588×10^{-21}	2.5645×10^{-05}
			2	4	-8.4703×10^{-22}	3.9933×10^{-05}
		Inhibition Curve Fit	1	4	-1.6941×10^{-21}	1.0769×10^{-04}
			2	4	-5.0822×10^{-21}	5.6960×10^{-05}
	IVT	Aromatase Activity	1	4	1.6941×10^{-20}	1.5990×10^{-04}
			2	4	-1.6941×10^{-21}	8.4987×10^{-05}
Positive Control	Battelle	Inhibition Curve Fit	1	4	0.0305	0.0010
			2	4	0.0294	0.0008
	IVT	Aromatase Activity	1	4	0.0159	0.0003
			2	4	0.0147	0.0004
Negative Control	Battelle	Inhibition Curve Fit	1	4	0.0639	0.0012
			2	4	0.0562	0.0020
	IVT	Aromatase Activity	1	4	0.0296	0.0004
			2	4	0.0279	0.0004

Table 6. Aromatase Activity Variance Component Estimates for Replicate within Source and Repetition Within Replicate and Two-Sample T-test Results. By Control Type.

Control Type	Variance/Degree of Freedom [a,b]		Source Effect [d]
	Repetition Within Replicate	Among Replicate Within Source (p-value)[c]	
Full Enzyme Activity Control	5.338×10^{-6} /df=18	7.9×10^{-5} /df=4 (p=0.0822)	p=0.0289
Background Activity Control	6.802×10^{-9} /df=18	0 /df=4 (p=NA)	p=1.0000
Positive Control	4.559×10^{-7} /df=12	5.415×10^{-7} /df=2 (p= NA)	p=0.0030
Negative Control	1.47×10^{-6} /df=12	1.5×10^{-5} /df=2 (p=0.1645)	p=0.0154

a. The variance estimates were based on the aromatase activity values for the individual repetition results within each replicate.
b. Variance estimate for the random replicate (within laboratory) effect were estimated based on a one-way random effects analysis of variance. The residual of the ANOVA was repetition (within replicate) effect.
c. p-value is based on the Wald Z-test result.
d. Based on two-sample t-test.

Table 7. Protein Concentration Summary Statistics (mg/mL)and Two-Sample T-test Result. By Microsome Source.

Microsomes Prepared by	Data Type	Replicate	Protein Concentration (mg/mL)	Summary Statistics			Two-Sample T-Test p-Value[a]
				N	Mean	Std Dev	
Battelle	Control Activity	1	20.270	6	19.911	1.664	<0.0001
		2	17.724				
	Curve Fit	1	20.067				
		2	18.607				
	Protein Determination	1	22.579				
		2	20.217				
IVT	Control Activity	1	7.987	4	8.569	0.529	
		2	8.304				
	Protein Determination	1	8.804				
		2	9.181				

a. Two-sample t-test was based on equal variances and 8 degrees of freedom.

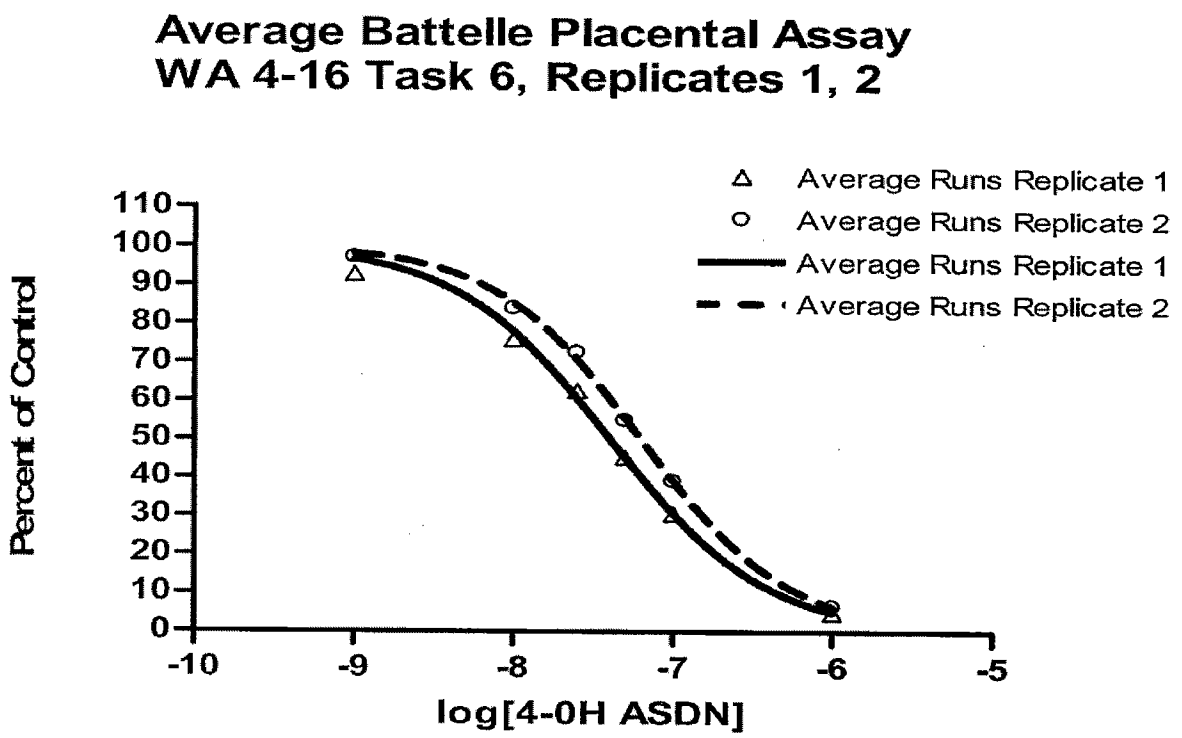


Figure 1. Concentration Response Curves and Averages of Repetitions Within 4-OH ASDN Concentrations. Placental Aromatase Assay. By Replicate. Microsomes Prepared by Battelle Laboratories.

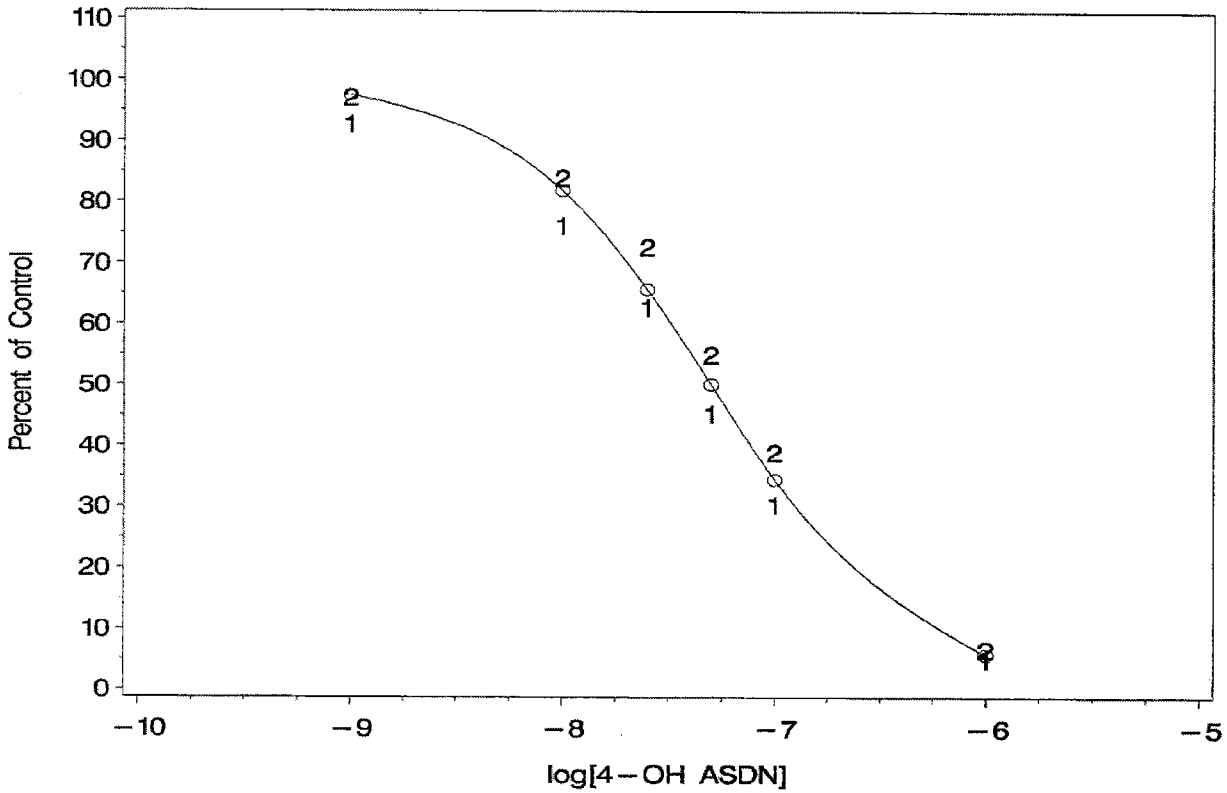


Figure 2. Overall Average Concentration Response Curve Across Replicates and Average Responses Across Repetitions Within 4-OH ASDN Concentrations. Placental Aromatase Assay. Parameters of Average Curve Based on One-Way Analysis of Variance Across Replicate Parameter Values. Microsomes Prepared by Battelle Laboratories.

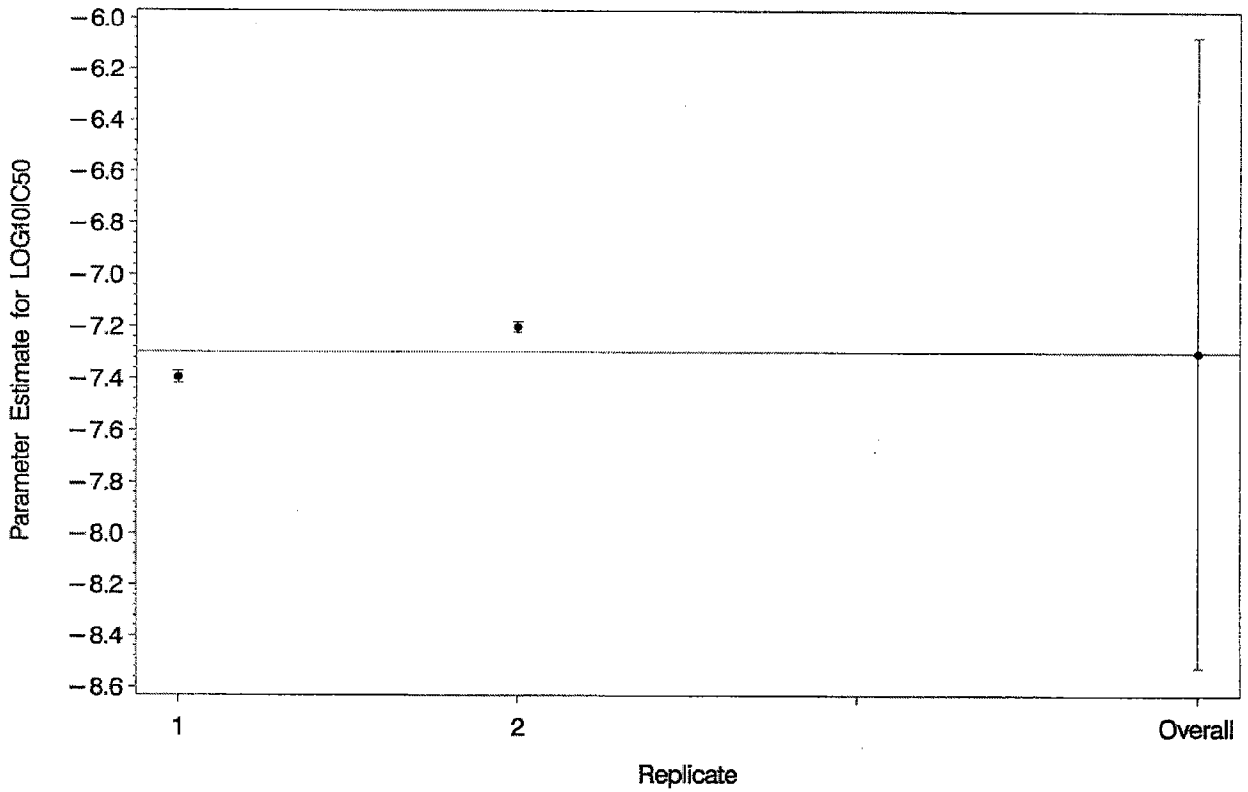


Figure 3. $Log_{10}IC_{50}$ Parameter Estimates and Their Associated 95% Confidence Intervals for Each Replicate and Average Across Replicates. Placental Aromatase Assay. The Solid Reference Line Corresponds to the Average Across Replicates. Microsomes Prepared by Battelle Laboratories.

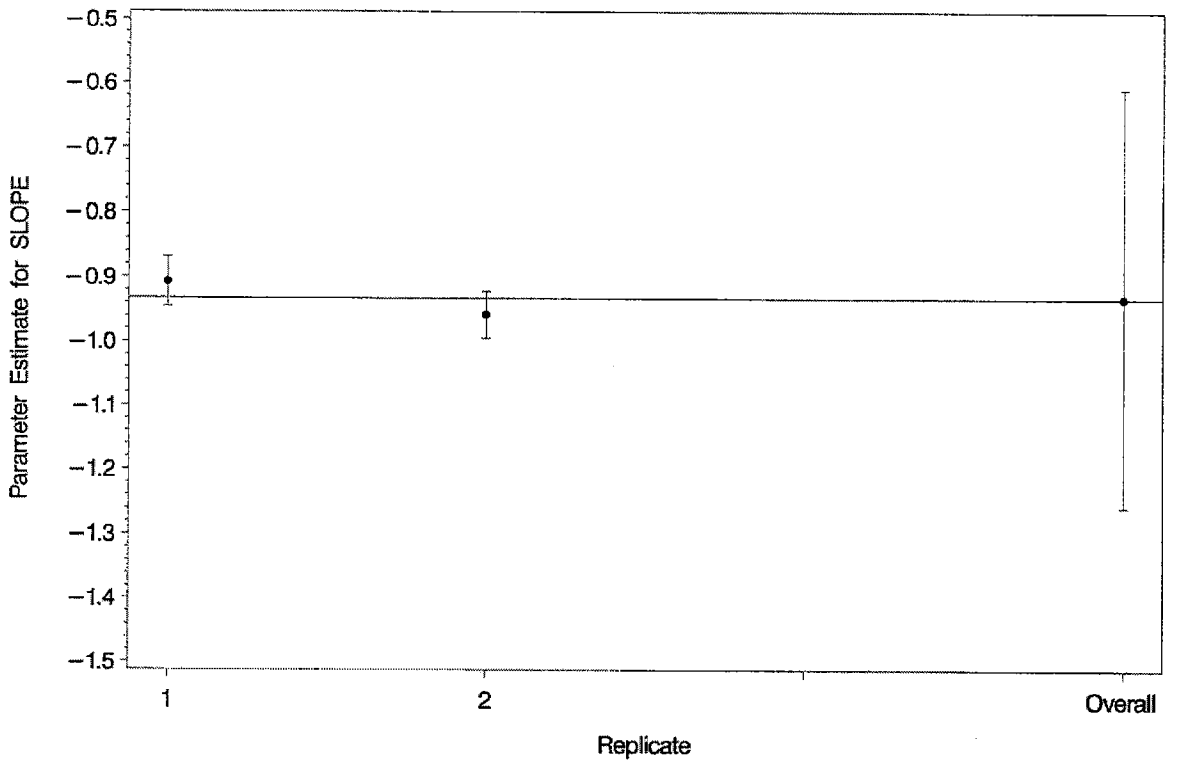


Figure 4. Slope (β) Parameter Estimates and Their Associated 95% Confidence Intervals for Each Replicate and Across Replicates. Placental Aromatase Assay. The Solid Reference Line Corresponds to the Average Across Replicates. Microsomes Prepared by Battelle Laboratories.

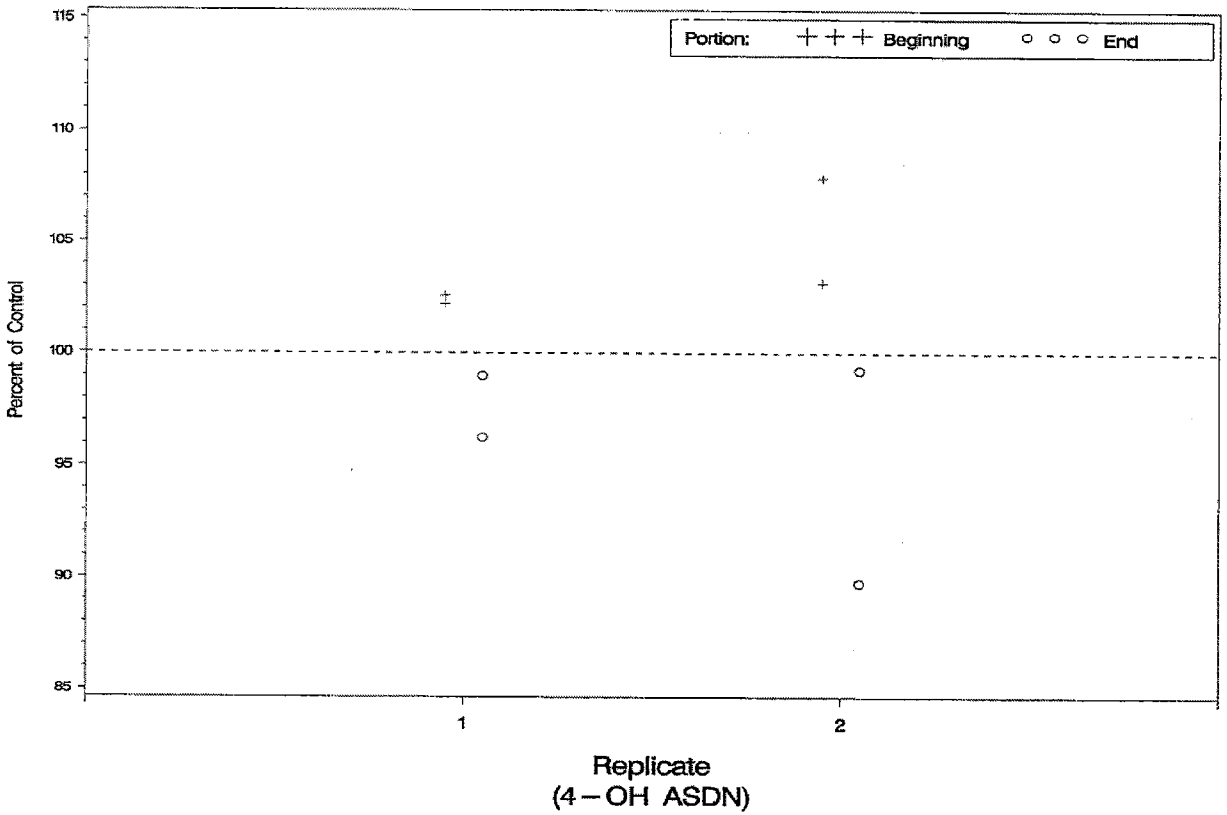


Figure 5. Full Enzyme Activity Control Data Associated with Inhibition Concentration Tests. Percent of Control by Replicate and Portion of Replicate (Beginning or End). Placental Aromatase Assay. Reference Line at 100%. Microsomes Prepared by Battelle Laboratories.

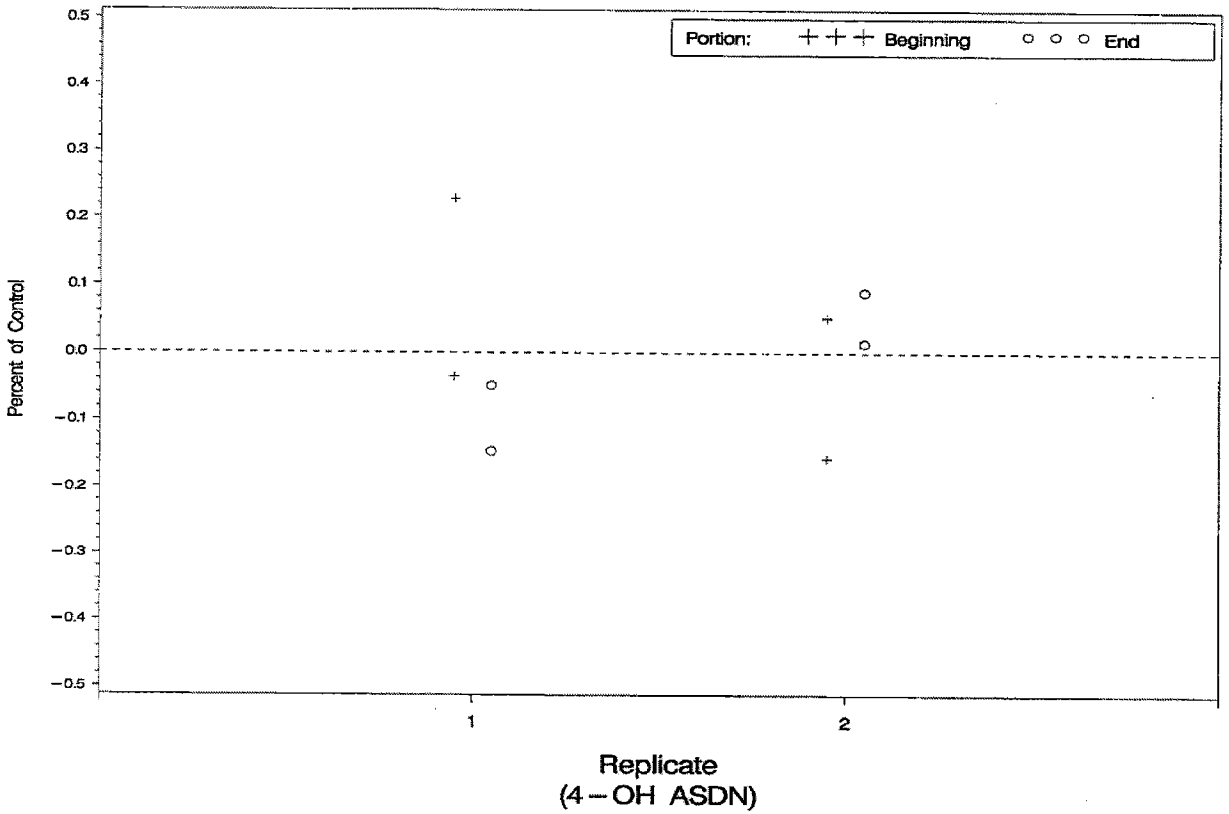


Figure 6. Background Activity Control Data Associated with Inhibition Concentration Tests. Percent of Control by Replicate and Portion of Replicate (Beginning or End). Placental Aromatase Assay. Reference Line at 0%. Microsomes Prepared by Battelle Laboratories.

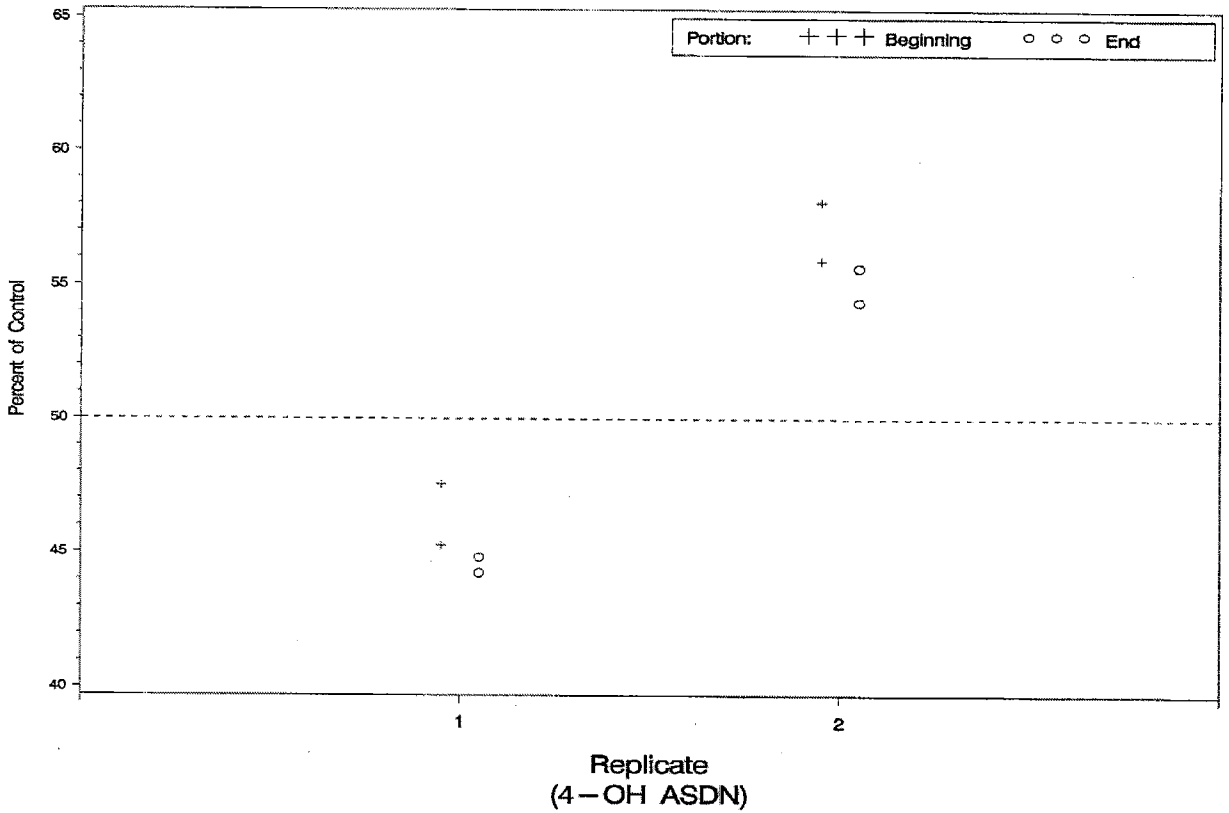


Figure 7. Positive Control Data Associated with Inhibition Concentration Tests. Percent of Control by Replicate and Portion of Replicate (Beginning or End). Placental Aromatase Assay. Reference Line at 50%. Microsomes Prepared by Battelle Laboratories.

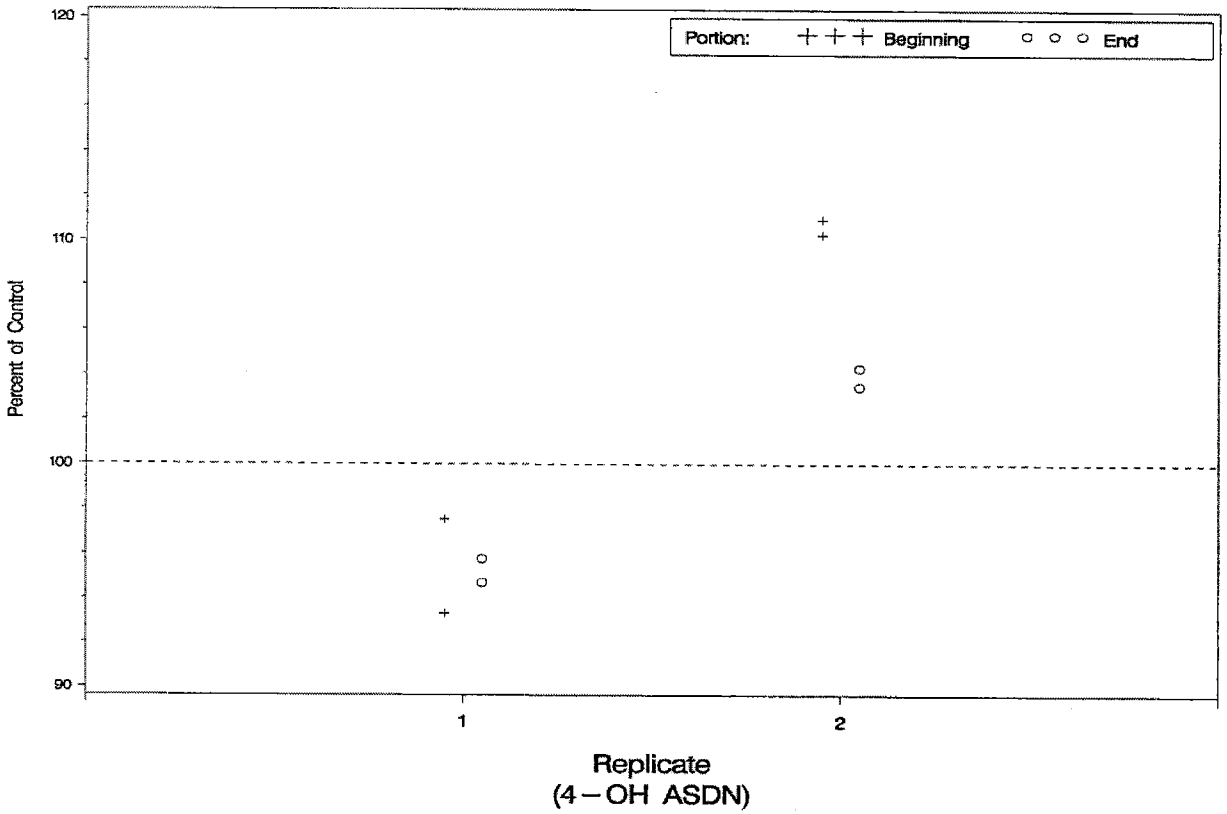


Figure 8. Negative Control Data Associated with Inhibition Concentration Tests. Percent of Control by Replicate and Portion of Replicate (Beginning or End). Placental Aromatase Assay. Reference Line at 100%. Microsomes Prepared by Battelle Laboratories.

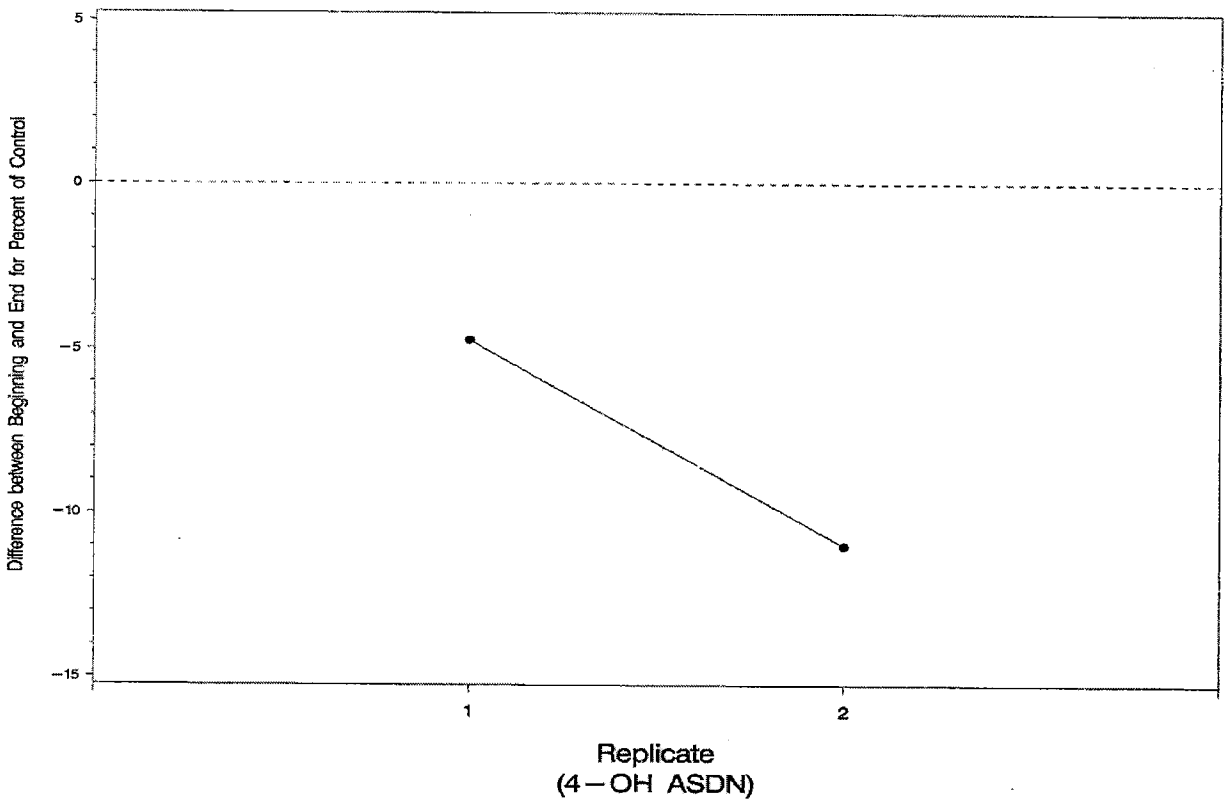


Figure 9. Full Enzyme Activity Control Data Associated with Inhibition Concentration Tests. Difference Between the Average of the Two End Percent of Control Responses and the Average of the Two Beginning Responses by Replicate (End Minus Beginning). By Replicate. Reference Line at 0%. Microsomes Prepared by Battelle Laboratories.

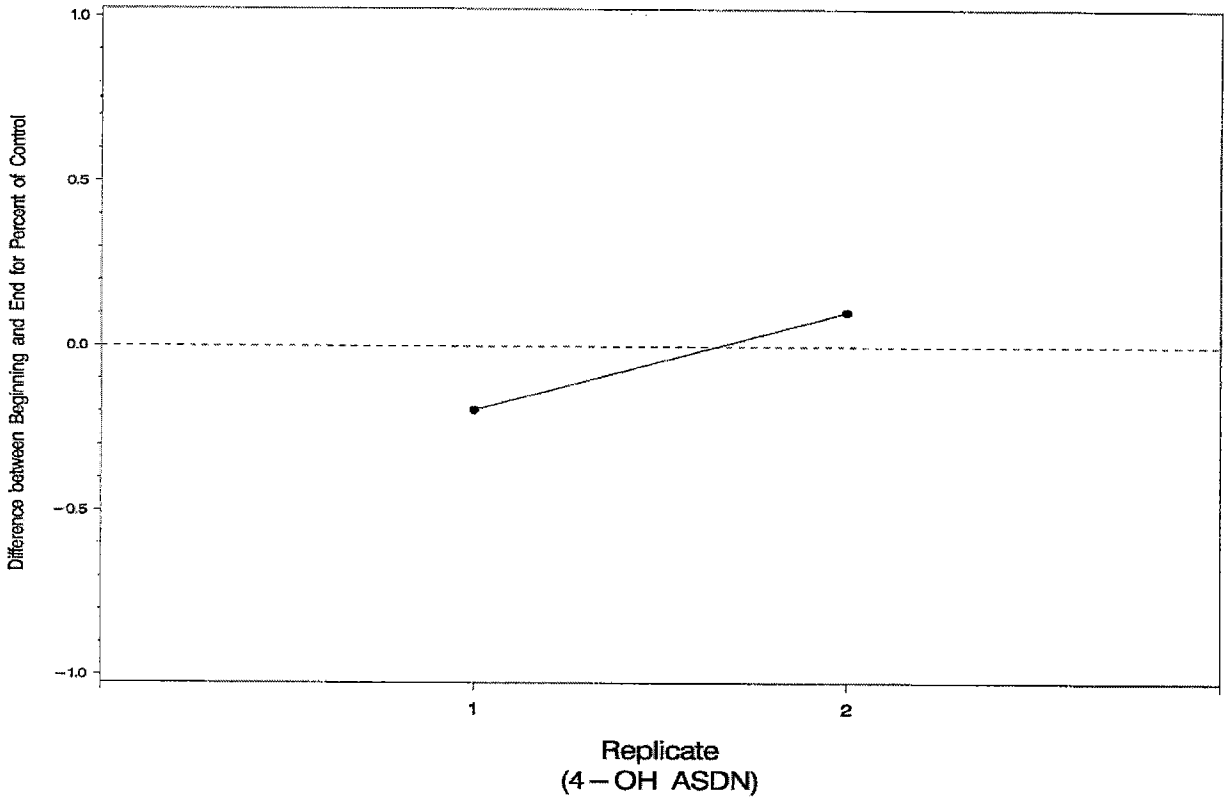


Figure 10. Background Activity Control Data Associated with Inhibition Concentration Tests. Difference Between the Average of the Two End Percent of Control Responses and the Average of the Two Beginning Responses by Replicate (End Minus Beginning). By Replicate. Reference Line at 0%. Microsomes Prepared by Battelle Laboratories.

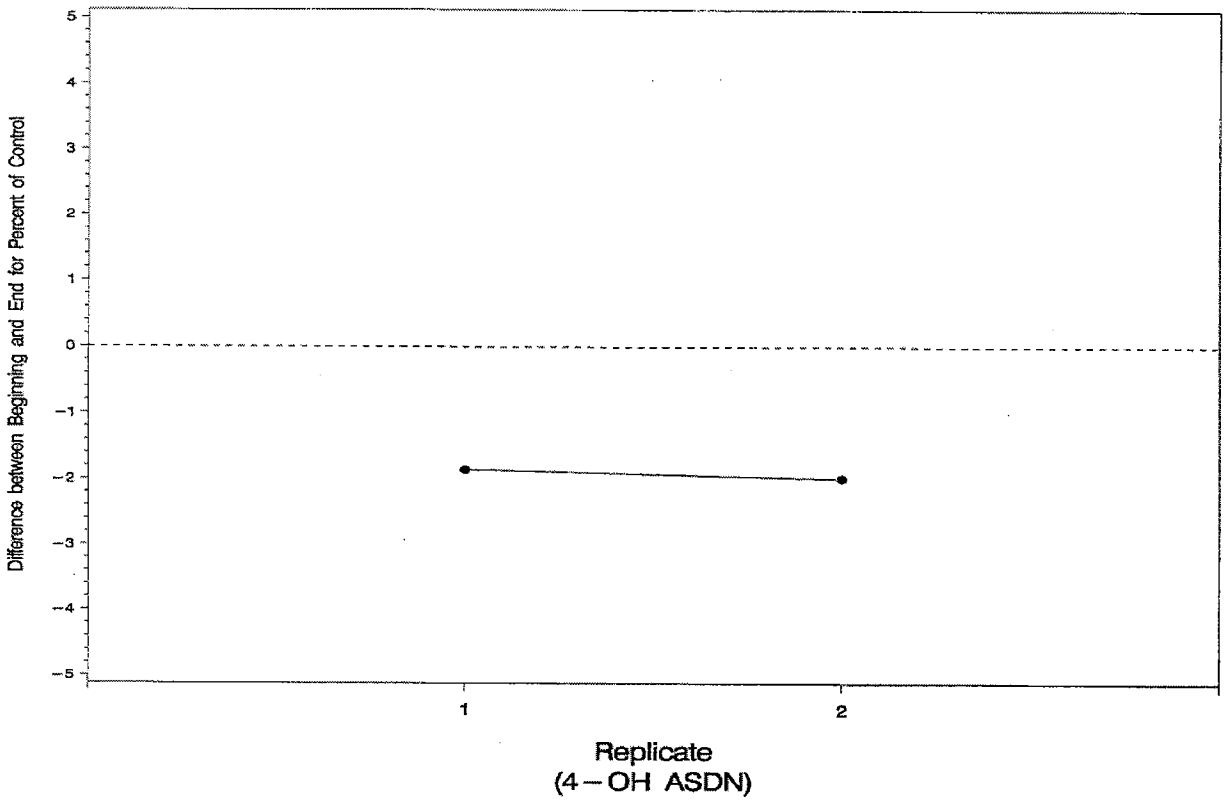


Figure 11. Positive Control Data Associated with Inhibition Concentration Tests. Difference Between the Average of the Two End Percent of Control Responses and the Average of the Two Beginning Responses by Replicate (End Minus Beginning). By Replicate. Reference Line at 0%. Microsomes Prepared by Battelle Laboratories.

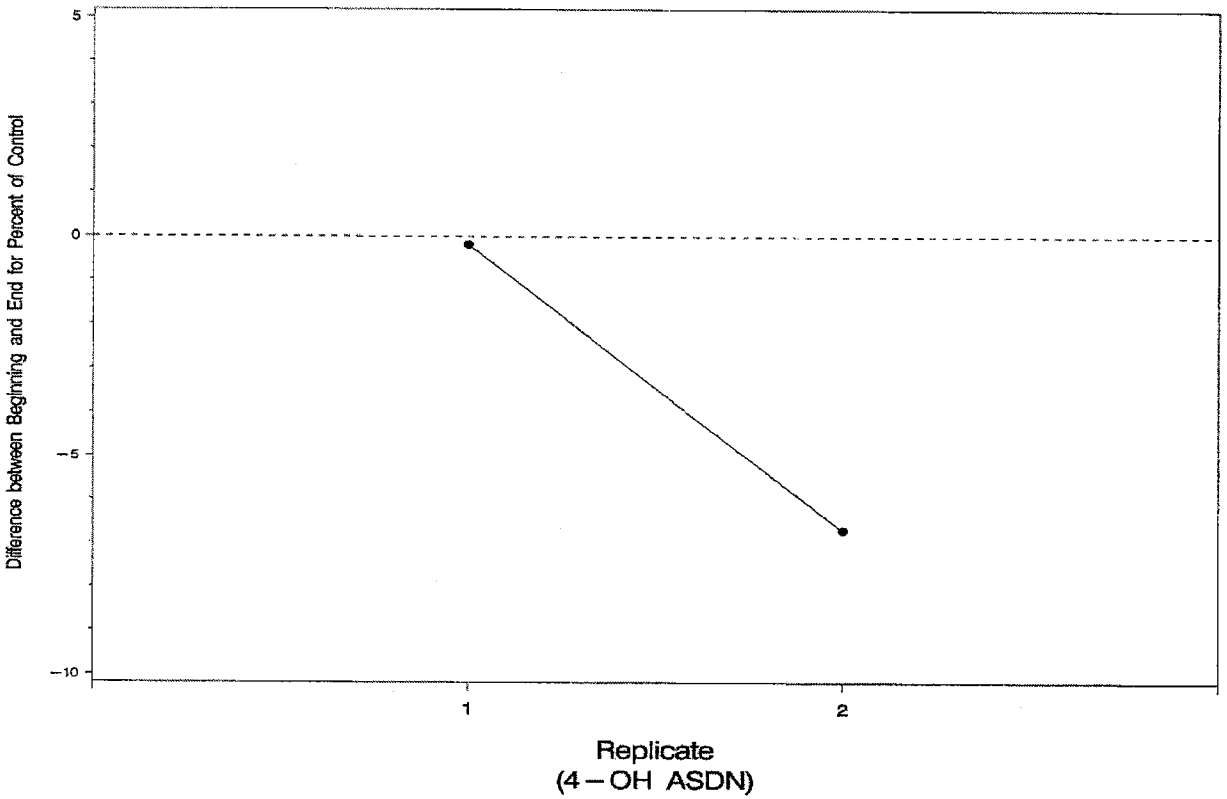


Figure 12. Negative Control Data Associated with Inhibition Concentration Tests. Difference Between the Average of the Two End Percent of Control Responses and the Average of the Two Beginning Responses by Replicate (End Minus Beginning). By Replicate. Reference Line at 0%. Microsomes Prepared by Battelle Laboratories.

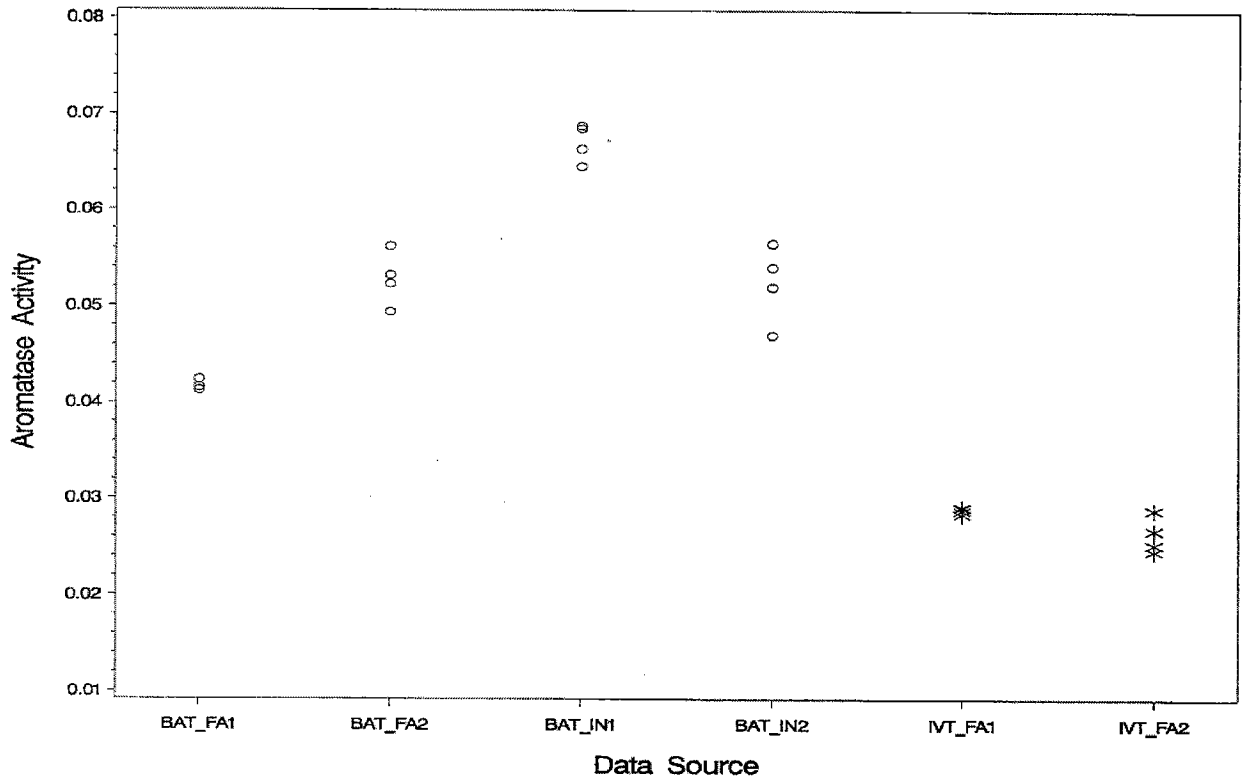


Figure 13. Aromatase Activity (nmol/mg protein/min). Full Enzyme Activity Controls. By Source and Replicate.

Note: BAT_FA1 and BAT_FA2 – Battelle prepared microsomes, analyzed for aromatase activity, Replicates 1 and 2
BAT_IN1 and BAT_IN2 – Battelle prepared microsomes, analyzed for inhibition curve fits, Replicates 1 and 2
IVT_FA1 and IVT_FA2 – In Vitro prepared microsomes, analyzed for aromatase activity, Replicates 1 and 2

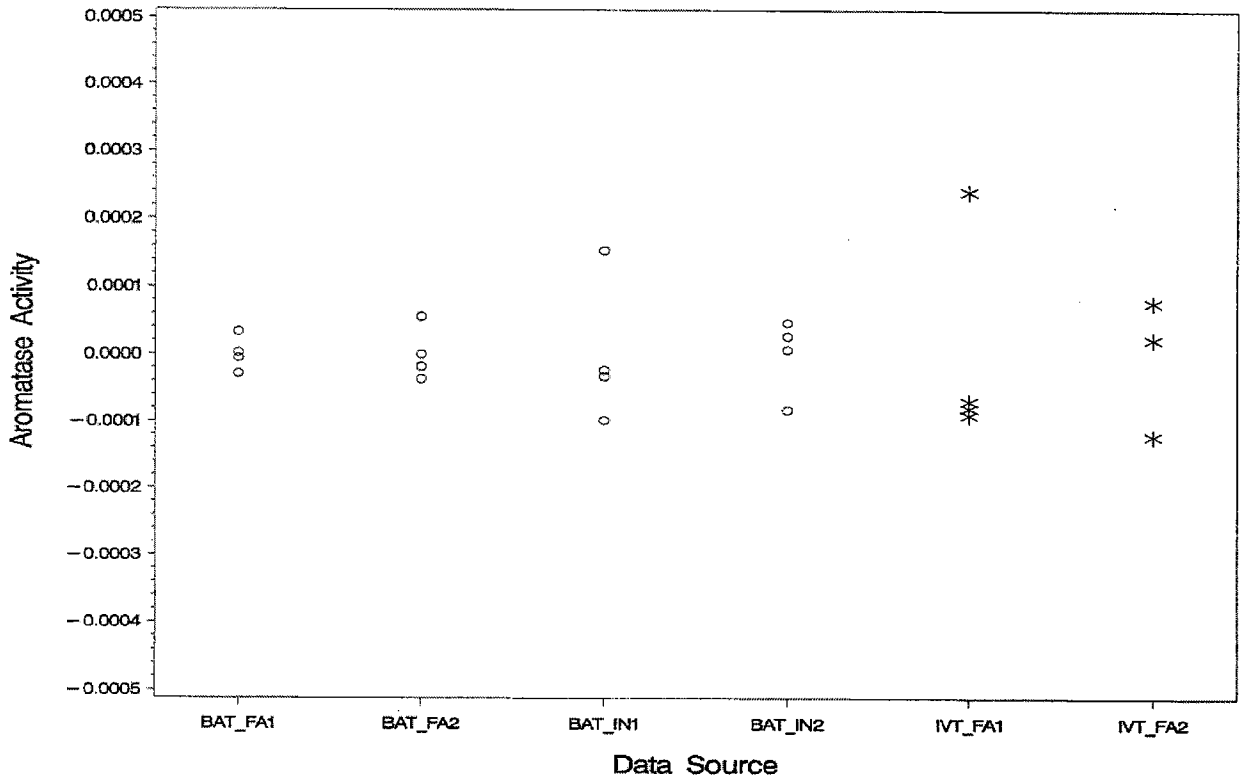


Figure 14. Aromatase Activity (nmol/mg protein/min). Background Activity Controls. By Source and Replicate.

Note: BAT_FA1 and BAT_FA2 – Battelle prepared microsomes, analyzed for aromatase activity, Replicates 1 and 2
BAT_IN1 and BAT_IN2 – Battelle prepared microsomes, analyzed for inhibition curve fits, Replicates 1 and 2
IVT_FA1 and IVT_FA2 – In Vitro prepared microsomes, analyzed for aromatase activity, Replicates 1 and 2

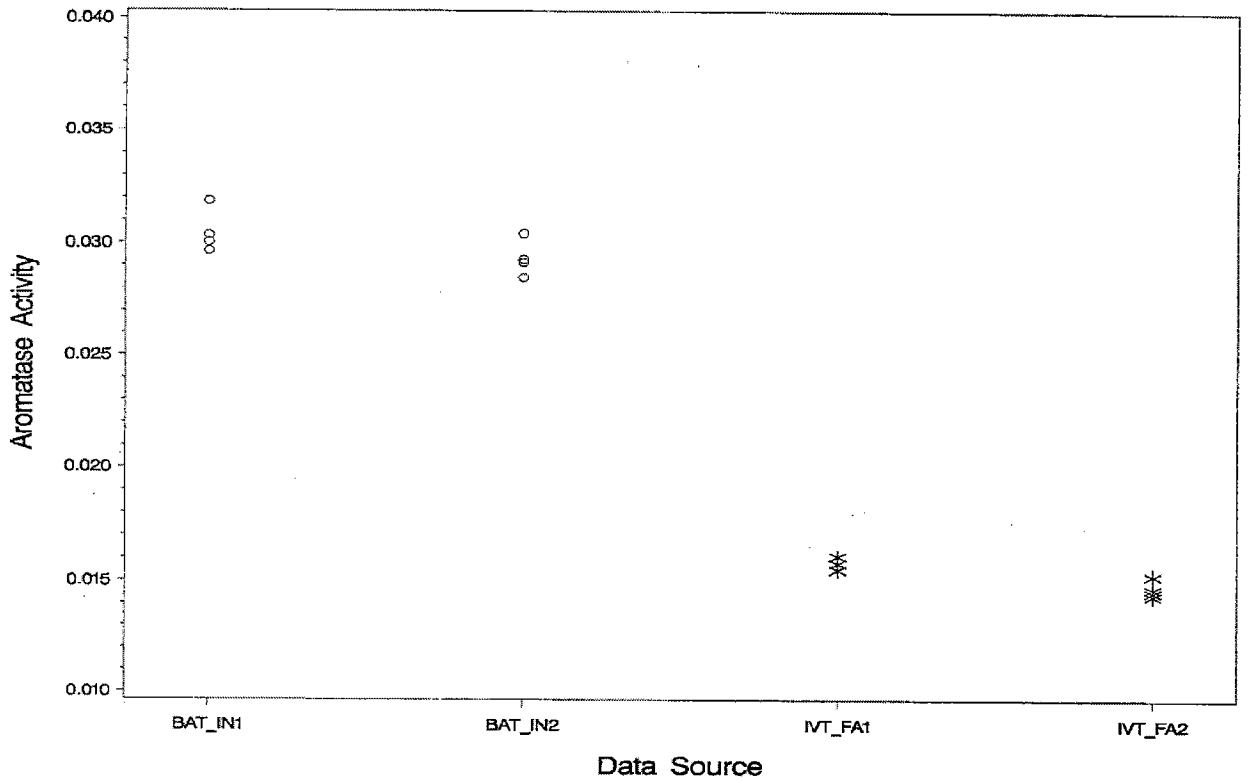


Figure 15. Aromatase Activity (nmol/mg protein/min). Positive Controls. By Source and Replicate.

Note: BAT_IN1 and BAT_IN2 – Battelle prepared microsomes, analyzed for inhibition curve fits, Replicates 1 and 2
IVT_FA1 and IVT_FA2 – In Vitro prepared microsomes, analyzed for aromatase activity, Replicates 1 and 2

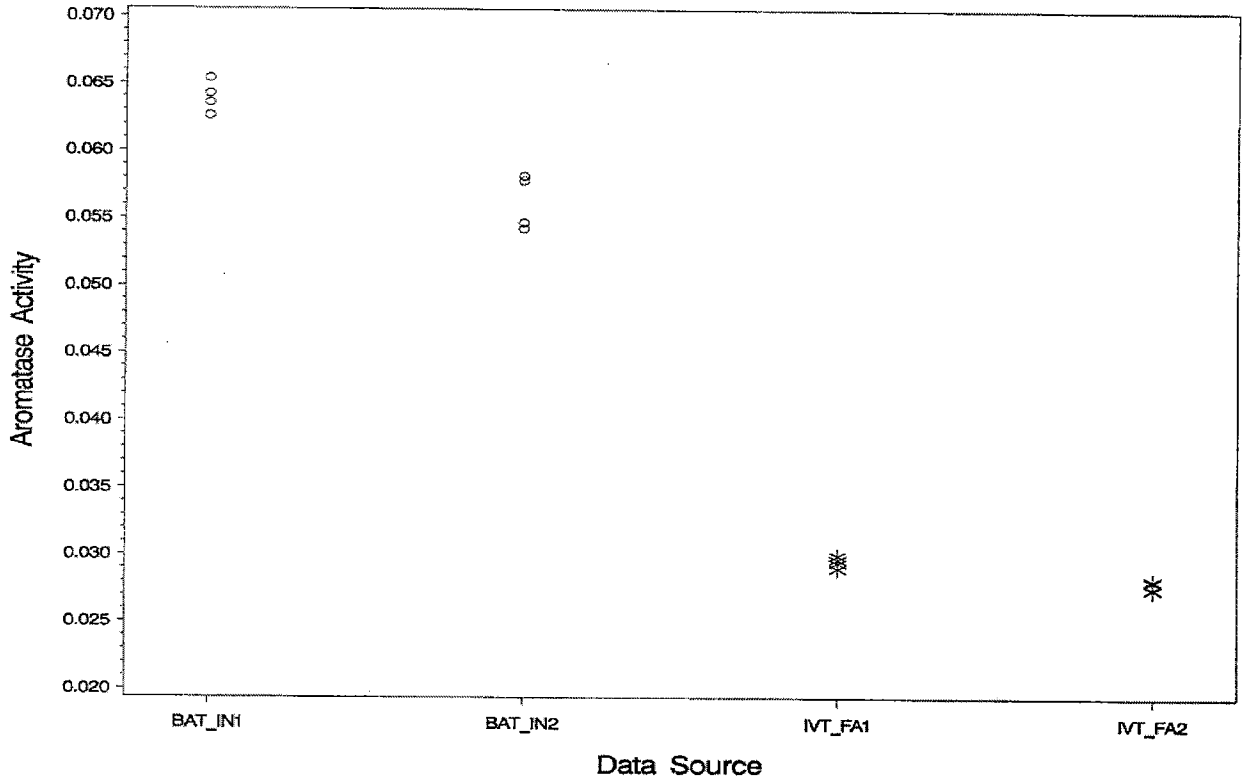


Figure 16. Aromatase Activity (nmol/mg protein/min). Negative Controls. By Source and Replicate.

Note: BAT_IN1 and BAT_IN2 – Battelle prepared microsomes, analyzed for inhibition curve fits, Replicates 1 and 2
IVT_FA1 and IVT_FA2 – In Vitro prepared microsomes, analyzed for aromatase activity, Replicates 1 and 2

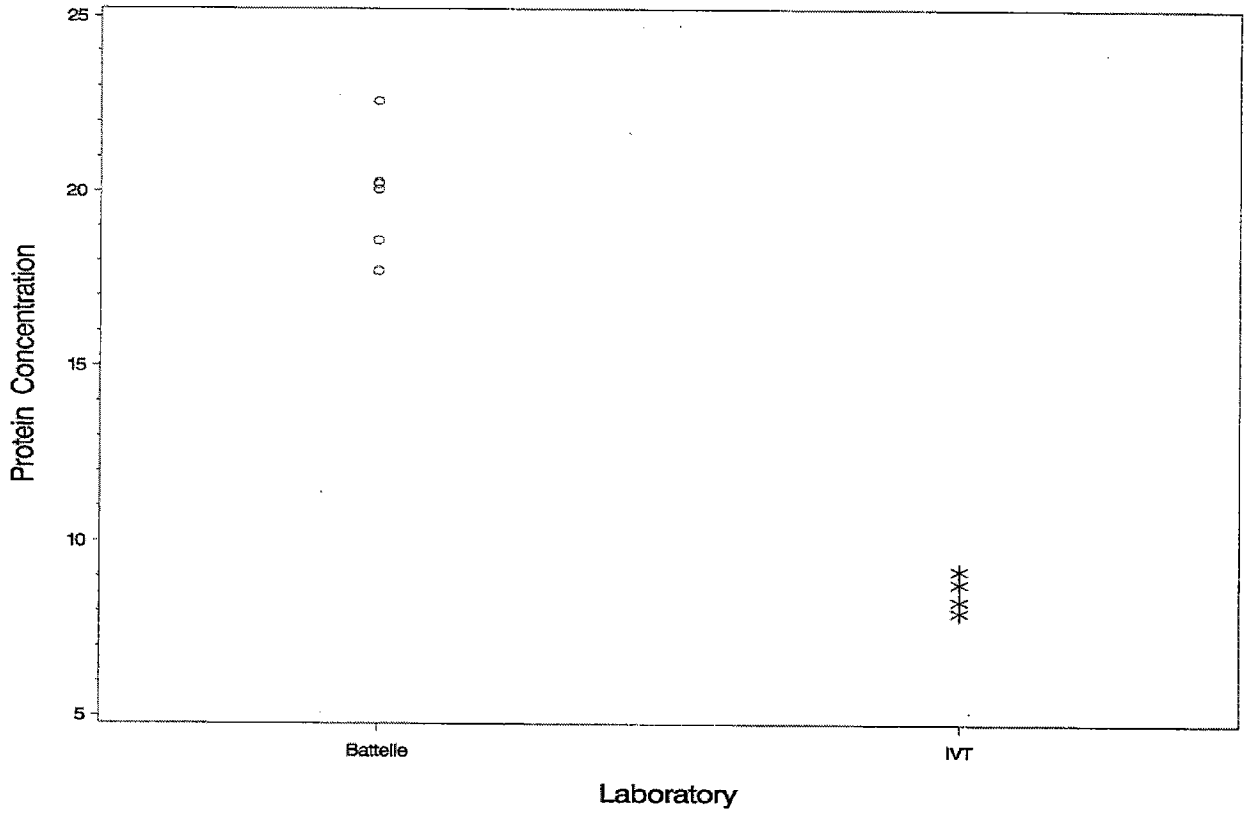


Figure 17. Protein Concentrations (mg/mL). By Source.

Table A-1. Percent of Control Activity in Placental Aromatase Assay Inhibition Study by Replicate, 4-OH ASDN Concentration Within Replicate, and Repetition Within Concentration. Based on Microsomes Prepared by Battelle Laboratories.

Replicate	Log [4-OH ASDN]	Percent of Control[1]		
		Repetition 1	Repetition 2	Repetition 3
1	-6.00	4.80	5.33	4.74
	-7.00	30.45	30.00	30.70
	-7.30	44.59	46.14	45.11
	-7.60	62.60	62.79	62.78
	-8.00	77.21	75.47	75.56
	-9.00	92.74	91.52	93.85
2	-6.00	6.30	6.62	6.93
	-7.00	37.74	39.79	39.29
	-7.30	53.82	54.41	56.74
	-7.60	73.87	70.62	73.48
	-8.00	84.61	82.38	84.84
	-9.00	93.42	98.25	101.56

[1] Percent of control values were calculated by dividing the background corrected aromatase activity values by the average of the four full enzyme activity control values within the same replicate and multiplying by 100 percent.

Table A-2. Full Enzyme Activity Control, Background Activity Control, Positive Control, and Negative Control Corrected Aromatase Activity and Percent of Control Data Associated with the Inhibition Curve Tests. By Replicate and Portion (Beginning or End). Placental Aromatase Assay. Based on Microsomes Prepared by Battelle Laboratories.

Aromatase Activity	Replicate	Portion	Corrected Aromatase Activity	Percent of Control[1]
Full Enzyme Activity Control	1	Beginning	0.06851	102.170
		Beginning	0.06877	102.559
		End	0.06639	99.003
		End	0.06455	96.267
	2	Beginning	0.05655	107.869
		Beginning	0.05407	103.134
		End	0.04705	89.745
		End	0.05203	99.252
Background Activity Control	1	Beginning	0.00015	0.229
		Beginning	-0.00002	-0.035
		End	-0.00010	-0.146
		End	-0.00003	-0.049
	2	Beginning	0.00003	0.052
		Beginning	-0.00008	-0.156
		End	0.00001	0.014
		End	0.00005	0.090
Positive Control	1	Beginning	0.03187	47.526
		Beginning	0.03034	45.248
		End	0.03005	44.817
		End	0.02966	44.234
	2	Beginning	0.03045	58.080
		Beginning	0.02930	55.902
		End	0.02850	54.368
		End	0.02917	55.637

[1] Percent of control values were calculated by dividing the background corrected aromatase activity values by the average of the four full enzyme activity control values within the same replicate and multiplying by 100 percent.

Aromatase Activity	Replicate	Portion	Corrected Aromatase Activity	Percent of Control[1]
Negative Control	1	Beginning	0.06258	93.322
		Beginning	0.06540	97.531
		End	0.06351	94.711
		End	0.06422	95.767
	2	Beginning	0.05781	110.274
		Beginning	0.05817	110.956
		End	0.05469	104.327
		End	0.05425	103.494

[1] Percent of control values were calculated by dividing the background corrected aromatase activity values by the average of the four full enzyme activity control values within the same replicate and multiplying by 100 percent.

Table A-3. Aromatase Activity of Full Enzyme Activity Controls. By Microsome Source and Test Type.

Microsomes Prepared By	Data Source	Replicate	Repetition	Aromatase Activity
Battelle	Control Activity	1	1	0.0424
			2	0.0424
			3	0.0413
			4	0.0416
		2	1	0.0562
			2	0.0532
			3	0.0523
			4	0.0494
	Curve Fit	1	1	0.0685
			2	0.0688
			3	0.0664
			4	0.0646
		2	1	0.0565
			2	0.0541
			3	0.0470
			4	0.0520
IVT	Control Activity	1	1	0.0291
			2	0.0284
			3	0.0290
			4	0.0288
		2	1	0.0289
			2	0.0267
			3	0.0252
			4	0.0246

Table A-4. Aromatase Activity of Background Activity Controls. By Microsome Source and Test Type.

Microsomes Prepared By	Data Source	Replicate	Repetition	Aromatase Activity
Battelle	Control Activity	1	1	-2.911×10^{-05}
			2	-5.821×10^{-06}
			3	3.299×10^{-05}
			4	1.940×10^{-06}
		2	1	-3.697×10^{-05}
			2	0
			3	5.546×10^{-05}
			4	-1.849×10^{-05}
	Curve Fit	1	1	1.537×10^{-04}
			2	-2.329×10^{-05}
			3	-9.780×10^{-05}
			4	-3.260×10^{-05}
		2	1	2.730×10^{-05}
			2	-8.191×10^{-05}
			3	7.446×10^{-06}
			4	4.716×10^{-05}
IVT	Control Activity	1	1	2.395×10^{-04}
			2	-8.983×10^{-05}
			3	-7.985×10^{-05}
			4	-6.986×10^{-05}
		2	1	2.252×10^{-05}
			2	-1.216×10^{-04}
			3	7.657×10^{-05}
			4	2.252×10^{-05}

Table A-5. Aromatase Activity of Positive Controls. By Microsome Source and Test Type.

Microsomes Prepared By	Data Source	Replicate	Repetition	Aromatase Activity
Battelle	Curve Fit	1	1	0.0319
			2	0.0303
			3	0.0301
			4	0.0297
		2	1	0.0304
			2	0.0293
			3	0.0285
			4	0.0292
IVT	Control Activity	1	1	0.0158
			2	0.0161
			3	0.0161
			4	0.0155
		2	1	0.0147
			2	0.0153
			3	0.0145
			4	0.0144

Table A-6. Aromatase Activity of Negative Controls. By Microsome Source and Test Type.

Microsomes Prepared By	Data Source	Replicate	Repetition	Aromatase Activity
Battelle	Curve Fit	1	1	0.0626
			2	0.0654
			3	0.0635
			4	0.0642
		2	1	0.0578
			2	0.0582
			3	0.0547
			4	0.0543
IVT	Control Activity	1	1	0.0300
			2	0.0297
			3	0.0291
			4	0.0295
		2	1	0.0276
			2	0.0281
			3	0.0283
			4	0.0275

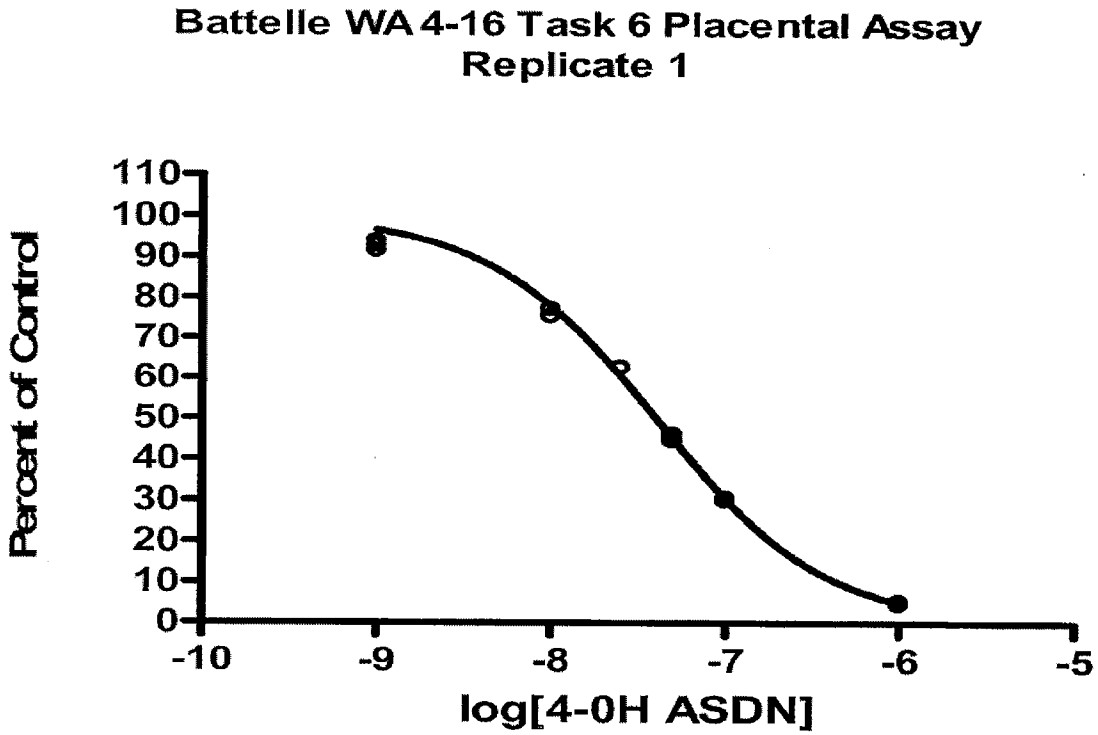


Figure A-1. **Replicate 1. Individual Percent of Control Values Vs. (Base 10) Logarithm of 4-OH ASDN Inhibitor Concentration. Concentration Response Curve Fitted to Average Responses Within Concentrations. Placental Aromatase Assay. Microsomes Prepared by Battelle Laboratories.**

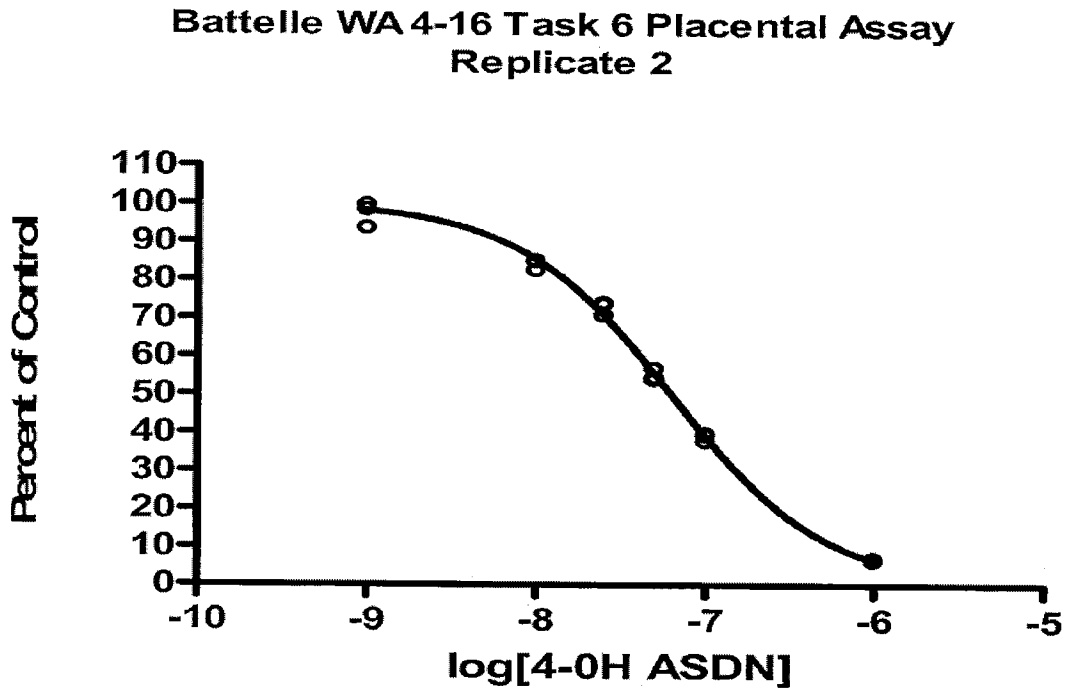


Figure A-2. Replicate 2. Individual Percent of Control Values Vs. (Base 10) Logarithm of 4-OH ASDN Inhibitor Concentration. Concentration Response Curve Fitted to Average Responses Within Concentrations. Placental Aromatase Assay. Microsomes Prepared by Battelle Laboratories