DRAFT FINAL REPORT

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

VOLUME II (Appendices B-E)

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

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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

APPENDIX B

IN VITRO TECHNOLOGIES REPORT

DRAFT TASK REPORT

PLACENTAL AROMATASE VALIDATION STUDY

WA 4-16 Task 6: Preparation and Characterization of Human Placental Microsomes

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

Sponsor:

Battelle Memorial Institute
505 King Avenue
Columbus, OH 43201-2639

Performing Laboratory:

In Vitro Technologies, Inc.
1450 South Rolling Road
Baltimore, MD 21227

DRAFT REPORT

Title:	PLACENTAL AROMATASE VALIDATION STUDY WA 4-16 Task 6: Preparation and Characterization of Human Placental Microsomes
Authors:	Aruna Koganti, Ph.D.
Performing Laboratory:	In Vitro Technologies, Inc. 1450 South Rolling Road Baltimore, MD 21227
Sponsor:	Battelle Memorial Institute 505 King Avenue Columbus, OH 43201-2639
Sponsor Representatives:	David Houchens, Ph.D. EDSP Program Manager Battelle Jerry D. Johnson, Ph.D. Diplomate, A.B.T. Work Assignment Leader Battelle
Study Initiation Date:	29 March 2005
Experimental Dates:	29 April 2005–31 May 2005
Draft Task Report Date	24 February 2006

Author: Approved:

Signature Date Signature Date

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STATEMENT OF COMPLIANCE

This study was conducted to the standards of U.S. FDA 21 CFR Part 58. Exception: The computer systems at In Vitro Technologies, Inc. are not validated. Therefore, this study was not in compliance with U.S. FDA 21 CFR Part 58, Section 58.63. This study was conducted under my scientific guidance and management.

Aruna Koganti, Ph.D.		
Study Director	Signature	Date

QUALITY ASSURANCE STATEMENT

This study was inspected in accordance with In Vitro Technologies standard operating procedures. Based on audits conducted, the results reported herein accurately reflect the methods used and the data collected for this study. All findings were reported to the Study Director and In Vitro Technologies Management.

Inspection/Audit Dates:	Study Phase Audited:	Date(s) reported to Study Director and Management:
25 March 2005	Protocol Review	25 March 2005
13 April 2005	Microsome preparation	15 April 2005
29 April 2005	Protein assay	29 April 2005
12 May 2005	Aromatase assay	12 May 2005
13 May 2005	Data entry	13 May 2005
20 May 2005	Aromatase assay	20 May 2005
31 May 2005	Aromatase assay	01 June 2005
31 January–02 February 2006	Data and report	03 February 2006

Quality Assurance	Date

PARTICIPATION

The following principal staff participated in the conduct of this study:

Study Director:	Aruna Koganti, Ph.D.
Scientists:	Eric J Balsley, B.Sc.
	Timothy Moeller, M.Sc.
Technical Writers:	Blaise Considine, B.A.
	Steve Sorrow, B.A.

DATA RETENTION

In Vitro Technologies will retain all supporting documentation in the In Vitro Technology archives, including raw data and written records, for a period of up to five years following submission of the final report to Battelle Memorial Institute. At the end of this period, Battelle will be notified to determine whether the data (excluding proprietary information) will be transferred, retained, or destroyed.

1.0 Executive Summary

The objectives of this study were to prepare human placental microsomes, analyze the microsomal preparation for protein content and uninhibited aromatase activity, and evaluate effects of the known aromatase inhibitor 4-hydroxy androstenedione (4-OH ASDN). This study was part of a multi-laboratory effort for the evaluation of the placental aromatase assay.

This study was conducted in two stages. In Stage 1, In Vitro Technologies procured a human placenta, prepared microsomes, and determined the protein concentration (two independent replicate experiments) and aromatase activity (uninhibited, two independent replicate experiments) of the microsomes that were prepared. The average protein concentration was 7.82 ± 0.927 mg/mL and the aromatase activity was 0.0382 ± 0.00540 nmol estrogen formed/mg protein/min for the replicate experiments. In addition, In Vitro Technologies ran two independent replicate experiments to determine the response of the microsomal aromatase to six concentrations of 4-OH ASDN using the above microsomal preparations. The IC_{50} value for 4-OH ASDN in the placental microsomes prepared by In Vitro Technologies was calculated to be 53.6 and 60.2 nM in these experiments. The data from these experiments were sent to Battelle for review and submission to the Environmental Protection Agency (EPA). Approval from the EPA Work Assignment Manager was obtained before In Vitro Technologies proceeded to Stage 2.

In Stage 2, the two laboratories that procured/prepared and characterized the microsomes in the first stage distributed their microsomes to the other participating laboratories; i.e., Battelle distributed microsomes to In Vitro Technologies, RTI, and WIL, whereas In Vitro Technologies distributed microsomes to Battelle, RTI, and WIL. In this way, each laboratory used microsomes prepared by both laboratories in their experiments to determine the protein concentration and uninhibited aromatase enzyme activity. Upon receipt of the microsomes from Battelle, In Vitro Technologies independently determined the protein concentration and aromatase activity (uninhibited) of these microsomes. The protein concentration was determined to be 24.5 ± 5.01 mg/mL and the aromatase activity was 0.0464 ± 0.00470 nmol estrogen formed/mg protein/min in the replicate experiments. The data from these experiments were sent to Battelle for review and submission to the EPA.

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 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 Method Validation 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 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.

2.2 Task Description and Objectives

The objectives of this study were to prepare human placental microsomes, analyze the microsomal preparation for protein content and uninhibited aromatase activity, and evaluate effects of the known aromatase inhibitor, 4-hydroxy androstenedione (4-OH ASDN). This task was conducted in two stages. This study is part of a multi-laboratory effort for the evaluation of the placental aromatase assay. The test system for this study was human placental microsomes. This test system was selected because it provides a biological source of the aromatase enzyme. Since the assay was evaluated for its potential to serve as a screening assay, the use of human tissue enhanced its predictive potential.

3.0 Materials and Methods

3.1 Substrate

The substrate for the aromatase assay was androstenedione (ASDN). Non-radiolabeled and radiolabeled ASDN were used. The non-radiolabeled ASDN (lot number 024K0809) was obtained from Sigma, St. Louis, MO by the Sponsor's Chemical Repository 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 number 3538496), was obtained from Perkin Elmer Life Science, Boston 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. The results of this analysis are presented in the report contained in Appendix 7.

Since the specific activity of the stock [^{3}H]ASDN was too high for use directly in the assay, a solution containing a mixture of nonradiolabeled and radiolabeled ASDN was prepared such that the final concentration of ASDN in the assay was 100 nM and the amount of tritium added to each incubation was approximately 0.1 µCi. This substrate solution had 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. A 1:100 dilution (10 µCi/mL) of the radiolabeled stock in buffer was prepared. A 1 mg/mL solution of ASDN in 95% ethanol was prepared. Dilutions were prepared in buffer to a final concentration of 1 µg/mL. The 1 µg/mL solution of ASDN (4.5 mL), 800 µL of the [^{3}H]ASDN dilution, and 2.7 mL of buffer were combined to make 8 mL of substrate solution (enough for 80 tubes). The weight of each component added to the substrate solution was recorded. After mixing the solution well, aliquots (approximately 20 µL) were weighed and combined with scintillation cocktail for radiochemical content analysis. The addition of 100 µL of the substrate solution to each 2 mL assay volume yielded a final [^{3}H]ASDN concentration of 100 nM with 0.1 µCi/tube.

3.2 Test and 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. These chemistry activities and results are described in the Sponsor's Chemistry reports, which are appended to this document (Appendix 7).

Table 1. Test and Control Substances

Chemical name	Chemical code	Mfr. Purity	CAS No.	Molecular formula	Molecular weight	Stock Solution ID	Target Stock Formulation Concentration	Vehicle	Storage Conditions
4-Hydroxy androstenedione	270-0035	99%	566-48-3	$C_{19}H_{26}O_3$	302.4 g/mol	063K4069	10 mM	95% ethanol	4° C
Lindane	270-0034	99.6%	58-89-9	$C_6H_6Cl_6$	290.8 g/mol	14419EB	100 mM	Dimethyl sulfoxide	4° C

4-OH ASDN is a known aromatase inhibitor. Positive control stock solutions were prepared and analyzed by Battelle and distributed to the laboratories (Lot Number 063K4069). The stock was received on 24 March 2005 and stored at 4°C until use. 4-OH ASDN was formulated in 95% ethanol. The total volume of positive control formulation used in each assay was 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 were prepared in ethanol on the day of use such that the target concentration of inhibitor was achieved by the addition of 20 µL of the dilution to a 2 mL assay volume. The target concentrations for the positive control are listed in Table 2.

A known aromatase non-inhibitor, lindane, was used as the negative control substance. Battelle provided a stock solution for lindane, formulated in DMSO (Lot Number 14419EB). The stock was received on 24 March 2005 and stored at 4°C until use. Fresh dilutions of the stock solution were prepared in DMSO on the day of use. Dilutions were prepared such that the target concentration of control substance, 1×10^{-6} M, was achieved by the addition of 20 µL of the dilution to a 2 mL assay volume.

Table 2. 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}

Sample type	Repetitions (test tubes)	Description	Control or Test chemical concentration (M final)
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

3.3 Microsomes

Caution: Microsomes can be denatured by detergents. Therefore, it was important to ensure that all glassware, etc. that was used in the preparation or usage of microsomes was free of detergent residue. New disposable test tubes, bottles, vials, pipettes and pipette tips were used directly in the assay. Durable lab ware that may have been exposed to detergents was 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 was kept well-chilled on ice and work commenced quickly. The placenta was placed on a tray that was set 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 were dissected, removed, and discarded. The spongy tissue was cut into small portions and placed on ice in pre-chilled (refrigerated) Buffer A. Batches of the tissue were sequentially removed to a beaker and minced with scissors. Buffer A was added to an approximate 2:1 w:v ratio and the mixture was homogenized using a Polytron homogenizer. Some fibrous material was resistant to homogenization and this tissue was removed from the homogenate or allowed to remain with the knowledge that it was removed in the centrifugation step to follow. The homogenate was transferred to centrifuge tubes and kept on ice until all of the tissue was processed or until the capacity of the centrifuge rotor was reached. Tissue homogenization continued in batches as described until all tissue was processed. The tissue homogenate was 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 in an appropriate centrifuge (Sorvall RC2-B) at 4°C. The supernatant was removed by pipetting and transferred to ultracentrifuge tubes (recommended approximate capacity 26 mL). The supernatant was centrifuged at a setting of 33,000 rpm (approximately 100,000 × g) in an appropriate ultracentrifuge (Beckman L7-55 Ultracentrifuge) for one hour at approximately 4°C to obtain the crude microsomal pellet. The supernatant was decanted and the microsomal pellet was dislodged from the wall of the tube by gentle swirling with a few mL of Buffer B. Care was taken to not dislodge the clear pellet that was often visible under the microsomal pellet. The microsomal pellet (along with the buffer) were poured into a Potter-Elvejhem homogenizer and resuspended in Buffer B. The suspension was transferred to ultracentrifuge tubes. The suspensions of multiple pellets were combined in a single ultracentrifuge tube. The samples were centrifuged at a setting of 33,000 rpm (approximately 100,000 × *g*, Beckman L7-55 Ultracentrifuge) for one hour 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 wall of the tube by gentle swirling in a few mL of Buffer C. All microsomal pellets were combined into a single lot and resuspended in Buffer C using a Potter Elvejhem homogenizer. An appropriate final volume of suspended microsomes ranged from 20 to 30 mL, depending on the amount of protein isolated from the placenta. The concentration of microsomes in this suspension was 55.3 mg/mL, which was measured at this point using the protein assay. The microsomes

were further diluted to a concentration of approximately 11.5 mg/mL and divided into aliquots (approximately 200 µL/tube) into labeled tubes (NUNC cryotubes), flash frozen in liquid nitrogen, and stored at approximately –70 ± 10°C until removal for use.

3.3.1 Microsome type information

Placental microsomes (In Vitro Technologies lot BAA) were obtained from a 26-year-old Caucasian female who delivered a baby boy. The mother had no reported medical history, except to note that she was a non-smoker.

3.4 Other assay components

Chemical	Supplier	Lot Number
NADPH	Sigma	103K7046
Propylene glycol	Fisher	042343
Sodium phosphate dibasic	JT Baker	A43465
Sodium phosphate monobasic	JT Baker	A28H21
Methylene chloride	Sigma	367-A0070003
95% ethanol	Battelle	04H23QB
Dimethyl sulfoxide	Battelle	2969A24437
DC Protein Kit Reagent A	BioRad	725838A
DC Protein Kit Reagent B	BioRad	210000116

3.4.1 NADPH

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

3.4.2 Buffer Preparation

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

Dissolved 6.90 ± 0.55 g of sodium phosphate monobasic (NaH_2PO_4; JT Baker, catalog number 4011-01, 137.99 g/mol) in 1 L deionized water to prepare 0.05 M NaH_2PO_4. Dissolved 7.10 ± 0.57 g of sodium phosphate dibasic (Na_2HPO_4; JT Baker, catalog number 4062-01, 141.96 g/mol) in 1 L deionized water to prepare 0.05 M Na_2HPO_4. Combined these solutions to a final pH of 7.0. To complete preparation of Buffer A, 85.58 ± 1.36 g sucrose (JT Baker, catalog number 4097-04, 342.3 g/mol) and 4.88 ± 0.48 g nicotinamide (Sigma, catalog number N3376, 122.1 g/mol) was dissolved in 1 L 0.05 M sodium phosphate buffer, pH 7.0. The buffer was stored in the refrigerator (2–8°C) until use.

Buffer B (Assay Buffer): 0.1 M sodium phosphate (pH 7.4)

Dissolved 13.80 ± 0.55 g NaH_2PO_4 (JT Baker, catalog number 4011-01, 137.99 g/mol) in 1 L distilled, deionized water to prepare 0.1 M NaH_2PO_4. Dissolved 14.20 ± 0.56 g Na_2HPO_4 (JT Baker, catalog number 4062-01, 141.96 g/mol) in 1 L distilled, deionized water to prepare 0.1 M Na_2HPO_4. Combined these solutions to a final pH of 7.4. The assay buffer was stored in the refrigerator (2–8°C) until use.

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

Dissolved 17.12 ± 0.27 g sucrose and 1.54 ± 0.12 mg dithiothreitol (Sigma, catalog number D5545, 154.3 g/mol) in about 100 mL 0.1 M sodium phosphate buffer, pH 7.4 (prepared as described above). The volume was adjusted to 160 mL with additional 0.1 M sodium phosphate buffer, pH 7.4. Added glycerol (Sigma, catalog number G7893, 92 g/mol) to a total solution volume of 200 mL.

3.5 Protein Determination

The protein concentration of each microsome preparation prepared in this task was measured by all participating laboratories. The protein concentration of the microsome preparation was determined on each day of use of the microsomes in the aromatase assay and at other times as appropriate.

For experiments evaluating protein concentration and uninhibited enzyme activity of microsomes prepared at In Vitro Technologies, a six-point standard curve was prepared, ranging from 0.125 to 1.5 mg protein/mL using bovine serum albumin (BSA). Protein was determined using a DC Protein Assay kit purchased from BioRad (Hercules, CA). Quality control standards (0.125, 0.5, and 1 mg/mL BSA) obtained from Pierce (Woburn, MA) were run in duplicate with each assay. To a 25 µL aliquot of unknown or standard, 125 µL of BioRad DC Protein Kit Reagent A was added and mixed. Next, 1 mL of BioRad DC Protein Kit Reagent B was added to each standard or unknown and the samples were vortex mixed. The samples remained at room temperature for at least 15 minutes to allow for color development. The absorbances were stable for approximately 1 hour. Each sample (unknown and standards) was transferred to quartz cuvettes and the absorbance (at 750 nm) was measured using a spectrophotometer. The protein concentration of the microsomal sample was determined by interpolation of the absorbance value using the curve developed using the protein standards.

For experiments evaluating protein concentration and uninhibited enzyme activity of microsomes prepared at Battelle and for experiments evaluating the effect of 4-OH ASDN, a six-point standard curve was prepared, ranging from 0.005 to 0.25 mg protein/mL using BSA. Protein was determined using a DC Protein Assay kit. Quality control standards (0.01 and 0.1 mg/mL BSA) prepared from standards obtained from Pierce (Woburn, MA) were run in duplicate with each assay. To a 200 µL aliquot of unknown or standard, 100 µL of DC Protein Kit Reagent A was added and mixed. Next, 0.8 mL of DC Protein Kit Reagent B was added to each standard or unknown and the samples were vortex mixed. The samples remained at room temperature for at least 15 minutes to allow for color development. The absorbances were stable for approximately 1 hour. Each sample (unknown and standards) 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 of the absorbance value using the curve developed using the protein standards

3.6 Cytochrome P450 Aromatase (CYP19) Activity

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

- 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 were in the full aromatase activity controls, except NADPH)
- positive control (all components that were 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 control (all components that were 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 were included with each replicate and were treated the same as the other samples. 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 set.

The assays were performed in 13 × 100 mm test tubes maintained at 37 ± 1°C in a shaking water bath. Each test tube was uniquely identified by applying a label to the test tube. Propylene glycol (100 µL), [^{3}H]ASDN, NADPH, and buffer (0.1 M sodium phosphate buffer, pH 7.4) was combined in the test tubes (total volume of 1 mL). The final concentrations for the assay components are presented in Table 3. The tubes and the microsomal suspension were 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 was 2 mL, and the tubes were incubated for 15 min. The incubations were stopped by the addition of methylene chloride (2 mL); the tubes were vortex-mixed for approximately 5 seconds and placed on ice. The tubes were vortex-mixed an additional 20 to 25 seconds. The tubes were centrifuged using a Jouan CR422 centrifuge with GH-3.8 rotor and a Sorvall RT7 centrifuge with RTH-750 rotor for 10 minutes at a setting of 1,000 rpm. The methylene chloride layer was removed and discarded; the aqueous layers were extracted again with methylene chloride (2 mL). This extraction procedure was performed one additional time, each time discarding the methylene chloride layer. The aqueous layer was transferred to vials and duplicate aliquots (0.5 mL) were transferred to 20-mL liquid scintillation counting vials. Liquid scintillation cocktail (Formula 989, Perkin Elmer, 10 mL) was added to each counting vial and shaken to mix the solution. The radiochemical content of each aliquot was determined using liquid scintillation spectrometry (LSS). Radioactivity found in the aqueous fractions represented ^3H_2O formed.

Table 3. 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

3.7 Data Analysis

The data analysis described in the following paragraphs addresses all of the experiments of this task. The laboratories were only responsible for performing the data analysis that corresponded to the experiments they were assigned to conduct.

Relevant data were entered into the latest version of the spreadsheet, Aromatase_Master_Version1.3.xls, for calculation of aromatase activity and percent of control. The spreadsheets with the data in them are included in Appendix 5. The instructions for the spreadsheet are described in Appendix A of the protocol.

Statistical Analyses

Statistical analysis, as described below, was carried out by Battelle. The resulting data were sent to In

Vitro Technologies and were included in the final report in Appendix 8.

Concentration Response Fits for the Control Chemicals

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 the positive and negative controls were run prior to the repetitions of the graded concentrations of 4-OH ASDN. 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 repeat tube (full enzyme activity controls, background activity controls, positive, and negative controls, and each 4-OH ASDN concentration), the Excel spreadsheet included total observed (uncorrected) disintegrations 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, was necessarily equal to 0 within each replicate.

For each tube, percent of control was 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 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. In agreement with past convention, logarithms were common logarithms (i.e., base 10). X denoted 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 DPM across the repeat tubes with the same 4-OH ASDN concentration
β = slope of the concentration response curve (β is 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 was fitted to relate percent of control activity to logarithm of concentration within each replicate:

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

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

The response curve was 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%. Model fits were carried out using Prism software (Version 4.02).

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} (10^{μ}) and slope (β). The estimated IC_{50} for 4-OH ASDN was a (weighted) geometric mean across the replicates. The estimated overall standard error was 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 were 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 are displayed in a table. The "Status" of each response curve is 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.

Graphical and Analysis of Variance Comparisons Among Concentration Response Curve Fits

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

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

For each replicate, β and μ were treated as a random variable with mean (βavg, μavg). X and Y ($0< Y <100$) denoted logarithm of concentration and percent of control, as defined above. The average response curve was:

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

Slope (β) and $\log_{10}IC_{50}$ (μ) were 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 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.

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

To assess whether this was the case, the control responses were adjusted for background DPM, 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 were necessarily 0% and the average of the four full enzyme activity controls within a replicate were necessarily 100%. 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 were 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 displayed the extent of consistency across replicates with respect to average value and variability and provided comparisons of beginning versus end of each replicate.

Two-factor analysis of variance models were 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 were:

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

The residual error variation corresponded to repetition within replicate and portion (with four degrees of freedom). The response was percent of control. Since 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, by the way in which "percent of control" was defined, the variation associated with the replicate was necessarily constrained to be 0.

If the daily replicates were in control, then the portion main effect and the portion by replicate interaction should have been insignificant. If the portion by replicate interaction was significant, then the nature of the effect was assessed by comparing the portion effect within each replicate to the portion effect averaged across replicates.

Microsomal Characterization Within and Between Laboratories

Laboratory 2 (Battelle) supplied microsomes to laboratories 1 (RTI), 3 (WIL), and 4 (In Vitro Technologies). Laboratory 4 supplied microsomes to laboratories 1, 2, and 3. Each laboratory independently determined protein concentration and aromatase activity of each microsomal preparation, as discussed in the protocol. Each test laboratory compared 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 were sent to the Data Coordination Center where an interlaboratory comparison was carried out.

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

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

Statistical Software

Concentration response curves were fitted to the data using the non-linear regression analysis features in the Prism statistical analysis package, Version 4.02. 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.

Interlaboratory Statistical Analysis

The lead laboratory and each of the participating laboratories carried 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 "intra-laboratory" statistical analyses for In Vitro Technologies was conducted by the Battelle Data Coordination Center. The Data Coordination Center carried out the "inter-laboratory" statistical analysis. This analysis combined 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.

4.0 Results

4.1 Radiochemical Purity – The measured radiochemical purity of the [^{3}H]ASDN was 97%. The radiochemical purity report is included in Appendix 7 in this report.

4.2 Stock Formulation Analysis – Lindane and 4-OH ASDN stocks prepared in DMSO and 95% ethanol were found to be stable for 168 and 173 days at approximately 5°C, respectively. The formulation stability reports provided by Battelle are included in Appendix 7.

4.3 Protein Analysis – The protein concentration of placental microsomes prepared by In Vitro Technologies (Lot BAA) was determined to be 8.202 and 7.934 mg/mL in two separate analyses. The protein concentration of placental microsomes prepared by Battelle (lot 6-041305) was determined to be 31.187 and 25.136 mg/mL in two separate analyses.

Experiment ID	Microsome Lot ID	Replicate	Assay Date	Measured Protein stock concentration (mg/mL)	Overall (Mean ± sd)	% CV
Placental Microsomes (protein determination experiments)	BAA	2	29 April 2005	8.202	7.82 ± 0.927	11.9
	BAA	3	29 April 2005	7.934		
Placental Microsomes (activity determination experiments)	BAA	1	11 May 2005	8.951		
	BAA	2	12 May 2005	8.086		
Placental Microsomes (inhibition experiments)	BAA	2	27 May 2005	6.172		
	BAA	3	31 May 2005	7.545		
Placental Microsomes (protein determination experiments)	6-041305	1	18 May 2005	31.187	24.5 ± 5.01	20.4
	6-041305	2	20 May 2005	25.136		
Placental Microsomes (activity determination experiments)	6-041305	1	18 May 2005	22.478		
	6-041305	2	20 May 2005	19.379		

Note: Results from replicate 1 of the lot BAA protein determination experiments were not considered accurate since, the estimated sample concentration was close to the estimated value of the low QC sample (0.097 mg/mL), and the deviation of the estimated value of the low QC sample compared to the nominal value of the low QC sample (0.125 mg/mL) was not acceptable. No data was obtained from

replicate 1 of lot BAA inhibition experiments. This experiment was terminated due to insufficient amount of thawed microsomes.

4.4 Aromatase Activity (nmol/mg protein/min) – Full Enzyme Activity Control

Experiment ID	Microsome Lot ID	Replicate	FEAC Beginning		FEAC End		Within Replicate (Mean ±sd)	% CV	Overall (Mean ±sd)	% CV
Placental Microsomes (activity determination experiments)	BAA	1	0.0373	0.0344	0.0308	0.0328	0.0338 ± 0.00275	8.12	0.0382 ± 0.00540	14.1
	BAA	2	0.0331	0.0353	0.0341	0.0344	0.0342 ± 0.000907	2.65		
Placental Microsomes (inhibition experiments)	BAA	2	0.0447	0.0490	0.0452	0.0460	0.0462 ± 0.00193	4.17		
	BAA	3	0.0392	0.0394	0.0371	0.0382	0.0385 ± 0.00106	2.75		
Placental Microsomes (activity determination experiments)	6-041305	1	0.0429	0.0437	0.0406	0.0419	0.0423 ± 0.00134	3.16	0.0464 ± 0.00470	10.1
	6-041305	2	0.0479	0.0528	0.0510	0.0505	0.0506 ± 0.00202	4.00		

4.5 Percent of Control—Inhibition Experiments with Placental Microsomes (Lot BAA)

Test chemical	Replicate	Log[test chemical]	Percent of Control			Mean ± sd	% CV
			Tube 1	Tube 2	Tube 3		
4-OH ASDN	2	-6.00	5.72	5.79	6.03	5.85 ± 0.163	2.78
		-7.00	37.05	31.10	35.13	34.4 ± 3.04	8.82
		-7.30	52.78	53.69	53.54	53.3 ± 0.488	0.91
		-7.60	69.17	66.09	71.66	69.0 ± 2.79	4.05
		-8.00	87.47	84.68	81.62	84.6 ± 2.93	3.46
		-9.00	102.11	98.79	98.19	99.7 ± 2.11	2.12
	3	-6.00	7.64	5.14	5.88	6.22 ± 1.28	20.6
		-7.00	41.08	38.14	37.72	39.0 ± 1.83	4.70
		-7.30	54.34	55.39	57.99	55.9 ± 1.88	3.36
		-7.60	66.81	69.70	70.91	69.1 ± 2.11	3.05
		-8.00	89.96	86.95	87.18	88.0 ± 1.68	1.90
		-9.00	102.90	99.46	104.33	102 ± 2.50	2.45

Note: Replicate 1 could not be completed due to insufficient amount of thawed microsomes.

4.6 IC_{50}

Test chemical	Replicate	Log[IC_{50}]	SE log[IC_{50}]	IC_{50} (nM)	Slope	SE slope	Status	Overall IC_{50} (Mean nM)
4-OH ASDN	2	-7.271	0.02360	53.6	-1.027	0.06353	complete	56.9
	3	-7.221	0.02510	60.2	-0.9567	0.06015	complete	

4.7 Statistical Analysis—The statistical analysis was conducted by Battelle and the report is presented in Appendix 8.

5.0 Discussion

Placental microsomes prepared by In Vitro Technologies (Lot BAA) and placental microsomes prepared by Battelle (Lot # 6-041305) were evaluated for their protein content and aromatase activity and for use in inhibition experiments. The quality control samples at the low concentration were not within 10% of nominal concentration during the first replicate experiment to determine protein concentration for lot BAA.

6.0 Conclusion

Placental microsomes prepared by In Vitro Technologies (Lot # BAA) and placental microsomes prepared by Battelle (Lot # 6-041305) were evaluated for their protein content and aromatase activity. The average protein concentrations were 7.82 ± 0.927 and 24.5 ± 5.01 mg/mL for lot BAA and lot 6-041305, respectively. The average aromatase activities of the two microsomal preparations were 0.0382 ± 0.00540 and 0.0464 ± 0.00470 nmol/mg protein/min for lot BAA and lot 6-041305, respectively. The IC_{50} value for 4-OH ASDN in placental microsomes (lot BAA) was determined to be 56.9 nM.

Appendix 1: In Vitro Technologies Protocol No. 1145

In Vitro Technologies, Inc.
Protocol No. 1145
Version: Final (28 March 2005)

Preparation and Characterization of Human Placental Microsomes (WA 4-16, Task 6)

Sponsor:	Battelle Memorial Institute 505 King Avenue Columbus, OH 43201-2639
Sponsor Representative:	David P. Houchens, Ph.D. E-mail: houchensd@battelle.org Tel.: (614) 424-3564 Fax: (614) 458-3564
Testing Facility:	In Vitro Technologies, Inc. 1450 South Rolling Road Baltimore, Maryland 21227 Tel.: (410) 455-1242 Fax: (410) 455-1245 E-mail: contractservices@invitrotech.com
Study Director:	Aruna Koganti, Ph.D.
EPA Contract Number:	68-W-01-023 (Battelle Prime Contractor)
In Vitro Technologies Study Number:	270-1145-09
Proposed Experimental Start Date:	28 March 2005
Proposed Experimental End Date:	25 April 2005

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Objectives

The objectives of this study are to prepare human placental microsomes, analyze the microsomal preparation for protein content and uninhibited aromatase activity, and to conduct a study with the known aromatase inhibitor 4-hydroxy androstenedione (4-OH ASDN). This task is to be conducted in two stages as described below (see Description of Study). This study is part of a multi-laboratory effort for the evaluation of the placental aromatase assay.

Test System Identification

The test system for this study is human placental microsomes provided by Battelle and In Vitro Technologies. The lot or batch number of the microsomes preparation and any other unique identifier assigned by Battelle and In Vitro Technologies will be recorded in study documentation and will be reported in the study report. All tubes used in the incubations will contain unique labels.

Test System Justification

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. Since the assay is being evaluated for its potential to serve as a screening assay, the use of human tissue enhances its predictive potential.

Description of Study

The study will be conducted in two stages. In Stage 1, In Vitro Technologies will procure a human placenta, prepare microsomes, and determine the protein concentration (two independent replicate experiments) and aromatase activity (uninhibited, two independent replicate experiments) of the microsomes that are prepared. In addition, In Vitro Technologies will run two independent replicates of a study to determine the response of the microsomal aromatase to six concentrations of 4-OH ASDN using the above 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 laboratories can proceed to Stage 2.

In Stage 2, the two laboratories 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 Technologies, RTI, and WIL, whereas In Vitro Technologies will distribute microsomes to Battelle, RTI, and WIL. In this way, each laboratory will use microsomes prepared by both laboratories in their tests. Battelle

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and In Vitro Technologies will include with the shipped microsomes the protein concentration and aromatase activity determinations. Upon receipt of the microsomes, each laboratory will independently determine 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.

Test Method

The *in vitro* test method involves combining microsomes, substrate, 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.

Experimental Methods

Materials

Battelle will provide the following materials:

- Placental microsomes
- 4-Androstene-3, 17-dione (ASDN, CAS no. 63-05-8)
- [1β-^{3}H] Androstenedione ([^{3}H] ASDN, 25.3 Ci/mmol, 1 mCi/ml)
- 4-Hydroxy androstenedione (4-OH ASDN, CAS no. 566-48-3; 302.4 g/mol, Sigma) stock solution (10 mM) in 95% ethanol. 4-OH ASDN is the test substance for this study.
- Lindane (CAS no. 58-89-9; 290.8 g/mol) stock solution (100 mM) in dimethylsulfoxide (DMSO)
- β-Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH, Sigma, catalog no. 1630, molecular weight. 833.4 g/mol)
- 95 % Ethanol
- Dimethyl sulfoxide (DMSO)

The following will be prepared at In Vitro Technologies or will be supplied by In Vitro Technologies:

- Placental microsomes
- 0.1 M Sodium phosphate buffer (pH 7.4)
- Dithiothreitol (Sigma, catalog number D5545, 154.3 g/mol)
- Sucrose (JT Baker, catalog number 4097-04, 342.3 g/mol)
- Glycerol (Sigma, catalog number G7893, 92 g/mol)
- Propylene glycol (JT Baker, catalog number 9402-01, 76.1 g/mol)

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- Liquid scintillation cocktail (Formula 989, Perkin Elmer)
- DC Protein Assay kit (BioRad)

The lot numbers and the purity of the materials received and used in this study will be included in the study report.

Positive Control Preparation

4-Hydroxyandrostenedione (4-OH ASDN) is a known aromatase inhibitor. Positive control stock solutions will be prepared and analyzed by Battelle and distributed to the laboratories. 4-OH ASDN will be formulated in 95 % ethanol. The total volume of positive control 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. The target concentrations for the positive control are listed in Table 2.

Negative Control Preparation

A known aromatase non-inhibitor, lindane, will be used as the negative control substance. Battelle will provide a stock solution for lindane, formulated in DMSO. Fresh dilutions of the stock solution will be prepared in DMSO on the day of use. Dilutions will be prepared such that the target concentration of control substance, 1×10^{-6} M, can be achieved by the addition of 20 µL of the dilution to a 2 mL assay volume.

Substrate Preparation

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 (ASDN, [^{3}H]ASDN) will be provided by Battelle. Battelle 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%.

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 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 95 % 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

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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 (approximately 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.

Human Placenta Procurement and Microsomes Preparation

Note: 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.

Placenta Procurement

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- to 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.

Buffer Preparation

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

Prepare 0.05 M sodium phosphate buffer, pH 7.0. Dissolve 6.90 ± 0.55 g of sodium phosphate monobasic (NaH_2PO_4; JT Baker, catalog number 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 (Na_2HPO_4; JT Baker, catalog number 4062-01, 141.96 g/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 to 8 °C). To complete preparation of Buffer A, dissolve 85.58 ± 1.36 g sucrose (JT Baker, catalog number 4097-04, 342.3 g/mol; or equivalent) and 4.88 ± 0.48 g nicotinamide (Sigma, catalog number N3376, 122.1 g/mol) in 1 L 0.05 M sodium phosphate buffer, pH 7.0. The buffer may be stored for up to one month in the refrigerator (2 to 8 °C).

Buffer B: 0.1 M sodium phosphate (pH 7.4)

Dissolve 13.80 ± 0.55 g NaH_2PO_4 (JT Baker, catalog number 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 Na_2HPO_4 (JT Baker, catalog number 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).

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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, catalog number 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, catalog number G7893, 92 g/mol) to a total solution volume of 200 mL.

Preparation of Microsomes

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, pipettes and pipette tips may be used directly in the assay. Durable lab ware 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 in an appropriate centrifuge (such as an sorvall RC2-B) at 4°C. The supernatant will be removed by pipetting and transferred to ultracentrifuge tubes (recommended approximate capacity is 26-mL). The supernatant 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 L7-55 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 (approximately

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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. 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 to 30 mL, depending 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 divided into aliquots (approximately 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.

Daily 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 to 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 hour 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.

Aromatase Assay Reagent Preparation

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

NADPH (β-nicotinamide adenine dinucleotide phosphate, reduced form, tetrasodium salt, Sigma, catalog number 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 100 µL of the stock is added to the 2 mL assay volume. NADPH must be prepared fresh each day and will be kept on ice.

Assays

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 six-point standard curve will be prepared, ranging from 0.11 to 1.5 mg protein/mL using bovine serum albumin (BSA). Protein will be determined by using a DC

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Protein Assay kit purchased from BioRad (Hercules, CA). Quality control 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 remain at room temperature for at least 15 minutes 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 (at 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.

Aromatase Assay

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 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, i.e. 5×10^{-8} M)
- negative control (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) will be run at the beginning and two at the end of each set.

The assays will be performed in 13 × 100 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 of 1 mL). The final concentrations for the assay components are presented in Table 1. 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 approximately 5 seconds and placed on ice. The tubes will be vortex-mixed an additional 20 to 25 seconds. The tubes will be centrifuged using a Jouan CR422 centrifuge with GH-3.8 rotor or equivalent for 10 minutes at a setting of 1,000 rpm. The methylene chloride layer will be removed and discarded; the aqueous layers will be 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

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(Formula 989, Perkin Elmer, 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 1. 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 ^3H_2O formed.

Determination of the Inhibition of Aromatase Activity by Reference Chemicals

In Vitro Technologies will conduct two independent replicates of the positive control experiment as outlined in Table 2. Each concentration of 4-OH ASDN will be run in triplicate tubes per replicate. The four types of control samples described in the Aromatase Assay section 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 the Aromatase Assay section 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 will remain at 2 mL.

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Table 2. 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

Description of Data Calculations

The data analysis described in the following paragraphs 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.

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. The instructions for the spreadsheet are described in Appendix A.

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Statistical Analyses

Statistical analysis, as described below, will be carried out by Battelle. The resulting data will be sent to In Vitro Technologies and will be included in the final report.

Concentration Response Fits for the Control 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. 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 spreadsheet will include total observed (uncorrected) disintegrations per minute (DPM) per tube and total aromatase activity per tube. The DPM and aromatase activity values will be corrected for the background DPM, 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) DPM 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 DPM 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:

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

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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. 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 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.

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:

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

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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.

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 DPM, 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% and the average of the four full enzyme activity controls within a replicate must necessarily be 100%. 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 four 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 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, then the portion main effect and the portion by replicate interaction should be insignificant. If the portion by replicate interaction is significant, then the

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nature of the effect will be assessed by comparing the portion effect within each replicate to the portion effect averaged across replicates.

Microsomal Characterization Within and Between Laboratories

Laboratory 2 (Battelle) will supply microsomes to laboratories 1 (RTI), 3 (WIL), and 4 (In Vitro Technologies), and laboratory 4 will supply microsomes to laboratories 1, 2, and 3. Each laboratory will independently 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 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
- 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, then estimates and confidence intervals of microsome preparation effect will be prepared, either averaged across laboratories or separately within laboratories, as appropriate.

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.

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 "intra-laboratory" statistical analyses

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for In Vitro Technologies will be conducted by the Battelle Data Coordination Center. 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.

Criteria for Data Acceptance

All data obtained will be reported.

Study Report

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 this protocol.

Data Retention

In Vitro Technologies will retain all supporting documentation, including raw data and written records, for a period of up to five years following issuance of the final report. At the end of this period, Battelle will be notified to determine whether the data (excluding proprietary information) will be transferred, retained, or destroyed. Study records to be maintained will include:

- 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

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Protocol Approval

This protocol has been reviewed and approved by the following:

Sponsor Representatives

David P. Houchens, Ph.D.
Program Manager
Endocrine Disruptor
Screening Program
Battelle Memorial Institute

Signature: David P. Houchens Date: 3/28/05

Jerry D. Johnson, Ph.D.
Work Assignment Leader
Endocrine Disruptor
Screening Program
Battelle Memorial Institute

Signature: Jerry D. Johnson Date: 3-28-05

Study Director

The study will be conducted to the standards of U.S. FDA 21 CFR Part 58. The study will be conducted under my scientific guidance and management. I have reviewed the procedures outlined in this protocol.

Aruna Koganti, Ph.D.
Study Director
In Vitro Technologies

Signature: K. Aruna Prabha Date: 29 March 2005

Review

Terri L. Pollock, B.A.
Quality Assurance Manager
Battelle Memorial Institute

Signature: Terri L Pollock Date: 3-28-05

Sharon Isbell
Director, Quality Systems
In Vitro Technologies

Signature: Sharon Isbell Date: 29 March 2005

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APPENDIX A

NOTES FOR USE OF THE SPREADSHEET: AROMATASE_MASTER_VERSION1.1.XLS

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Substrate Specific Activity Worksheet

This worksheet calculates:

1. The radiochemical content (DPM/mL) of the substrate solution
2. The new specific activity of the [^{3}H]ASDN in the substrate solution

The first item is based on the results of liquid scintillation counting analysis of weighed aliquots of the substrate solution.

The second item is calculated by:

1. determining the mass of ASDN (both radiolabeled and nonradiolabeled)/g of solution. This calculation uses both the measured mass of nonradiolabeled ASDN used in the solution preparation and also the specific activity of the stock [^{3}H]ASDN.

2. the radiochemical content (mCi/g) of the solution is then divided by the mass of ASDN/g solution to arrive at the new specific activity for [^{3}H]ASDN in the substrate solution.

Data to be input include:

- substrate solution aliquot weights (g) and DPM results
- weight (mg) of ASDN used in original stock and volume (mL) of the original stock
- all dilution factors for the dilution of ASDN stock to the solution that was finally used in substrate preparation.
- weight (g) of ASDN dilution used to prepare substrate solution and total weight (g) of substrate solution, and
- specific activity of the stock [^{3}H]ASDN (μCi/mmol)

Protein Worksheet

This worksheet calculates protein content based on absorbance data of standards and unknown samples obtained when samples are analyzed using a commercially available kit.

Data to be input include the concentration of protein standard stock solution (mg/10 mL), protein stock ID, Sample IDs, absorbance data (in triplicate) for standards and unknowns and appropriate dilution factors.

Absorbance values are corrected for blank absorbance. A calibration curve is prepared by linear regression of the standards data (corrected absorbance vs. mg protein measured). The concentration of protein in the unknowns is calculated based on the standard curve.

Microsomes and Chemical Dilutions Worksheet

This worksheet calculates the concentration of protein in the final microsomes dilution. It also serves as the data input center for the reference chemical concentrations used in the assay.

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Data input include volumes used in the preparation of microsomes dilutions. Also entered is the protein concentration of the stock microsomes. Normally, this value will be determined using the protein worksheet described above.

Reference chemical concentrations are entered in molar units of the final concentrations used in the assay.

Activity Calculation Worksheet

The primary aim of this worksheet is to calculate aromatase activity for each sample in a set based on measured DPM, protein concentration and incubation time.

The function of each section is described below:

Section 1 (Columns A–B)

This section contains fields for sample identification

Section 2 (Columns C–I)

This section calculates the total DPM that remain in the incubation mixture after extraction (this is a measure of the ^3H_2O formed in the reaction).

Data input:

1. Aliquot volume
2. DPM measured for each aliquot of each sample.

Output:

The worksheet calculates the average DPM/mL for each repetition, the average DPM/mL for each sample, and the total DPM contained in the sample (based on the aliquots and total sample volumes)

Section 3: (Columns J–L)

This section calculates the percent turnover of the substrate to product.

Data input: Volume of substrate solution used in each assay tube

Linked Data: Column K links to radiochemical content value for the substrate that is calculated in the substrate specific activity worksheet

Output: Percent conversion to product

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Section 4 (Columns M–N)

This section calculates the nmol ^3H_2O formed

Data input: None

Linked Data: Column N links to specific activity value for the substrate that is calculated in the substrate specific activity worksheet

Calculations: Column M corrects the total DPM in each tube for background DPM determined in negative control tubes

Column N Converts DPM data to nmol using the substrate specific activity

Data output: nmol ^3H_2O formed

Section 5 (Columns O–R)

This section calculates aromatase activity in each tube.

Data input: Volume of diluted microsomes used in assay tube and incubation time

Output: Aromatase activity (nmol/mg protein/min)

Results Summary Worksheet

This worksheet summarizes the results.

Section 1 (Columns A–D, Rows 3–15)

This section summarized control data.

Data input: none

Output: average and SD for control samples for beginning, end and overall portions

Section 2 (Columns A–F, Rows 18–42)

This section summarizes activity values according to inhibitor level and replicate

Data input: None

Output: Log[test inhibitor]

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Section 3 (Columns H–L, Rows 18–28)

This section calculates percent of control values for each reference chemical concentration and replicate and organizes the data in a format suitable for importation into Prism Software.

Data input: None

Output: Percent of control values with data arranged in a format suitable for importation into Prism Software.

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Appendix 2: Protocol Amendment

PROTOCOL AMENDMENT FORM

IVT Study Number: 270-1145-09 Document Number: PAM05-011

Date of Sponsor's Verbal Approval: 18 May 2005

Briefly describe the amendment:
The protocol is amended to change 1) the 'Daily Use of Microsomes' section and 2) the 'Protein Assay' section. The details of the changes are attached. The new procedures will be used in protein determination and aromatase activity determination experiments to be conducted with microsomes provided by Battelle. The new procedure for 'Protein Assay' will also be used for protein determination in the experiments evaluating aromatase activity inhibition by 4-OHASDN.

Briefly describe the reason for the amendment:
The changes to the 'Daily Use of Microsomes' section are made in order to allow all required experiments (four protein assays and two aromatase assays) to be conducted with two vials of microsomes provided by Battelle.

The changes to the 'Protein Assay' section are made to improve the accuracy of the protein determination.

These changes were requested by the Sponsor.

Approved by: Jerry D. Johnson Date: 5-26-05
Sponsor Representative

Approved by: K. Aruna Prabha Date: 27 May 2005
Study Director

Effective Date: 04 June 2002

Attachment to Protocol Amendment for study 270-1145-09
Document Number: PAM05-011

Daily Use of Microsomes

1. Thaw and rehomogenize the microsomes as described in the protocol.
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 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.

Protein Assay

Low Protein Concentration Assay

Standard curve range: 5-250 μg/mL

Protein Assay Kit: Biorad Dc Protein Assay Kit.

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 2 mg/mL BSA (Pierce)
250	875	125
125	937.5	62.5
50	975	25
25	1975	25
10	995	5
5	1995	5

Assay procedure

1. Pipet 200 μL unknown, standard, 0.1M sodium phosphate buffer (blank) or QC sample into each tube.
2. Add 100 μL Biorad Dc Reagent A to each tube.
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.

Appendix 3: Protocol Deviation

PROTOCOL DEVIATION FORM

IVT Study Number: 270-1145-09 Document Number: PDV06-014

Date of Deviation: 28 April 05, 29 April 05, 11 May 05, 12 May 05

Scientist (if applicable): AK/TM/EJB

Date Sponsor Notified (if applicable): n/a

Describe the deviation:
The protocol states that the standard curve concentrations should range from 0.11 to 1.5 mg/mL. The standard curve concentrations used ranged from 0.125 to 1.5 mg/mL.

Describe the corrective action:
None

Assessment:
This deviation does not impact the scientific outcome of the study, since the sample concentrations were within 0.125 to 1.5 mg/mL.

Form Completed by: K. Anuradha Reddy (Signature) 23 Feb 2006 (Date)

Approved by: K. Anuradha Reddy (Study Director) Date: 23 Feb 2006

Appendix 4: Quality Assurance Project Plan for Task 6

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

Thomas McClintock 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.

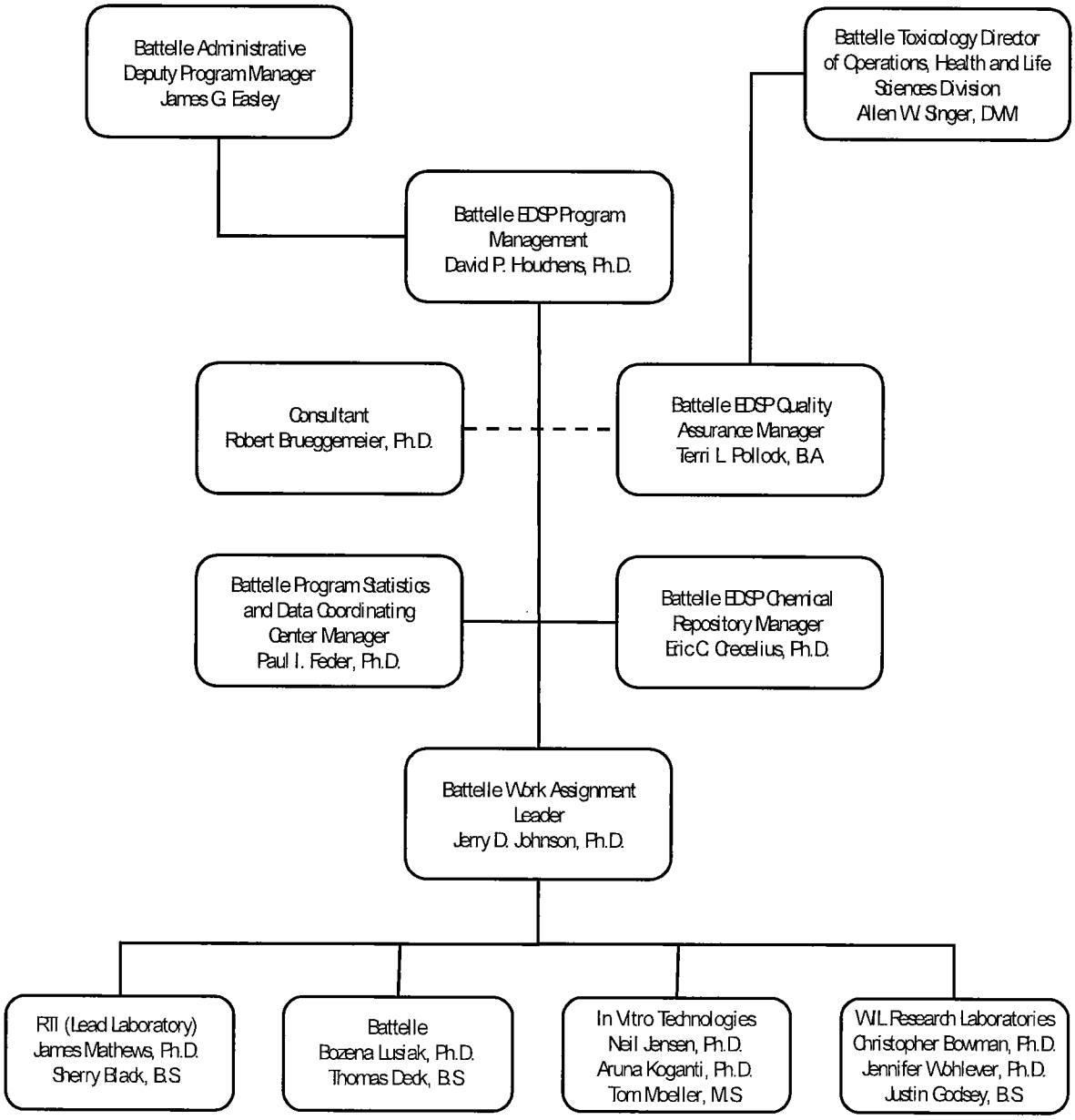

Figure 4-1. WA 4-16 Project Organization Overview

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 □L 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
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.

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 $\log_{10}IC_{50}$ (□), the within replicate standard error of □, 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.

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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 5: Aromatase Spreadsheets

Aromatase Assay Spreadsheet

Assay Date	4/29/2005	Test Chemical ID		# Concentrations tested			
Technician ID	TM	Replicate #	2	Microsome type	Placental	Microsome ID BAA	

Assay Date	4/29/2005	Test Chemical ID	0	# Concentrations tested	0							
Technician ID	TM	Replicate #	2	Microsome type	Placental	Microsome ID	BAA					

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	1.5	1	0.75	0.5	0.25	0.125	0	2	1	FI92021
	0.420	0.325	0.268	0.193	0.116	0.073	0.032			
	0.457	0.340	0.277	0.204	0.115	0.076	0.032			
	0.453	0.341	0.275	0.202	0.114	0.081	0.036			

Samples:	Lot BAA	QC 0.125	QC 0.5	QC 1.0
	0.159	0.075	0.198	0.336
	0.149	0.079	0.204	0.356
	0.157	0.078	0.202	0.349

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
1.5	75	100	0.00150	25	0.0375	0.443	0.410	0.0358
1	50	100	0.00100	25	0.0250	0.335	0.302	0.0259
0.75	37.5	100	0.00075	25	0.0188	0.273	0.240	0.0202
0.5	25	100	0.00050	25	0.0125	0.199	0.166	0.0134
0.25	12.5	100	0.00025	25	0.0063	0.115	0.082	0.0056
0.125	6.25	100	0.00013	25	0.0031	0.077	0.044	0.0021

Variables	Regression results	
m, b	0.092	-0.002
se_m, se_b	0.005	0.001
r^2, se_y	0.990	0.001
F, df	407	4
ss_{reg}, ss_{resid}	0.001	0.000

Regression results are calculated using the function LINEST

Blank	0.033	r^2=	0.990
		m=	0.092
		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
Lot BAA	0.159	0.126	0.010	25	150	3300	0.009	0.008	8.202
Lot BAA	0.149	0.116	0.009	25	150	3300	0.008		
Lot BAA	0.157	0.123	0.009	25	150	3300	0.008		
QC 0.125	0.075	0.041	0.002	25	25	25	0.000	0.000	0.088
QC 0.125	0.079	0.046	0.002	25	25	25	0.000		
QC 0.125	0.078	0.045	0.002	25	25	25	0.000		
QC 0.5	0.198	0.165	0.013	25	25	25	0.001	0.001	0.544
QC 0.5	0.204	0.170	0.014	25	25	25	0.001		
QC 0.5	0.202	0.169	0.014	25	25	25	0.001		
QC 1.0	0.336	0.303	0.026	25	25	25	0.001	0.001	1.079
QC 1.0	0.356	0.323	0.028	25	25	25	0.001		
QC 1.0	0.349	0.316	0.027	25	25	25	0.001		

Aromatase Assay Spreadsheet

Assay Date	4/29/2005	Test Chemical ID		# Concentrations tested		
Technician ID	TM	Replicate #	3	Microsome type	Placental	Microsome ID BAA

Assay Date	4/29/2005	Test Chemical ID	0	# Concentrations tested	0						
Technician ID	TM	Replicate #	3	Microsome type	Placental	Microsome ID	BAA				

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	1.5	1	0.75	0.5	0.25	0.125	0	2	1	FI92021
	0.418	0.290	0.225	0.171	0.087	0.055	0.026			
	0.409	0.284	0.221	0.169	0.086	0.054	0.026			
	0.412	0.294	0.222	0.170	0.098	0.069	0.026			

Samples:	Lot BAA	QC 0.125	QC 0.5	QC 1.0
	0.130	0.069	0.188	0.293
	0.118	0.069	0.180	0.302
	0.122	0.068	0.184	0.303

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	
1.5	75	100	0.00150	25	0.0375	0.413	0.387	0.0372	m, b	0.097	0.000
1	50	100	0.00100	25	0.0250	0.289	0.264	0.0252	se_m, se_b	0.002	0.000
0.75	37.5	100	0.00075	25	0.0188	0.223	0.197	0.0187	r^2, se_y	0.998	0.001
0.5	25	100	0.00050	25	0.0125	0.170	0.144	0.0135	F, df	2132	4
0.25	12.5	100	0.00025	25	0.0063	0.091	0.065	0.0058	ss_{reg}, ss_{resid}	0.001	0.000
0.125	6.25	100	0.00013	25	0.0031	0.059	0.033	0.0028			

Regression results are calculated using the function LINEST

Blank	0.026	r^2=	0.998		
		m=	0.097		
		b=	0.000		

	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
Lot BAA	0.130	0.104	0.010	25	150	3300	0.008	0.008	7.934
Lot BAA	0.118	0.092	0.008	25	150	3300	0.007		
Lot BAA	0.122	0.096	0.009	25	150	3300	0.008		
QC 0.125	0.069	0.043	0.004	25	25	25	0.000	0.000	0.148
QC 0.125	0.069	0.043	0.004	25	25	25	0.000		
QC 0.125	0.068	0.042	0.004	25	25	25	0.000		
QC 0.5	0.188	0.163	0.015	25	25	25	0.001	0.001	0.597
QC 0.5	0.180	0.154	0.014	25	25	25	0.001		
QC 0.5	0.184	0.158	0.015	25	25	25	0.001		
QC 1.0	0.293	0.267	0.025	25	25	25	0.001	0.001	1.045
QC 1.0	0.302	0.276	0.026	25	25	25	0.001		
QC 1.0	0.303	0.277	0.026	25	25	25	0.001		

Aromatase Assay Spreadsheet

Assay Date	5/11/2005	Test Chemical ID	NA	# Concentrations tested	NA		
Technician ID	TM	Replicate #	1	Microsome type	Placental	Microsome ID	BAA

Assay Date	5/11/2005	Test Chemical ID	NA		# Concentrations tested	NA					
Technician ID	TM	Replicate #	1	Microsome type	Placental	Microsome ID	BAA				

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	1.5	1	0.75	0.5	0.25	0.125	0	2	1	FI92021
	0.420	0.303	0.231	0.167	0.090	0.054	0.024			
	0.416	0.315	0.238	0.173	0.083	0.060	0.025			
	0.423	0.300	0.244	0.175	0.091	0.059	0.026			

Samples:	Lot BAA	QC 0.125	QC 0.5	QC 1.0
	0.108	0.065	0.177	0.299
	0.102	0.068	0.164	0.302
	0.103	0.064	0.175	0.298

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	
1.5	75	100	0.00150	25	0.0375	0.420	0.394	0.0364	m, b	0.093	0.000
1	50	100	0.00100	25	0.0250	0.306	0.281	0.0258	se_m, se_b	0.003	0.001
0.75	37.5	100	0.00075	25	0.0188	0.238	0.212	0.0194	r^2, se_y	0.995	0.001
0.5	25	100	0.00050	25	0.0125	0.172	0.146	0.0133	F, df	869	4
0.25	12.5	100	0.00025	25	0.0063	0.088	0.063	0.0055	ss_{reg}, ss_{resid}	0.001	0.000
0.125	6.25	100	0.00013	25	0.0031	0.058	0.033	0.0027			
			Blank	0.025							

Regression results are calculated using the function LINEST

					r^2=	0.995					
					m=	0.093					
					b=	0.000					

	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	
Lot BAA	0.108	0.082	0.007	25	150	4800	0.009	0.009		8.951
Lot BAA	0.102	0.077	0.007	25	150	4800	0.009			
Lot BAA	0.103	0.077	0.007	25	150	4800	0.009			
QC 0.125	0.065	0.040	0.003	25	25	25	0.000	0.000		0.137
QC 0.125	0.068	0.043	0.004	25	25	25	0.000			
QC 0.125	0.064	0.039	0.003	25	25	25	0.000			
QC 0.5	0.177	0.152	0.014	25	25	25	0.001	0.001		0.533
QC 0.5	0.164	0.139	0.013	25	25	25	0.001			
QC 0.5	0.175	0.149	0.014	25	25	25	0.001			
QC 1.0	0.299	0.274	0.025	25	25	25	0.001	0.001		1.009
QC 1.0	0.302	0.277	0.025	25	25	25	0.001			
QC 1.0	0.298	0.273	0.025	25	25	25	0.001			

Assay Date	5/11/2005	Test Chemical ID	NA	# Concentrations tested	NA	Microsome type	Placental	Microsome ID	BAA	Technician ID	TM	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 (initial)	% 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 (nmol estrogen formed/mg protein/min
Full activity control	1	2	0.5	1	4002.966	8005.932	8123.206	16246.412	0.1	190159	8.54	14913	0.0157	1	0.014	15	0.0373
			0.5	2	4120.24	8240.48			0.1								
	2	2	0.5	1	3674.235	7348.47	7542.269	15084.538	0.1	190159	7.93	13752	0.0144	1	0.014	15	0.0344
			0.5	2	3868.034	7736.068			0.1								
	3	2	0.5	1	3434.678	6869.356	6811.576	13623.152	0.1	190159	7.16	12290	0.0129	1	0.014	15	0.0308
			0.5	2	3376.898	6753.796			0.1								
	4	2	0.5	1	3645.556	7291.112	7218.507	14437.014	0.1	190159	7.59	13104	0.0138	1	0.014	15	0.0328
			0.5	2	3572.951	7145.902			0.1								
Background control	1	2	0.5	1	266.2337	532.4674	589.1414	1178.2828	0.1	190159	0.62	-155	-0.0002	1	0.014	15	-0.0004
			0.5	2	322.9077	645.8154			0.1								
	2	2	0.5	1	490.229	980.456	954.2392	1908.4784	0.1	190159	1.00	576	0.0006	1	0.014	15	0.0014
			0.5	2	464.0102	928.0204			0.1								
	3	2	0.5	1	236.2766	472.5532	522.3937	1044.7874	0.1	190159	0.55	-288	-0.0003	1	0.014	15	-0.0007
			0.5	2	286.1171	572.2342			0.1								
	4	2	0.5	1	334.249	668.498	600.173	1200.346	0.1	190159	0.63	-133	-0.0001	1	0.014	15	-0.0003
			0.5	2	265.924	531.848			0.1								
Positive control	1	2	0.5	1	1847.796	3695.592	3754.851	7509.702	0.1	190159	3.95	6177	0.0065	1	0.014	15	0.0155
			0.5	2	1907.055	3814.11			0.1								
	2	2	0.5	1	1723.891	3447.782	3415.004	6830.008	0.1	190159	3.59	5497	0.0058	1	0.014	15	0.0138
			0.5	2	1691.113	3382.226			0.1								
	3	2	0.5	1	1695.953	3391.906	3287.859	6575.718	0.1	190159	3.46	5243	0.0055	1	0.014	15	0.0131
			0.5	2	1591.906	3183.812			0.1								
	4	2	0.5	1	1963.62	3927.24	3600.112	7200.224	0.1	190159	3.79	5867	0.0062	1	0.014	15	0.0147
			0.5	2	1636.492	3272.984			0.1								
Negative Control	1	2	0.5	1	3525.896	7051.792	6784.839	13569.676	0.1	190159	7.14	12237	0.0129	1	0.014	15	0.0306
			0.5	2	3258.942	6517.884			0.1								
	2	2	0.5	1	3339.834	6679.668	6583.403	13166.806	0.1	190159	6.92	11834	0.0124	1	0.014	15	0.0296
			0.5	2	3243.569	6487.138			0.1								
	3	2	0.5	1	3493.573	6987.146	6742.031	13484.062	0.1	190159	7.09	12151	0.0128	1	0.014	15	0.0304
			0.5	2	3248.458	6496.916			0.1								
	4	2	0.5	1	3240.377	6480.754	6297.59	12595.18	0.1	190159	6.62	11262	0.0118	1	0.014	15	0.0282
			0.5	2	3057.213	6114.426			0.1								
NA	1-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	1-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	1-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	2-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	2-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	2-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	3-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	3-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	3-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	4-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	4-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	4-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	5-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	5-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	5-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	6-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	6-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	6-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	7-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	7-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	7-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	8-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	8-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	8-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													

Aromatase Assay Spreadsheet

Assay Date	5/12/2005	Test Chemical ID	NA	# Concentrations tested	NA		
Technician ID	TM	Replicate #	2	Microsome type	Placental	Microsome ID	BAA

Assay Date	5/12/2005	Test Chemical ID	NA		# Concentrations tested	NA					
Technician ID	TM	Replicate #	2	Microsome type	Placental	Microsome ID	BAA				

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	1.5	1	0.75	0.5	0.25	0.125	0	2	1	FI92021
	0.424	0.338	0.269	0.183	0.114	0.072	0.029			
	0.450	0.346	0.271	0.186	0.121	0.070	0.031			
	0.458	0.329	0.258	0.183	0.118	0.071	0.029			

Samples:	Lot BAA	QC 0.125	QC 0.5	QC 1.0
	0.118	0.076	0.191	0.329
	0.117	0.079	0.202	0.343
	0.116	0.077	0.201	0.338

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
1.5	75	100	0.00150	25	0.0375	0.444	0.414	0.0360
1	50	100	0.00100	25	0.0250	0.338	0.308	0.0263
0.75	37.5	100	0.00075	25	0.0188	0.266	0.236	0.0198
0.5	25	100	0.00050	25	0.0125	0.184	0.154	0.0124
0.25	12.5	100	0.00025	25	0.0063	0.118	0.088	0.0064
0.125	6.25	100	0.00013	25	0.0031	0.071	0.041	0.0022

				Regression results	
			Variables		
Blank	0.030		m, b	0.091	-0.002
			se_m, se_b	0.004	0.001
			r^2, se_y	0.992	0.001
			F, df	522	4
			ss_{reg}, ss_{resid}	0.001	0.000

Regression results are calculated using the function LINEST

r^2= 0.992
m= 0.091
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
Lot BAA	0.118	0.088	0.006	25	150	4800	0.008	0.008	8.086
Lot BAA	0.117	0.087	0.006	25	150	4800	0.008		
Lot BAA	0.116	0.086	0.006	25	150	4800	0.008		
QC 0.125	0.076	0.046	0.003	25	25	25	0.000	0.000	0.110
QC 0.125	0.079	0.049	0.003	25	25	25	0.000		
QC 0.125	0.077	0.048	0.003	25	25	25	0.000		
QC 0.5	0.191	0.161	0.013	25	25	25	0.001	0.001	0.547
QC 0.5	0.202	0.172	0.014	25	25	25	0.001		
QC 0.5	0.201	0.172	0.014	25	25	25	0.001		
QC 1.0	0.329	0.299	0.026	25	25	25	0.001	0.001	1.048
QC 1.0	0.343	0.313	0.027	25	25	25	0.001		
QC 1.0	0.338	0.308	0.026	25	25	25	0.001		

Assay Date	5/12/2005	Test Chemical ID	NA	# Concentrations tested	NA	Microsome type	Placental	Microsome ID	BAA	Technician ID	TM	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)	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 (initial)	% 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 (nmol estrogen formed/mg protein/min
Full activity control	1	2	0.5	1	3305.515	6611.03	6647.934	13295.868	0.1	192490	6.91	12097	0.0126	1	0.013	15	0.0331
			0.5	2	3342.419	6684.838			0.1								
	2	2	0.5	1	3586.626	7173.252	7049.446	14098.892	0.1	192490	7.32	12900	0.0134	1	0.013	15	0.0353
			0.5	2	3462.82	6925.64			0.1								
	3	2	0.5	1	3533.688	7067.376	6817.35	13634.7	0.1	192490	7.08	12436	0.0129	1	0.013	15	0.0341
			0.5	2	3263.662	6557.324			0.1								
	4	2	0.5	1	3426.216	6852.432	6878.34	13756.68	0.1	192490	7.15	12558	0.0130	1	0.013	15	0.0344
			0.5	2	3452.124	6904.248			0.1								
Background control	1	2	0.5	1	260.9617	521.9234	527.1954	1054.3908	0.1	192490	0.55	-144	-0.0001	1	0.013	15	-0.0004
			0.5	2	266.2337	532.4674			0.1								
	2	2	0.5	1	297.3369	594.6738	663.6738	1327.3476	0.1	192490	0.69	129	0.0001	1	0.013	15	0.0004
			0.5	2	366.3369	732.6738			0.1								
	3	2	0.5	1	205.6062	411.2124	449.6472	899.6944	0.1	192490	0.47	-299	-0.0003	1	0.013	15	-0.0008
			0.5	2	244.241	488.482			0.1								
	4	2	0.5	1	405.9404	811.8808	756.104	1512.208	0.1	192490	0.79	314	0.0003	1	0.013	15	0.0009
			0.5	2	350.1636	700.3272			0.1								
Positive control	1	2	0.5	1	1791.584	3583.168	3787.018	7574.036	0.1	192490	3.93	6376	0.0066	1	0.013	15	0.0175
			0.5	2	1995.434	3990.868			0.1								
	2	2	0.5	1	2049.512	4099.024	4149.465	8298.93	0.1	192490	4.31	7101	0.0074	1	0.013	15	0.0195
			0.5	2	2099.953	4199.906			0.1								
	3	2	0.5	1	1905.821	3811.642	3735.199	7470.398	0.1	192490	3.88	6272	0.0065	1	0.013	15	0.0172
			0.5	2	1829.376	3658.756			0.1								
	4	2	0.5	1	2089.413	4178.826	4020.045	8040.09	0.1	192490	4.18	6842	0.0071	1	0.013	15	0.0187
			0.5	2	1930.632	3861.264			0.1								
Negative Control	1	2	0.5	1	3666.29	7332.58	7306.593	14613.186	0.1	192490	7.59	13415	0.0139	1	0.013	15	0.0367
			0.5	2	3640.303	7280.606			0.1								
	2	2	0.5	1	3819.551	7639.102	7724.771	15449.542	0.1	192490	8.03	14251	0.0148	1	0.013	15	0.0390
			0.5	2	3905.22	7810.44			0.1								
	3	2	0.5	1	3706.112	7412.224	7388.236	14776.472	0.1	192490	7.68	13578	0.0141	1	0.013	15	0.0372
			0.5	2	3682.124	7364.248			0.1								
	4	2	0.5	1	3769.827	7539.654	7686.717	15373.434	0.1	192490	7.99	14175	0.0147	1	0.013	15	0.0388
			0.5	2	3916.89	7833.78			0.1								
NA	1-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	1-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	1-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	2-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	2-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	2-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	3-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	3-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	3-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	4-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	4-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	4-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	5-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	5-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	5-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	6-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	6-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	6-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	7-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	7-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	7-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	8-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	8-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	8-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													

Aromatase Assay Spreadsheet

Assay Date	5/27/2005	Test Chemical ID	4-OH ASDI	# Concentrations tested	6		
Technician ID	EJB	Replicate #	2	Microsome type	Placental	Microsome ID	BAA

Assay Date	5/27/2005	Test Chemical ID	4-OH ASDN	# Concentrations tested	6						
Technician ID	EJB	Replicate #	2	Microsome type	Placental	Microsome ID	BAA				

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	0.25	0.125	0.05	0.025	0.01	0.005	0	2	1	FI92021
	0.583	0.360	0.177	0.115	0.065	0.044	0.035			
	0.594	0.379	0.188	0.118	0.068	0.045	0.036			
	0.594	0.370	0.192	0.122	0.076	0.049	0.038			

Samples:	Unknown	QC 0.01	QC 0.1
	0.100	0.047	0.241
	0.100	0.047	0.255
	0.099		

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	
0.25	125	1000	0.00025	25	0.0063	0.590	0.554	0.0060	m, b	0.011	0.000
0.125	62.5	1000	0.00013	25	0.0031	0.370	0.334	0.0035	se_m, se_b	0.001	0.000
0.05	25	1000	0.00005	25	0.0013	0.186	0.150	0.0014	r^2, se_y	0.988	0.000
0.025	25	2000	0.00003	25	0.0006	0.118	0.082	0.0007	F, df	337	4
0.01	5	1000	0.00001	25	0.0003	0.069	0.033	0.0001	ss_{reg}, ss_{resid}	0.000	0.000
0.005	5	2000	0.00001	25	0.0001	0.046	0.010	-0.0001			

Regression results are calculated using the function LINEST

Blank	0.036	r^2=	0.988	
		m=	0.011	
		b=	0.000	

	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
Unknown	0.100	0.064	0.000	25	150	48000	0.006	0.006	6.172
Unknown	0.100	0.064	0.000	25	150	48000	0.006		
Unknown	0.099	0.063	0.000	25	150	48000	0.006		
QC 0.01	0.047	0.011	0.000	25	25	25	0.000	0.000	-0.004
QC 0.01	0.047	0.011	0.000	25	25	25	0.000		
QC 0.01					25	25			
QC 0.1	0.241	0.205	0.002	25	25	25	0.000	0.000	0.086
QC 0.1	0.255	0.219	0.002	25	25	25	0.000		
QC 0.1					25	25			
					25	25			
					25	25			
					25	25			

Assay Date	5/27/2005	Test Chemical ID	4-OH ASDN	# Concentrations tested	6	Microsome type	Placental	Microsome ID	BAA	Technician ID	EJB	Replicate #	2

Microsome Dilution Details		
Dilution A	0.15	mL microsome Stock used
	48	mL total volume
	320	dilution factor
Dilution B	1	mL microsome Dilution A used
	1	mL total volume
	1	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	320	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	6.172417
Protein Concentration (dilution added to assay, mg/mL):	0.019289

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	5/27/2005	Test Chemical ID	4-OH ASDN	# Concentrations tested	6	Microsome type	Placental	Microsome ID	BAA	Technician ID	EJB	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)	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 (initial)	% 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 (nmol estrogen formed/mg protein/min
Full activity control	1	2	0.5	1	3034.429	6068.858	5934.003	11868.006	0.1	175272	6.77	11380	0.0129	1	0.010	15	0.0447
			0.5	2	2899.574	5799.148			0.1								
	2	2	0.5	1	3291.538	6583.076	6483.413	12966.826	0.1	175272	7.40	12479	0.0142	1	0.010	15	0.0490
			0.5	2	3191.875	6383.75			0.1								
	3	2	0.5	1	2938.717	5877.434	6003.112	12006.224	0.1	175272	6.85	11518	0.0131	1	0.010	15	0.0452
			0.5	2	3064.395	6128.79			0.1								
	4	2	0.5	1	3126.571	6257.142	6099.376	12198.752	0.1	175272	6.96	11711	0.0133	1	0.010	15	0.0460
			0.5	2	2970.805	5941.61			0.1								
Background control	1	2	0.5	1	113.1298	226.2596	218.9567	437.9134	0.1	175272	0.25	-50	-0.0001	1	0.010	15	-0.0002
			0.5	2	105.8269	211.6538			0.1								
	2	2	0.5	1	112.3077	224.6154	209.27607	418.55214	0.1	175272	0.24	-69	-0.0001	1	0.010	15	-0.0003
			0.5	2	96.96837	193.93674			0.1								
	3	2	0.5	1	152.4175	304.835	270.9344	541.8688	0.1	175272	0.31	54	0.0001	1	0.010	15	0.0002
			0.5	2	118.5169	237.0338			0.1								
	4	2	0.5	1	141.1025	282.205	276.778	553.556	0.1	175272	0.32	66	0.0001	1	0.010	15	0.0003
			0.5	2	135.6755	271.351			0.1								
Positive control	1	2	0.5	1	1625.401	3250.802	3275.224	6550.448	0.1	175272	3.74	6062	0.0069	1	0.010	15	0.0238
			0.5	2	1649.823	3299.646			0.1								
	2	2	0.5	1	1558.946	3117.892	3040.682	6081.364	0.1	175272	3.47	5593	0.0064	1	0.010	15	0.0220
			0.5	2	1481.736	2963.472			0.1								
	3	2	0.5	1	1633.818	3267.636	3115.402	6230.804	0.1	175272	3.55	5743	0.0065	1	0.010	15	0.0225
			0.5	2	1481.584	2963.168			0.1								
	4	2	0.5	1	1444.523	2889.046	2871.583	5743.166	0.1	175272	3.28	5255	0.0060	1	0.010	15	0.0206
			0.5	2	1427.06	2854.12			0.1								
Negative Control	1	2	0.5	1	3287.081	6574.162	6564.944	13129.888	0.1	175272	7.49	12642	0.0144	1	0.010	15	0.0496
			0.5	2	3277.663	6555.726			0.1								
	2	2	0.5	1	3075.013	6150.026	6443.092	12886.184	0.1	175272	7.35	12399	0.0141	1	0.010	15	0.0487
			0.5	2	3368.079	6736.158			0.1								
	3	2	0.5	1	3266.928	6533.856	6316.061	12632.122	0.1	175272	7.21	12144	0.0138	1	0.010	15	0.0477
			0.5	2	3049.133	6098.266			0.1								
	4	2	0.5	1	3138.021	6276.042	6292.862	12585.724	0.1	175272	7.18	12098	0.0137	1	0.010	15	0.0475
			0.5	2	3154.841	6309.682			0.1								
4-OH ASDN	1-1	2	0.5	1	276.1	552.2	580.4748	1160.9492	0.1	175272	0.66	673	0.0008	1	0.010	15	0.0026
			0.5	2	304.3746	608.7492			0.1								
	1-2	2	0.5	1	308.4109	616.8218	584.5278	1169.0556	0.1	175272	0.67	681	0.0008	1	0.010	15	0.0027
			0.5	2	276.1169	552.2338			0.1								
	1-3	2	0.5	1	307.5107	615.0214	598.9774	1197.9548	0.1	175272	0.68	710	0.0008	1	0.010	15	0.0028
			0.5	2	291.4667	582.9334			0.1								
	2-1	2	0.5	1	1267.067	2534.134	2424.912	4849.824	0.1	175272	2.77	4362	0.0050	1	0.010	15	0.0171
			0.5	2	1157.845	2315.69			0.1								
	2-2	2	0.5	1	1024.144	2048.288	2074.278	4148.556	0.1	175272	2.37	3661	0.0042	1	0.010	15	0.0144
			0.5	2	1050.134	2100.268			0.1								
	2-3	2	0.5	1	1172.667	2345.334	2311.579	4623.158	0.1	175272	2.64	4135	0.0047	1	0.010	15	0.0162
			0.5	2	1138.912	2277.824			0.1								
	3-1	2	0.5	1	1657.884	3315.768	3350.53	6701.06	0.1	175272	3.82	6213	0.0071	1	0.010	15	0.0244
			0.5	2	1692.646	3385.292			0.1								
	3-2	2	0.5	1	1747.51	3495.02	3404.062	6808.124	0.1	175272	3.88	6320	0.0072	1	0.010	15	0.0248
			0.5	2	1656.552	3313.104			0.1								
	3-3	2	0.5	1	1683.148	3366.296	3395.499	6790.998	0.1	175272	3.87	6303	0.0072	1	0.010	15	0.0247
			0.5	2	1712.351	3424.702			0.1								
	4-1	2	0.5	1	2162.956	4325.912	4315.138	8630.276	0.1	175272	4.92	8142	0.0092	1	0.010	15	0.0320
			0.5	2	2152.182	4304.364			0.1								
	4-2	2	0.5	1	2081.284	4162.568	4133.803	8267.606	0.1	175272	4.72	7780	0.0088	1	0.010	15	0.0305
			0.5	2	2052.519	4105.038			0.1								
	4-3	2	0.5	1	2203.36	4406.72	4462.057	8924.114	0.1	175272	5.09	8436	0.0096	1	0.010	15	0.0331
			0.5	2	2258.697	4517.394			0.1								
	5-1	2	0.5	1	2593.761	5187.522	5392.4055	10784.811	0.1	175272	6.15	10297	0.0117	1	0.010	15	0.0404
			0.5	2	2798.645	5597.289			0.1								
	5-2	2	0.5	1	2672.046	5344.092	5228.199	10456.398	0.1	175272	5.97	9968	0.0113	1	0.010	15	0.0391
			0.5	2	2556.153	5112.306			0.1								
	5-3	2	0.5	1	2439.579	4879.158	5048.098	10096.196	0.1	175272	5.76	9608	0.0109	1	0.010	15	0.0377
			0.5	2	2608.519	5217.038			0.1								
	6-1	2	0.5	1	3152.67	6305.34	6254.246	12508.492	0.1	175272	7.14	12021	0.0137	1	0.010	15	0.0472
			0.5	2	3101.576	6203.152			0.1								
	6-2	2	0.5	1	3065.293	6170.586	6058.803	12117.606	0.1	175272	6.91	11630	0.0132	1	0.010	15	0.0457
			0.5	2	2973.51	5947.02			0.1								
	6-3	2	0.5	1	3105.714	6211.428	6023.363	12046.726	0.1	175272	6.87	11559	0.0131	1	0.010	15	0.0454
			0.5	2	2917.649	5835.298			0.1								

Assay Date	5/27/2005	Test Chemical ID	4-OH ASDN	# Concentrations tested	Microsome 6 type	Placental	Microsome ID BAA	Technician ID EJB	Replicate #	2

Control Type	Portion	Average	SD
Full activity	Beginning	0.0468	0.0031
Full activity	End	0.0456	0.0005
Full activity	Overall	0.0462	0.0019
Background	Beginning	-0.0002	5.37519E-05
Background	End	0.0002	3.24467E-05
Background	Overall	0.0000	0.000273252
Positive	Beginning	0.0229	0.0013
Positive	End	0.0216	0.0014
Positive	Overall	0.0222	0.0013
Negative	Beginning	0.0492	0.0007
Negative	End	0.0476	0.0001
Negative	Overall	0.0484	0.0010

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
4-OH ASDN	1	1	1.00E-06	-6.00	0.0026
4-OH ASDN	1	2	1.00E-06	-6.00	0.0027
4-OH ASDN	1	3	1.00E-06	-6.00	0.0028
4-OH ASDN	2	1	1.00E-07	-7.00	0.0171
4-OH ASDN	2	2	1.00E-07	-7.00	0.0144
4-OH ASDN	2	3	1.00E-07	-7.00	0.0162
4-OH ASDN	3	1	5.00E-08	-7.30	0.0244
4-OH ASDN	3	2	5.00E-08	-7.30	0.0248
4-OH ASDN	3	3	5.00E-08	-7.30	0.0247
4-OH ASDN	4	1	2.50E-08	-7.60	0.0320
4-OH ASDN	4	2	2.50E-08	-7.60	0.0305
4-OH ASDN	4	3	2.50E-08	-7.60	0.0331
4-OH ASDN	5	1	1.00E-08	-8.00	0.0404
4-OH ASDN	5	2	1.00E-08	-8.00	0.0391
4-OH ASDN	5	3	1.00E-08	-8.00	0.0377
4-OH ASDN	6	1	1.00E-09	-9.00	0.0472
4-OH ASDN	6	2	1.00E-09	-9.00	0.0457
4-OH ASDN	6	3	1.00E-09	-9.00	0.0454

Percent of control values				
	Log[test	Replicate		
Level	substance]	1	2	3
1	-6.00	5.72	5.79	6.03
2	-7.00	37.05	31.10	35.13
3	-7.30	52.78	53.69	53.54
4	-7.60	69.17	66.09	71.66
5	-8.00	87.47	84.68	81.62
6	-9.00	102.11	98.79	98.19

Aromatase Assay Spreadsheet

Assay Date	5/31/2005	Test Chemical ID	4-OH ASDI	# Concentrations tested	6		
Technician ID	EJB	Replicate #	3	Microsome type	Placental	Microsome ID	BAA

Assay Date	5/31/2005	Test Chemical ID	4-OH ASDN	# Concentrations tested	6						
Technician ID	EJB	Replicate #	3	Microsome type	Placental	Microsome ID	BAA				

Standards:								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
	0.25	0.125	0.05	0.025	0.01	0.005	0	2	1	FI92021
	0.604	0.361	0.173	0.102	0.057	0.046	0.026			
	0.613	0.378	0.175	0.105	0.059	0.045	0.031			
	0.624	0.382	0.183	0.107	0.061	0.044	0.031			

Samples:			
	Unknown	QC 0.01	QC 0.1
	0.103	0.047	0.254
	0.103		0.258
	0.099		

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	
0.25	125	1000	0.00025	25	0.0063	0.613	0.584	0.0060	m, b	0.011	0.000
0.125	62.5	1000	0.00013	25	0.0031	0.374	0.344	0.0035	se_m, se_b	0.000	0.000
0.05	25	1000	0.00005	25	0.0013	0.177	0.148	0.0014	r^2, se_y	0.992	0.000
0.025	25	2000	0.00003	25	0.0006	0.105	0.075	0.0006	F, df	495	4
0.01	5	1000	0.00001	25	0.0003	0.059	0.030	0.0001	ss_{reg}, ss_{resid}	0.000	0.000
0.005	5	2000	0.00001	25	0.0001	0.045	0.016	0.0000			

Regression results are calculated using the function LINEST

Blank	0.029	r^2=	0.992		
		m=	0.011		
		b=	0.000		

	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
Unknown	0.103	0.074	0.001	25	150	48000	0.008	0.008	7.545
Unknown	0.103	0.074	0.001	25	150	48000	0.008		
Unknown	0.099	0.070	0.001	25	150	48000	0.007		
QC 0.01	0.047	0.018	0.000	25	25	25	0.000	0.000	0.000
QC 0.01					25	25			
QC 0.01					25	25			
QC 0.1	0.254	0.225	0.002	25	25	25	0.000	0.000	0.089
QC 0.1	0.258	0.229	0.002	25	25	25	0.000		
QC 0.1					25	25			
					25	25			
					25	25			
					25	25			

Assay Date	5/31/2005	Test Chemical ID	4-OH ASDN	# Concentrations tested	6	Microsome type	Placental	Microsome ID	BAA	Technician ID	EJB	Replicate #	3

Microsome Dilution Details		
Dilution A	0.15	mL microsome Stock used
	48	mL total volume
	320	dilution factor
Dilution B	1	mL microsome Dilution A used
	1	mL total volume
	1	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	320	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	7.545251
Protein Concentration (dilution added to assay, mg/mL):	0.023579

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	5/31/2005	Test Chemical ID	4-OH ASDN	# Concentrations tested	6	Microsome type	Placental	Microsome ID	BAA	Technician ID	EJB	Replicate #	3

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 (initial)	% 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 (nmol estrogen formed/mg protein/min
Full activity control	1	2	0.5	1	2795.498	5590.996	5717.948	11435.896	0.1	157261	7.27	10879	0.0139	1	0.012	15	0.0392
			0.5	2	2922.45	5844.9			0.1								
	2	2	0.5	1	2879.422	5758.844	5753.963	11507.926	0.1	157261	7.32	10951	0.0139	1	0.012	15	0.0394
			0.5	2	2874.541	5749.082			0.1								
	3	2	0.5	1	2754.596	5509.192	5428.588	10857.176	0.1	157261	6.90	10300	0.0131	1	0.012	15	0.0371
			0.5	2	2673.992	5347.984			0.1								
	4	2	0.5	1	2831.758	5663.516	5577.564	11155.128	0.1	157261	7.09	10598	0.0135	1	0.012	15	0.0382
			0.5	2	2745.806	5491.612			0.1								
Background control	1	2	0.5	1	152.4175	304.835	302.161	604.322	0.1	157261	0.38	47	0.0001	1	0.012	15	0.0002
			0.5	2	149.7435	299.487			0.1								
	2	2	0.5	1	127.535	255.07	251.439	502.878	0.1	157261	0.32	-54	-0.0001	1	0.012	15	-0.0002
			0.5	2	123.904	247.808			0.1								
	3	2	0.5	1	134.6783	269.3566	272.0502	544.1004	0.1	157261	0.35	-13	0.0000	1	0.012	15	0.0000
			0.5	2	137.3719	274.7438			0.1								
	4	2	0.5	1	153.0937	306.1874	289.0927	578.1854	0.1	157261	0.37	21	0.0000	1	0.012	15	0.0001
			0.5	2	135.999	271.998			0.1								
Positive control	1	2	0.5	1	1796.861	3597.722	3428.477	6856.954	0.1	157261	4.36	6300	0.0080	1	0.012	15	0.0227
			0.5	2	1629.616	3259.232			0.1								
	2	2	0.5	1	1435.454	2870.908	2917.371	5834.742	0.1	157261	3.71	5277	0.0067	1	0.012	15	0.0190
			0.5	2	1481.917	2963.834			0.1								
	3	2	0.5	1	1403.853	2807.706	2820.313	5640.626	0.1	157261	3.59	5083	0.0065	1	0.012	15	0.0183
			0.5	2	1416.46	2832.92			0.1								
	4	2	0.5	1	1614.547	3229.094	3143.716	6287.432	0.1	157261	4.00	5730	0.0073	1	0.012	15	0.0206
			0.5	2	1529.169	3058.338			0.1								
Negative Control	1	2	0.5	1	3084.149	6168.298	6236.256	12472.512	0.1	157261	7.93	11915	0.0152	1	0.012	15	0.0429
			0.5	2	3152.107	6304.214			0.1								
	2	2	0.5	1	3156.876	6313.752	6151.763	12303.526	0.1	157261	7.82	11746	0.0150	1	0.012	15	0.0423
			0.5	2	2994.887	5989.774			0.1								
	3	2	0.5	1	2891.086	5782.172	5906.502	11813.004	0.1	157261	7.51	11256	0.0143	1	0.012	15	0.0405
			0.5	2	3015.416	6030.832			0.1								
	4	2	0.5	1	3011.423	6022.846	5974.361	11948.722	0.1	157261	7.60	11391	0.0145	1	0.012	15	0.0410
			0.5	2	2962.938	5925.876			0.1								
4-OH ASDN	1-1	2	0.5	1	344.9468	689.8935	686.6756	1373.3512	0.1	157261	0.87	816	0.0010	1	0.012	15	0.0029
			0.5	2	341.7288	683.4576			0.1								
	1-2	2	0.5	1	296.2938	592.5876	552.9984	1105.9968	0.1	157261	0.70	549	0.0007	1	0.012	15	0.0020
			0.5	2	256.7046	513.4092			0.1								
	1-3	2	0.5	1	320.8807	641.7614	592.9323	1185.8646	0.1	157261	0.75	626	0.0008	1	0.012	15	0.0023
			0.5	2	272.0516	544.1032			0.1								
	2-1	2	0.5	1	1216.673	2433.346	2472.722	4945.444	0.1	157261	3.14	4388	0.0056	1	0.012	15	0.0158
			0.5	2	1256.049	2512.098			0.1								
	2-2	2	0.5	1	1098.981	2197.962	2315.536	4631.072	0.1	157261	2.94	4074	0.0052	1	0.012	15	0.0147
			0.5	2	1216.555	2433.11			0.1								
	2-3	2	0.5	1	1153.677	2307.354	2293.062	4586.124	0.1	157261	2.92	4029	0.0051	1	0.012	15	0.0145
			0.5	2	1138.385	2276.77			0.1								
	3-1	2	0.5	1	1588.212	3176.424	3181.118	6362.236	0.1	157261	4.05	5805	0.0074	1	0.012	15	0.0209
			0.5	2	1591.906	3183.812			0.1								
	3-2	2	0.5	1	1599.056	3198.112	3236.753	6473.505	0.1	157261	4.12	5916	0.0075	1	0.012	15	0.0213
			0.5	2	1637.697	3275.394			0.1								
	3-3	2	0.5	1	1676.958	3353.916	3376.035	6752.07	0.1	157261	4.29	6195	0.0079	1	0.012	15	0.0223
			0.5	2	1699.077	3398.154			0.1								
	4-1	2	0.5	1	1907.618	3815.236	3847.006	7694.012	0.1	157261	4.89	7137	0.0091	1	0.012	15	0.0257
			0.5	2	1939.388	3878.776			0.1								
	4-2	2	0.5	1	1978.17	3956.34	4001.059	8002.118	0.1	157261	5.09	7445	0.0095	1	0.012	15	0.0268
			0.5	2	2022.889	4045.778			0.1								
	4-3	2	0.5	1	2016.873	4037.746	4066.005	8132.01	0.1	157261	5.17	7575	0.0096	1	0.012	15	0.0273
			0.5	2	2047.132	4094.264			0.1								
	5-1	2	0.5	1	2660.65	5321.3	5083.138	10166.276	0.1	157261	6.46	9609	0.0122	1	0.012	15	0.0346
			0.5	2	2422.488	4844.976			0.1								
	5-2	2	0.5	1	2505.043	5010.086	4922.357	9844.714	0.1	157261	6.26	9287	0.0118	1	0.012	15	0.0334
			0.5	2	2417.314	4834.628			0.1								
	5-3	2	0.5	1	2513.124	5026.248	4934.665	9869.33	0.1	157261	6.27	9312	0.0119	1	0.012	15	0.0335
			0.5	2	2421.541	4843.082			0.1								
	6-1	2	0.5	1	2816.766	5633.532	5774.333	11548.666	0.1	157261	7.34	10991	0.0140	1	0.012	15	0.0396
			0.5	2	2957.567	5915.134			0.1								
	6-2	2	0.5	1	2778.663	5557.326	5590.776	11181.552	0.1	157261	7.11	10624	0.0135	1	0.012	15	0.0383
			0.5	2	2812.113	5624.226			0.1								
	6-3	2	0.5	1	2887.533	5775.066	5850.717	11701.434	0.1	157261	7.44	11144	0.0142	1	0.012	15	0.0401
			0.5	2	2963.184	5926.368			0.1								

Assay Date	5/31/2005	Test Chemical ID	4-OH ASDN	# Concentrations tested		Microsome 6 type	Placental	Microsome ID BAA	Technician ID EJB	Replicate #	3

Control Type	Portion	Average	SD
Full activity	Beginning	0.0393	0.0002
Full activity	End	0.0376	0.0008
Full activity	Overall	0.0385	0.0011
Background	Beginning	0.0000	0.000258296
Background	End	0.0000	8.67869E-05
Background	Overall	0.0000	0.0001581
Positive	Beginning	0.0208	0.0026
Positive	End	0.0195	0.0016
Positive	Overall	0.0202	0.0019
Negative	Beginning	0.0426	0.0004
Negative	End	0.0408	0.0003
Negative	Overall	0.0417	0.0011

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
4-OH ASDN	1	1	1.00E-06	-6.00	0.0029
4-OH ASDN	1	2	1.00E-06	-6.00	0.0020
4-OH ASDN	1	3	1.00E-06	-6.00	0.0023
4-OH ASDN	2	1	1.00E-07	-7.00	0.0158
4-OH ASDN	2	2	1.00E-07	-7.00	0.0147
4-OH ASDN	2	3	1.00E-07	-7.00	0.0145
4-OH ASDN	3	1	5.00E-08	-7.30	0.0209
4-OH ASDN	3	2	5.00E-08	-7.30	0.0213
4-OH ASDN	3	3	5.00E-08	-7.30	0.0223
4-OH ASDN	4	1	2.50E-08	-7.60	0.0257
4-OH ASDN	4	2	2.50E-08	-7.60	0.0268
4-OH ASDN	4	3	2.50E-08	-7.60	0.0273
4-OH ASDN	5	1	1.00E-08	-8.00	0.0346
4-OH ASDN	5	2	1.00E-08	-8.00	0.0334
4-OH ASDN	5	3	1.00E-08	-8.00	0.0335
4-OH ASDN	6	1	1.00E-09	-9.00	0.0396
4-OH ASDN	6	2	1.00E-09	-9.00	0.0383
4-OH ASDN	6	3	1.00E-09	-9.00	0.0401

Percent of control values				
	Log[test	Replicate		
Level	substance]	1	2	3
1	-6.00	7.64	5.14	5.88
2	-7.00	41.08	38.14	37.72
3	-7.30	54.34	55.39	57.99
4	-7.60	66.81	69.70	70.91
5	-8.00	89.96	86.95	87.18
6	-9.00	102.90	99.46	104.33

Aromatase Assay Spreadsheet

Assay Date	5/18/2005	Test Chemical ID	NA	# Concentrations tested		
Technician ID	TM	Replicate #	1	Microsome type	Placental	Microsome ID 6-041305

Assay Date	5/18/2005	Test Chemical ID	NA	# Concentrations tested	0					
Technician ID	TM	Replicate #	1	Microsome type	Placental	Microsome ID	6-041305			

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	0.25	0.125	0.05	0.025	0.01	0.005	Blank	2	1	FI92021
	0.428	0.306	0.126	0.073	0.046	0.036	0.023			
	0.438	0.282	0.132	0.072	0.044	0.035	0.028			
	0.497	0.291	0.145	0.072	0.044	0.037	0.025			

Samples:	6-041305	QC 0.01	QC 0.1
	0.107	0.054	0.255
	0.104	0.044	0.257
	0.101		

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	
0.25	125	1000	0.00025	25	0.0063	0.455	0.429	0.0059	m, b	0.014	0.000
0.125	62.5	1000	0.00013	25	0.0031	0.293	0.268	0.0036	se_m, se_b	0.001	0.000
0.05	25	1000	0.00005	25	0.0013	0.134	0.109	0.0014	r^2, se_y	0.985	0.000
0.025	25	2000	0.00003	25	0.0006	0.072	0.047	0.0005	F, df	255	4
0.01	5	1000	0.00001	25	0.0003	0.044	0.019	0.0001	ss_{reg}, ss_{resid}	0.000	0.000
0.005	5	2000	0.00001	25	0.0001	0.036	0.011	0.0000			

Regression results are calculated using the function LINEST

Blank	0.025	r^2=	0.985		
		m=	0.014		
		b=	0.000		

	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	0.107	0.082	0.001	25	63	50000	0.033	0.031	31.187
6-041305	0.104	0.079	0.001	25	63	50000	0.031		
6-041305	0.101	0.075	0.001	25	63	50000	0.030		
QC 0.01	0.054	0.028	0.000	25	200	200	0.000	0.000	0.008
QC 0.01	0.044	0.019	0.000	25	200	200	0.000		
QC 0.01									
QC 0.1	0.255	0.230	0.003	25	200	200	0.000	0.000	0.125
QC 0.1	0.257	0.232	0.003	25	200	200	0.000		
QC 0.1									

Aromatase Assay Spreadsheet

Assay Date	5/20/2005	Test Chemical ID	NA	# Concentrations tested		
Technician ID	TM	Replicate #	2	Microsome type	Placental	Microsome ID 6-041305

Assay Date	5/20/2005	Test Chemical ID	NA	# Concentrations tested	0
Technician ID	TM	Replicate #	2	Microsome type	Placental
Microsome ID	6-041305				

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	0.25	0.125	0.05	0.025	0.01	0.005	Blank	2	1	FI92021
	0.495	0.313	0.144	0.085	0.048	0.033	0.025			
	0.525	0.310	0.136	0.073	0.045	0.038	0.024			
	0.509	0.288	0.135	0.072	0.043	0.033	0.024			

Samples:	6-041305	QC 0.01	QC 0.1
	0.096	0.051	0.253
	0.104	0.050	0.251
	0.081		

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	
0.25	125	1000	0.00025	25	0.0063	0.510	0.485	0.0061	m, b	0.013	0.000
0.125	62.5	1000	0.00013	25	0.0031	0.304	0.279	0.0034	se_m, se_b	0.000	0.000
0.05	25	1000	0.00005	25	0.0013	0.138	0.114	0.0013	r^2, se_y	0.994	0.000
0.025	25	2000	0.00003	25	0.0006	0.077	0.053	0.0006	F, df	671	4
0.01	5	1000	0.00001	25	0.0003	0.045	0.021	0.0002	ss_{reg}, ss_{resid}	0.000	0.000
0.005	5	2000	0.00001	25	0.0001	0.035	0.010	0.0000			

Regression results are calculated using the function LINEST

Blank	0.024	r^2=	0.994	
		m=	0.013	
		b=	0.000	

	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	0.096	0.072	0.001	25	63	50000	0.026	0.025	25.136
6-041305	0.104	0.079	0.001	25	63	50000	0.029		
6-041305	0.081	0.057	0.001	25	63	50000	0.020		
QC 0.01	0.051	0.027	0.000	25	200	200	0.000	0.000	0.009
QC 0.01	0.050	0.025	0.000	25	200	200	0.000		
QC 0.01									
QC 0.1	0.253	0.229	0.003	25	200	200	0.000	0.000	0.112
QC 0.1	0.251	0.227	0.003	25	200	200	0.000		
QC 0.1									

Aromatase Assay Spreadsheet

Assay Date	5/18/2005	Test Chemical ID	NA	# Concentrations tested			
Technician ID	TM	Replicate #	1	Microsome type	Placental	Microsome ID	6-041305

Assay Date	5/18/2005	Test Chemical ID	NA	# Concentrations tested	0
Technician ID	TM	Replicate #	1	Microsome type	Placental

Microsome ID 6-041305

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	0.25	0.125	0.05	0.025	0.01	0.005	Blank	2	1	FI92021
	0.495	0.313	0.144	0.085	0.048	0.033	0.025			
	0.525	0.310	0.136	0.073	0.045	0.038	0.024			
	0.509	0.288	0.135	0.072	0.043	0.033	0.024			

Samples:	6-041305	QC 0.01	QC 0.1
	0.096	0.051	0.253
	0.104	0.050	0.251
	0.081		

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
0.25	125	1000	0.00025	25	0.0063	0.510	0.485	0.0061
0.125	62.5	1000	0.00013	25	0.0031	0.304	0.279	0.0034
0.05	25	1000	0.00005	25	0.0013	0.138	0.114	0.0013
0.025	25	2000	0.00003	25	0.0006	0.077	0.053	0.0006
0.01	5	1000	0.00001	25	0.0003	0.045	0.021	0.0002
0.005	5	2000	0.00001	25	0.0001	0.035	0.010	0.0000

			Variables	Regression results	
			m, b	0.013	0.000
			se_m, se_b	0.000	0.000
			r^2, se_y	0.994	0.000
			F, df	671	4
			ss_{reg}, ss_{resid}	0.000	0.000

Regression results are calculated using the function LINEST

Blank	0.024	r^2=	0.994	
		m=	0.013	
		b=	0.000	

	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	0.096	0.072	0.001	25	63	50000	0.026	0.025	25.136
6-041305	0.104	0.079	0.001	25	63	50000	0.029		
6-041305	0.081	0.057	0.001	25	63	50000	0.020		
QC 0.01	0.051	0.027	0.000	25	200	200	0.000	0.000	0.009
QC 0.01	0.050	0.025	0.000	25	200	200	0.000		
QC 0.01									
QC 0.1	0.253	0.229	0.003	25	200	200	0.000	0.000	0.112
QC 0.1	0.251	0.227	0.003	25	200	200	0.000		
QC 0.1									

Assay Date	5/18/2005	Test Chemical ID	NA	# Concentrations tested		0 Microsome type	Placental	Microsome ID	6-041305	Technician ID	EJB	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 (initial)	% 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 (nmol estrogen formed/mg protein/min
Full activity control	1	2	0.5	1	4298.111	8596.222	8555.214	17110.428	0.1	183687	9.31	16594	0.0181	1	0.014	15	0.0429
			0.5	2	4257.103	8514.206											
	2	2	0.5	1	4312.399	8624.798	8705.605	17411.21	0.1	183687	9.48	16894	0.0184	1	0.014	15	0.0437
			0.5	2	4393.206	8786.412											
	3	2	0.5	1	3964.509	7929.018	8107.213	15214.426	0.1	183687	8.83	15698	0.0171	1	0.014	15	0.0406
			0.5	2	4142.704	8285.408											
	4	2	0.5	1	4118.462	8236.924	8359.413	16718.826	0.1	183687	9.10	16202	0.0177	1	0.014	15	0.0419
			0.5	2	4240.951	8481.902											
Background control	1	2	0.5	1	124.7753	249.5506	264.8407	529.6814	0.1	183687	0.29	13	0.0000	1	0.014	15	0.0000
			0.5	2	140.0654	280.1308											
	2	2	0.5	1	142.759	285.518	249.7187	499.4374	0.1	183687	0.27	-17	0.0000	1	0.014	15	0.0000
			0.5	2	106.9597	213.9194											
	3	2	0.5	1	117.6556	235.3112	247.9041	495.8082	0.1	183687	0.27	-21	0.0000	1	0.014	15	-0.0001
			0.5	2	130.2485	260.497											
	4	2	0.5	1	140.0654	280.1308	271.091	542.182	0.1	183687	0.30	25	0.0000	1	0.014	15	0.0001
			0.5	2	131.0256	262.0512											
Positive control	1	2	0.5	1	2353.828	4707.656	4847.557	9695.114	0.1	183687	5.28	9178	0.0100	1	0.014	15	0.0237
			0.5	2	2493.729	4987.458											
	2	2	0.5	1	2168.978	4337.956	4472.76	8945.52	0.1	183687	4.87	8429	0.0092	1	0.014	15	0.0218
			0.5	2	2303.782	4607.564											
	3	2	0.5	1	2104.443	4208.886	4270.388	8540.776	0.1	183687	4.65	8024	0.0087	1	0.014	15	0.0208
			0.5	2	2165.945	4331.89											
	4	2	0.5	1	2322.776	4645.552	4580.428	9160.856	0.1	183687	4.99	8644	0.0094	1	0.014	15	0.0224
			0.5	2	2257.652	4515.304											
Negative Control	1	2	0.5	1	4077.785	8155.57	8600.306	17200.612	0.1	183687	9.36	16684	0.0182	1	0.014	15	0.0432
			0.5	2	4522.521	9045.042											
	2	2	0.5	1	4480.749	8961.498	8847.908	17695.816	0.1	183687	9.63	17179	0.0187	1	0.014	15	0.0444
			0.5	2	4367.159	8734.318											
	3	2	0.5	1	4349.779	8699.558	8740.497	17480.994	0.1	183687	9.52	16964	0.0185	1	0.014	15	0.0439
			0.5	2	4390.718	8781.436											
	4	2	0.5	1	4233.098	8466.196	8294.913	16589.826	0.1	183687	9.03	16073	0.0175	1	0.014	15	0.0416
			0.5	2	4061.815	8123.63											
NA	1-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	1-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	1-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	2-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
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	2-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
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				2													

Aromatase Assay Spreadsheet

Assay Date	5/20/2005	Test Chemical ID	NA	# Concentrations tested		
Technician ID	EJB	Replicate #	2	Microsome type	Placental	Microsome ID 6-041305

Assay Date	5/20/2005	Test Chemical ID	NA	# Concentrations tested	0						
Technician ID	EJB	Replicate #	2	Microsome type	Placental	Microsome ID	6-041305				

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	0.25	0.125	0.05	0.025	0.01	0.005	Blank	2	1	FI92021
	0.457	0.304	0.143	0.095	0.058	0.046	0.030			
	0.449	0.300	0.145	0.086	0.057	0.044	0.042			
	0.453	0.292	0.143	0.092	0.056	0.046	0.035			

Samples:	6-041305	QC 0.01	QC 0.1
	0.089	0.051	0.245
	0.088	0.053	0.263
	0.093		

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	
0.25	125	1000	0.00025	25	0.0063	0.453	0.417	0.0059	m, b	0.015	0.000
0.125	62.5	1000	0.00013	25	0.0031	0.299	0.263	0.0037	se_m, se_b	0.001	0.000
0.05	25	1000	0.00005	25	0.0013	0.143	0.107	0.0014	r^2, se_y	0.984	0.000
0.025	25	2000	0.00003	25	0.0006	0.091	0.055	0.0006	F, df	240	4
0.01	5	1000	0.00001	25	0.0003	0.057	0.021	0.0001	ss_{reg}, ss_{resid}	0.000	0.000
0.005	5	2000	0.00001	25	0.0001	0.045	0.009	0.0000			

Regression results are calculated using the function LINEST

Blank	0.036	r^2=	0.984	
		m=	0.015	
		b=	0.000	

	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	0.089	0.053	0.001	25	63	50000	0.019	0.019	19.379
6-041305	0.088	0.052	0.001	25	63	50000	0.019		
6-041305	0.093	0.057	0.001	25	63	50000	0.021		
QC 0.01	0.051	0.015	0.000	25	200	200	0.000	0.000	0.002
QC 0.01	0.053	0.017	0.000	25	200	200	0.000		
QC 0.01									
QC 0.1	0.245	0.209	0.003	25	200	200	0.000	0.000	0.120
QC 0.1	0.263	0.227	0.003	25	200	200	0.000		
QC 0.1									

Assay Date	5/20/2005	Test Chemical ID	NA	# Concentrations tested	0	Microsome type	Placental	Microsome ID	6-041305	Technician ID	EJB	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)	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 (initial)	% 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 (nmo estrogen formed/mg protein/min
Full activity control	1	2	0.5	1	3973.552	7947.104	7658.982	15717.964	0.1	174118	9.03	15208	0.0174	1	0.012	15	0.0479
			0.5	2	3885.43	7770.86											
	2	2	0.5	1	4280.827	8561.654	8632.034	17264.068	0.1	174118	9.92	16754	0.0192	1	0.012	15	0.0528
			0.5	2	4351.207	8702.414											
	3	2	0.5	1	4135.723	8271.446	8341.912	16683.824	0.1	174118	9.58	16173	0.0185	1	0.012	15	0.0510
			0.5	2	4206.189	8412.378											
	4	2	0.5	1	4263.599	8527.198	8269.683	16539.366	0.1	174118	9.50	16029	0.0184	1	0.012	15	0.0505
			0.5	2	4006.084	8012.168											
Background control	1	2	0.5	1	95.57258	191.14516	251.79938	503.59876	0.1	174118	0.29	-7	0.0000	1	0.012	15	0.0000
			0.5	2	156.2268	312.4536											
	2	2	0.5	1	125.6422	251.2844	239.7983	479.5966	0.1	174118	0.28	-31	0.0000	1	0.012	15	-0.0001
			0.5	2	114.1561	228.3122											
	3	2	0.5	1	167.252	334.504	298.1293	596.2586	0.1	174118	0.34	86	0.0001	1	0.012	15	0.0003
			0.5	2	130.8773	261.7546											
	4	2	0.5	1	119.5773	239.1546	231.0795	462.1572	0.1	174118	0.27	-48	-0.0001	1	0.012	15	-0.0002
			0.5	2	111.5013	223.0026											
Positive control	1	2	0.5	1	2625.447	5250.894	5180.186	10360.372	0.1	174118	5.95	9850	0.0113	1	0.012	15	0.0310
			0.5	2	2554.739	5109.478											
	2	2	0.5	1	2443.563	4887.126	4909.305	9818.61	0.1	174118	5.64	9308	0.0107	1	0.012	15	0.0293
			0.5	2	2465.742	4931.484											
	3	2	0.5	1	2520.007	5040.014	5036.765	10073.53	0.1	174118	5.79	9563	0.0110	1	0.012	15	0.0301
			0.5	2	2516.758	5033.516											
	4	2	0.5	1	2280.132	4560.264	4750.914	9501.828	0.1	174118	5.46	8991	0.0103	1	0.012	15	0.0283
			0.5	2	2470.782	4941.564											
Negative Control	1	2	0.5	1	4713.151	9426.302	9260.235	18520.47	0.1	174118	10.64	18010	0.0206	1	0.012	15	0.0568
			0.5	2	4547.084	9094.168											
	2	2	0.5	1	4730.317	9460.634	9165.69	18333.38	0.1	174118	10.53	17823	0.0204	1	0.012	15	0.0562
			0.5	2	4436.373	8872.746											
	3	2	0.5	1	4519.07	9038.14	8941.98	17883.96	0.1	174118	10.27	17374	0.0199	1	0.012	15	0.0548
			0.5	2	4422.91	8845.82											
	4	2	0.5	1	4570.26	9140.52	9091.398	18182.796	0.1	174118	10.44	17672	0.0202	1	0.012	15	0.0557
			0.5	2	4521.138	9042.276											
NA	1-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
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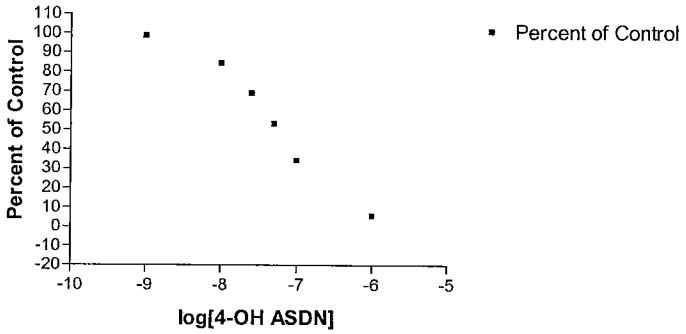
Appendix 6: Prism Output

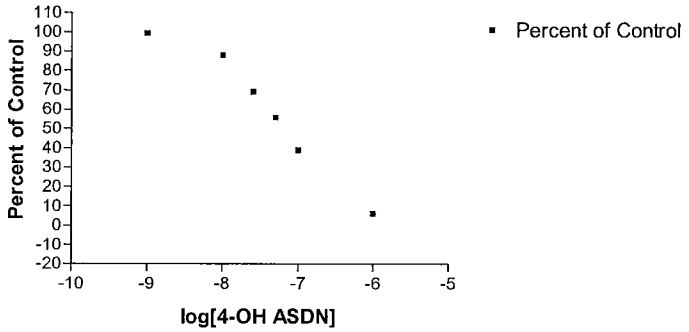
IVT 4-16 Task 6 Replicate 2



log[4-OH ASDN]	Percent of Control		
	Y1	Y2	Y3
-6.0	5.7	5.8	6.0
-7.0	37.0	31.1	35.1
-7.3	52.8	53.7	53.5
-7.6	69.2	66.1	71.7
-8.0	87.5	84.7	81.6
-9.0	99.5	98.8	98.2

	Percent of Control
Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	1.029
TOP	100.2
LOGEC50	-7.271
HILLSLOPE	-1.027
EC50	5.360e-008
Std. Error	
BOTTOM	1.858
TOP	1.450
LOGEC50	0.02360
HILLSLOPE	0.06353
95% Confidence Intervals	
BOTTOM	-2.956 to 5.014
TOP	97.08 to 103.3
LOGEC50	-7.321 to -7.220
HILLSLOPE	-1.164 to -0.8910
EC50	4.770e-008 to 6.022e-008
Goodness of Fit	
Degrees of Freedom	14
R^2	0.9966
Absolute Sum of Squares	59.03
Sy.x	2.053
Data	
Number of X values	6
Number of Y replicates	3
Total number of values	18
Number of missing values	0

IVT 4-16 Task 6 Replicate 3



log[4-OH ASDN]	Percent of Control		
	Y1	Y2	Y3
-6.0	7.6	5.1	5.9
-7.0	41.1	38.1	37.7
-7.3	54.3	55.4	58.0
-7.6	66.8	69.7	70.9
-8.0	90.0	86.9	87.2
-9.0	99.5	99.5	99.5

	Percent of Control
Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	-0.1778
TOP	101.9
LOGEC50	-7.221
HILLSLOPE	-0.9567
EC50	6.015e-008
Std. Error	
BOTTOM	2.064
TOP	1.445
LOGEC50	0.02510
HILLSLOPE	0.06015
95% Confidence Intervals	
BOTTOM	-4.605 to 4.249
TOP	98.85 to 105.0
LOGEC50	-7.275 to -7.167
HILLSLOPE	-1.086 to -0.8276
EC50	5.313e-008 to 6.809e-008
Goodness of Fit	
Degrees of Freedom	14
R^2	0.9970
Absolute Sum of Squares	52.51
Sy.x	1.937
Data	
Number of X values	6
Number of Y replicates	3
Total number of values	18
Number of missing values	0

Appendix 7: Sponsor Chemistry Reports

DRAFT 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

DRAFT 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
Study Initiation Date:	
Experimental Dates:	January 5, 2005
Draft Task Report Date	February 3, 2005

Author:		Approved:	
Sherry Black Research Chemist	Date	James Mathews, Ph. D, DABT Study Director	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).

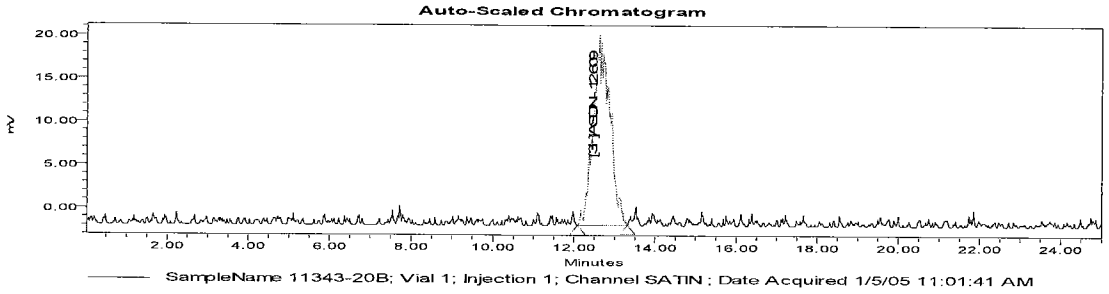
The radiochemical purity of the [^{3}H]ASDN (1:100 dilution in ethanol) was determined using high performance liquid chromatography (HPLC) and liquid scintillation counting. The HPLC system consists of a Waters 2690 Separations Module, a Waters 2487 Dual λ Absorbance Detector and a β-RAM Model 3 flow-through 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 [3H]ASDN



Conclusion

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

Battelle
The Business of Innovation

ANALYTICAL CHEMISTRY ACTIVITIES REPORT

LINDANE

CAS No.: 58-89-9

Lot No.: 14419EB (Sigma Aldrich)

Receipt Date: 1/6/05

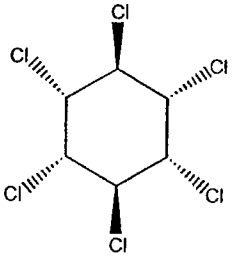
Amount Received: 10 g

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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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.

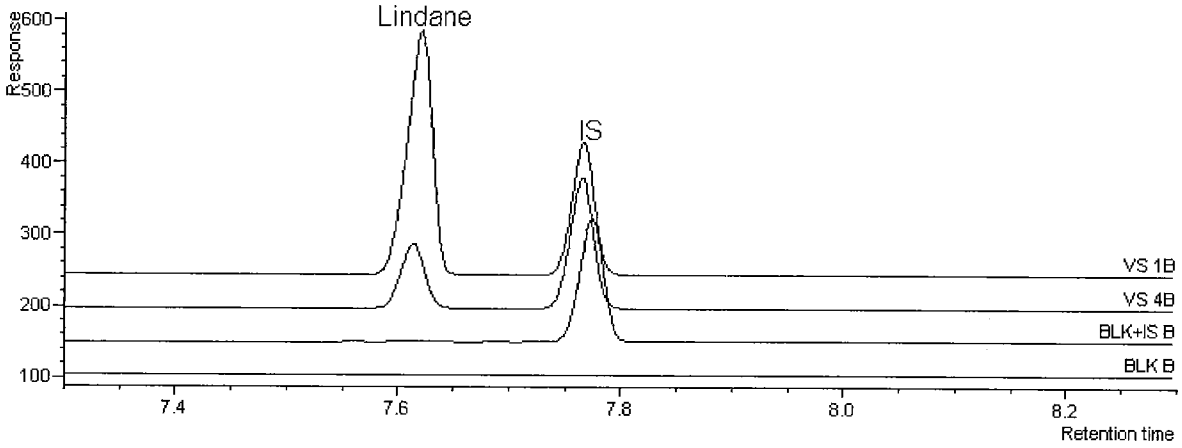

Figure 2 – Representative Overlaid Chromatograms from a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from the Validation (Shown Top to Bottom)

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.

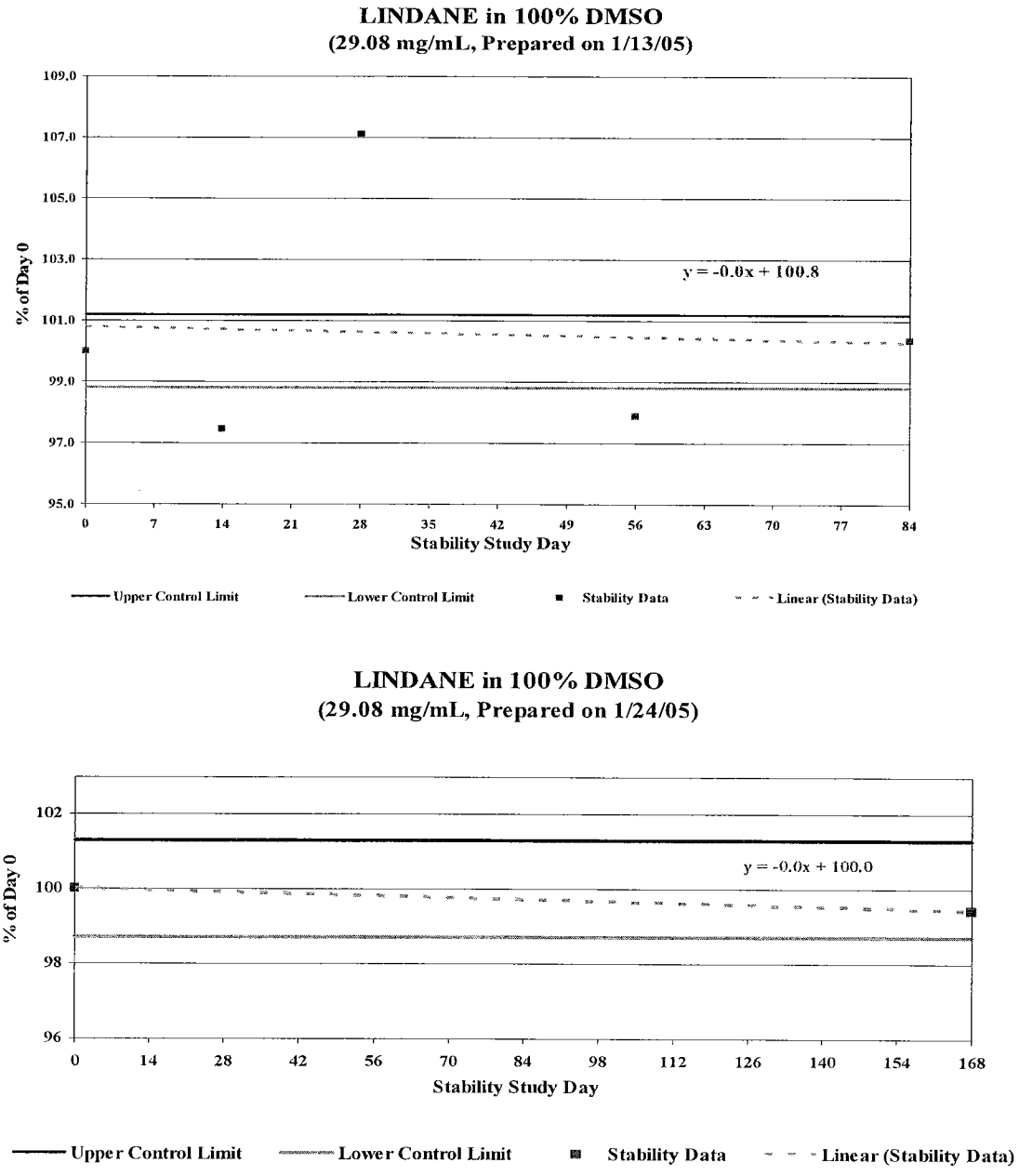

Figure 3 – Control Chart for the Storage Stability Study

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.

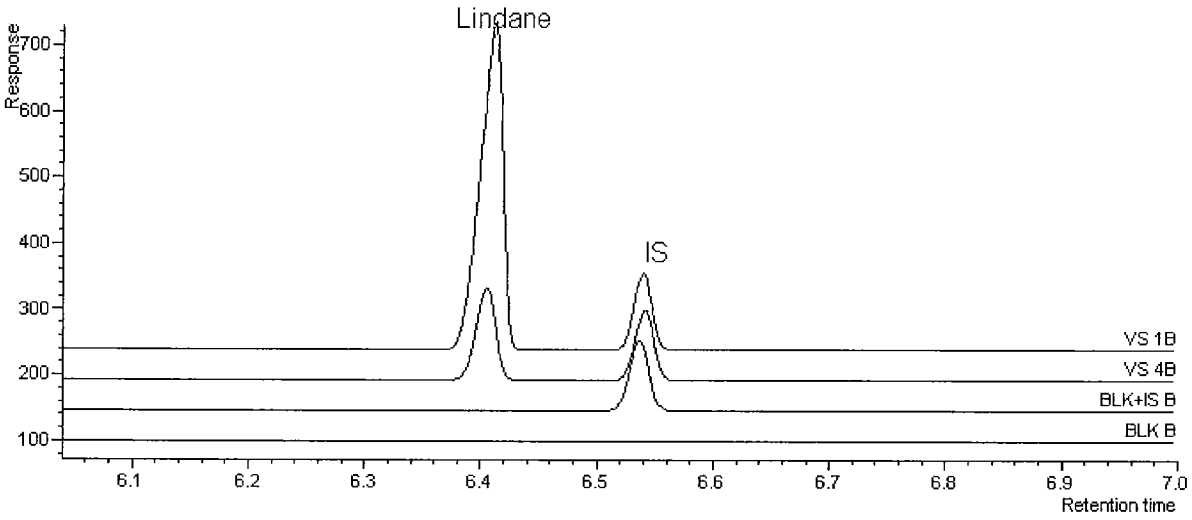

Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from a Formulation Analysis (Shown Top to Bottom)

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.

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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

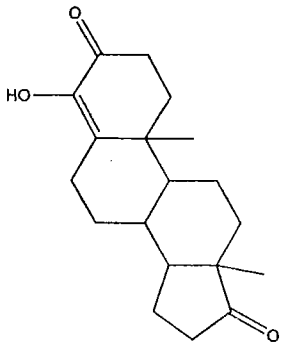
Amount Received: 3.1 g

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

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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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.

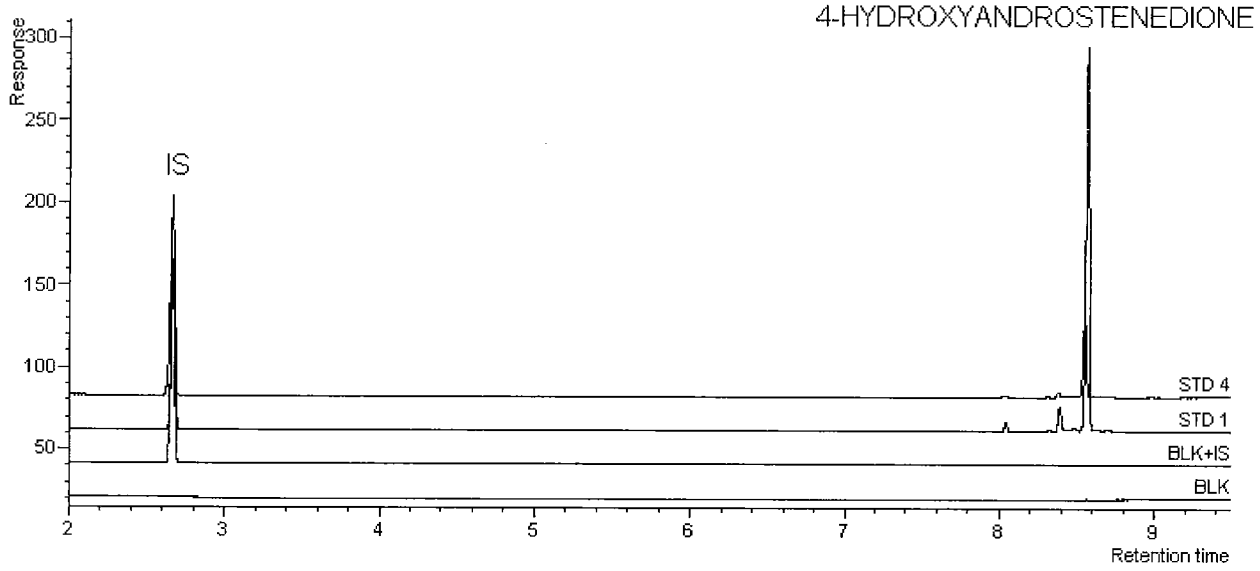

Figure 2 – Representative Overlaid Chromatograms from a Low and High Vehicle/Calibration Standard, Blank with IS, and Blank from the Validation (Shown Top to Bottom)

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.

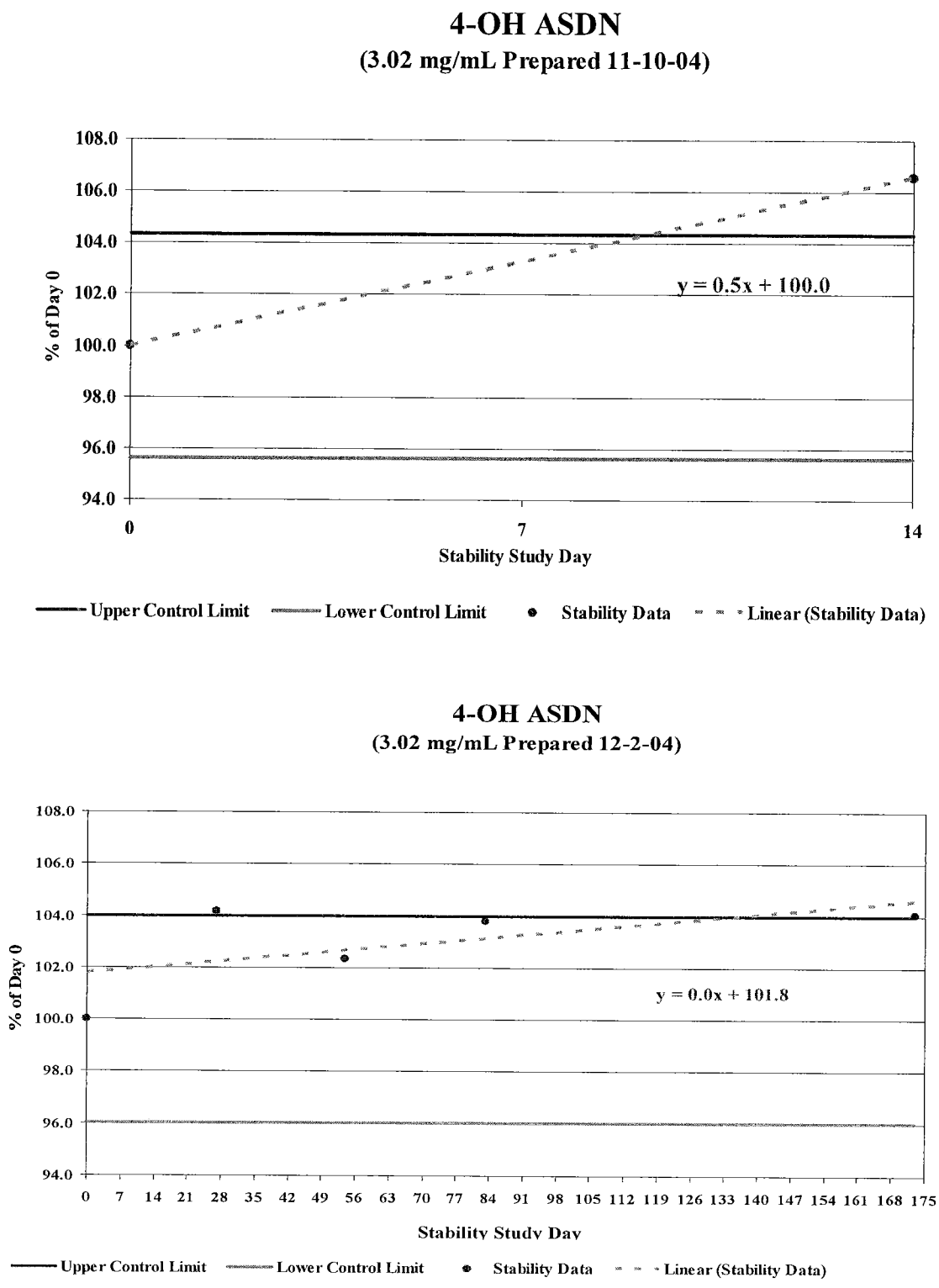

Figure 3 – Control Charts for the Storage Stability Studies

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.

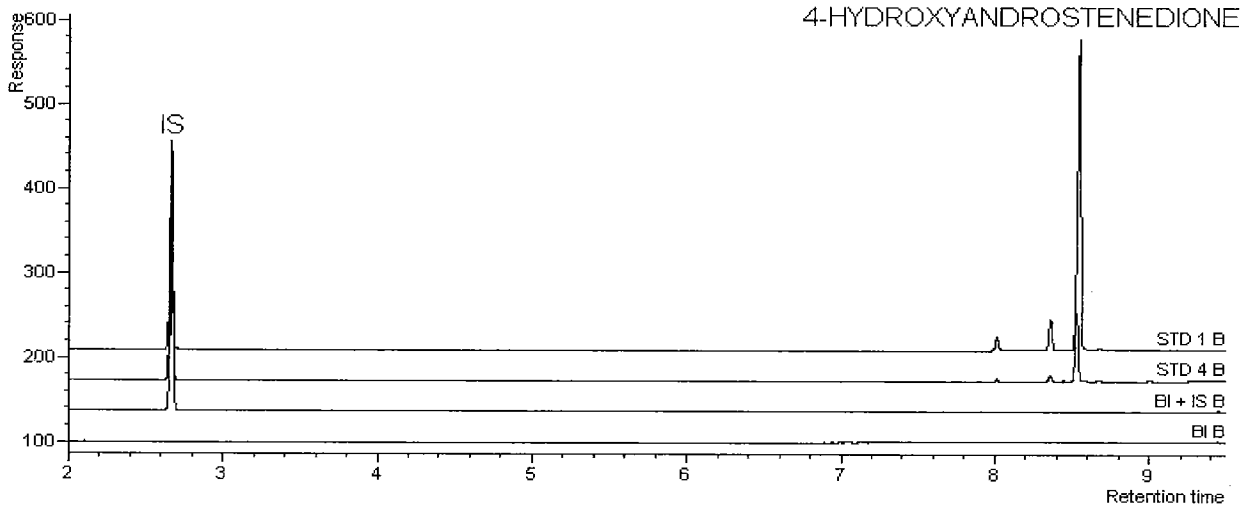

Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from Formulation Analysis

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 $\leq$ 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.

Appendix 8: Statistician Report

DRAFT REPORT

PREPARATION AND CHARACTERIZATION OF HUMAN PLACENTAL MICROSOMES

INTRALABORATORY STATISTICAL ANALYSIS OF IN VITRO TECHNOLOGIES, INC DATA

EPA CONTRACT NUMBER 68-W-01-023
WORK ASSIGNMENT 4-16, TASK 6

January 24, 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 In Vitro Technologies, Inc. Data

EPA CONTRACT NUMBER 68-W-01-023
WORK ASSIGNMENT 4-16, TASK 6

Ying-Liang Chou, Author _____ Date _____

Paul I. Feder, Reviewer _____ Date _____

Offsite Quality Assurance Statement

Printed: 1/23/200611:25:27AM

Study Number:270114509

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 Battelle Task Leader/Battelle Management	Date Reported to Offsite Study Director / Management
Audit study file	1/11/2006	1/11/2006	1/11/2006
Audit draft report	1/11/2006	1/11/2006	1/11/2006

Huang Kelly

Quality Assurance Unit

1-25-06

Date

This report discusses the methods and results of the intralaboratory statistical analysis of the In Vitro Technologies 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 or In Vitro Technologies (IVT) and were analyzed by IVT.

Summary and Conclusions

Three types of data were analyzed: 4-OH ASDN positive control inhibition, aromatase activity, and protein concentration. IVT developed the data discussed in this report. IVT analyzed IVT prepared microsomes and determined 4-OH ASDN positive control inhibition curve fits, aromatase activity levels, and protein concentrations. IVT also analyzed Battelle prepared microsomes and determined aromatase activity levels and protein concentrations. For the inhibition concentration data there were two independent replicates, with microsomes prepared by IVT. For the aromatase activity data there were four independent replicates with microsomes prepared by IVT and two independent replicates with microsomes prepared by Battelle. For the protein concentration data there were six independent replicates with microsomes prepared by IVT and four independent replicates with microsomes prepared by Battelle. 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 the $\log_{10}IC_{50}$ the 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.

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. 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.

3. 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.

4. 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 IVT prepared microsomes.

Introduction and Background

In Task 6 of the Placental Aromatase Validation Study, Battelle and In Vitro Technologies (IVT) 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.

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 IVT, based on the microsomes prepared by IVT and on those prepared by Battelle.

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 are displayed in Table A-2.

Aromatase Activity Data

There are four types of aromatase activity data: full enzyme activity control, background activity control, positive, and negative controls. The microsomes were prepared by Battelle or IVT. There were four independent replicates of IVT tests with IVT prepared microsomes. Two replicates corresponded to the inhibition curve tests and two replicates corresponded to the aromatase activity tests. These were two independent replicates of IVT tests with Battelle produced microsomes. Four repeat determinations were made in each replicate. The background corrected aromatase activity values were used 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 IVT on microsomes prepared by Battelle and

on microsomes prepared by IVT. Protein concentration determinations were made in the inhibition curve tests (IVT 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 IVT prepared microsomes and four replicate protein concentration determinations for the protein corresponding to the Battelle 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 IVT 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 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 concentration (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 activity 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 of 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 laboratory (the laboratory which prepared the microsomes) and the random effect was replicate within laboratory. 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 and in intermediate calculations.

Statistical Analysis Results

Concentration Response Inhibition Curve

Concentration response inhibition curves were fitted separately to the individual repetitions within each replicate and to the averages of the repetitions at each inhibitor 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 and 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 3 has a slightly higher estimated IC_{50} and a slightly more negative slope (Table 1), but the differences are very small.

The parameters of the mean concentration response curve, based on a random effects analysis of variance model fit with replicate as a random effect, are displayed in Table 1. The overall 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 are graphed, in Figure 3 for $\log_{10}IC_{50}$ and in Figure 4 for slope. In Figure 3, replicate 2 is seen to have a lower IC_{50} than the average and replicate 3 is seen to have a higher IC_{50} than the average. The substantially larger width of the of the confidence interval for the overall average in Figure 3 is due to having just one degree of freedom to estimate replicate-to-replicate variation. In Figure 4, replicates 2 and 3 show that their slopes were very close to the overall average.

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 is more than nine times the individual replicate within-replicate variances. For slope the replicate-to-replicate variation is zero while the individual replicate within-replicate variances are close to zero.

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 the 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. 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 3% and 4% lower than at the beginning for replicates 2 and 3 respectively (Figure 9). The standard error of the average of these differences was about 2.05 %. The difference between the beginning and the end, averaged across replicates, were not significant (p=0.1370). The estimated repetition variation was 8.4064 and was larger than the variation among replicates. The estimated variances for replicate and portion by replicate interaction were zero.

For the background activity controls the averages of the two measurements at the end were approximately 1% and 0.1% higher than at the beginning for replicates 2 and 3 respectively (Figure 10). The standard error of the average of these differences was about 0.33 %. The difference between the beginning and the end, averaged across replicates, was not significant (p=0.2455).
The estimated portion by replicate interaction and repetition variations were 0.0464 and 0.1301 respectively. The replicate variation was 0, by definition.

For the positive controls, the averages of the two measurements at the end were approximately 3% and 3.5% lower than at the beginning for replicates 2 and 3 respectively (Figure 11). The standard error of the average of these differences was about 2.85 %. The difference between the beginning and the end, averaged across replicates, was not significant (p=0.3139).
The estimated repetition variation was 16.1894 and was larger than the replicate variation, 5.1708. There was no portion by replicate interaction variation.

For the negative controls, the averages of the two measurements at the end were approximately 3.5% and 4.5% lower than at the beginning for replicates 2 and 3 respectively (Figure 12). The standard error of the average of these differences was about 0.72 %. The difference between the beginning and the end, averaged across replicates, was statistically significant (p=0.0024). The estimated replicate variation was 6.6706, which was larger than the repetition variation, 1.0401. There was no portion by replicate interaction variation.

With the exception of the negative controls the variation among repetitions within replicates was substantially larger than the variation among replicates.

Aromatase Activity

Mixed effects analysis of variance was carried out on the aromatase activity levels for each of the four control types (full enzyme activity, background activity, positive, and negative controls) pooled across the inhibition curve tests (In Vitro Technologies prepared microsomes) and the aromatase activity tests (In Vitro Technologies and Battelle prepared microsomes). The fixed effect was microsome source (the laboratory which prepared the microsomes) and the random effect was replicate within laboratory. The residual variation was based on the repetition within replicate effect. The response was aromatase activity (nmol/mg protein/min). Summary statistics are displayed in Table 5. Scatter plots for each of the control types are presented in Figures 13 to 16. Table 6 shows that the variance components were close to zero for each of the four control types. For each of the control types analysis of variance tests for microsome source effects were not significant at the 0.05 level.

Protein Concentration Determination

Table 7 displays protein concentration summary statistics and the results of a two-sample t-test to compare microsome sources. The two-sample t-test shows that the Battelle microsomes had significant higher protein concentrations than the In VitroTechnologies microsomes ($p=0.0062$). On average the protein concentrations in the Battelle microsomes were more than three times higher than that in the IVT 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 In Vitro Technologies.

Replicate	$Log_{10}IC_{50}$ (SE)	IC_{50} (GSE)[d]	Slope (SE)	Status
Individual Values[a]				
2	-7.259 (0.012)	$5.508x10^{-8}$ (1.028)	-0.982 (0.024)	C
3	-7.203 (0.012)	$6.265x10^{-8}$ (1.029)	-0.989 (0.023)	C
Mean[c]	-7.231 (0.028)	$5.875x10^{-8}$ (1.067)	-0.986 (0.017)	--
Average Values[b]				
2	-7.257 (0.012)	$5.531x10^{-8}$ (1.027)	-0.982 (0.023)	C
3	-7.201 (0.013)	$6.291x10^{-8}$ (1.030)	-0.982 (0.024)	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 In Vitro Technologies.

Parameter	Estimate (95% CI)		
	Replicate 2[a]	Replicate 3[a]	Mean[b]
$Log_{10}IC_{50}$	-7.259 (-7.285, -7.233)	-7.203 (-7.229, -7.177)	-7.231 (-7.587, -6.875)
Slope	-0.982 (-1.032, -0.932)	-0.989 (-1.038, -0.940)	-0.986 (-1.019, -0.952)

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 In Vitro Technologies.

Parameter	Variance/Degree of Freedom[a,b]			
	Replicate 2	Replicate 3	Overall	
			Among Replicate (p-value)[d]	Variance of Mean[c,e]
$Log_{10}IC_{50}$	0.000147 /df=16	0.000150 /df=16	0.001420 /df=1 (p=0.261)	0.000784 /df=1.00
Slope	0.000555 /df=16	0.000534 /df=16	0 /df=1 (p=NA)	0.000272 /df=31.99

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 among replicate variation were estimated based on a one-way heterogeneous variance 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 $2*((1/K)* \sum(S_r^2 + S_i^2))^2/(var(S_r^2) +(2/K^2)* \sum(Si^4 /d f_i))$, where S_r^2 is the among 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 Controls, Background Activity Controls, Positive Controls, And Negative Controls. Portion Effects and Variation Across Replicates of Portion Effects Within Replicates. In Vitro Technologies Inhibition Curve Analysis Controls (2 Replicates). Microsomes Prepared by In Vitro Technologies.

Parameter	Difference Between Beginning and End Portions (Beginning Minus End)		Variance Components[a]		
	Estimate (%) (Std. Error)	p-Value/ Degree of Freedom	Replicate	Replicate* Portion	Residual (Repetition)
Full Enzyme Activity Control	3.518 (2.050)	0.1370/ df=6	0	0	8.4064
Background Activity Control	-0.543 (0.334)	0.2455/ df=2	0	0.0464	0.1301
Positive Control	3.184 (2.845)	0.3139/ df=5	5.1708	0	16.1894
Negative Control	4.069 (0.721)	0.0024/ df=5	6.6706	0	1.0401

a. For background and full enzyme activity control responses the among replicate variation is constrained to be 0, since within each replicate percent of controls are constrained to sum to 0 and 100 respectively.

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	0.0423	0.0013
			2	4	0.0506	0.0020
	IVT	Aromatase Activity	1	4	0.0338	0.0028
			2	4	0.0342	0.0009
		Inhibition Curve Fit	2	4	0.0462	0.0019
			3	4	0.0385	0.0011
Background Activity Control	Battelle	Aromatase Activity	1	4	-1.863×10^{-19}	5.884×10^{-05}
			2	4	-1.355×10^{-20}	1.882×10^{-04}
	IVT	Aromatase Activity	1	4	1.084×10^{-19}	9.756×10^{-04}
			2	4	-4.608×10^{-19}	7.503×10^{-04}
		Inhibition Curve Fit	2	4	1.220×10^{-19}	2.733×10^{-04}
			3	4	-2.643×10^{-19}	1.581×10^{-04}
Positive Control	Battelle	Aromatase Activity	1	4	0.0222	0.0012
			2	4	0.0297	0.0012
	IVT	Aromatase Activity	1	4	0.0143	0.0010
			2	4	0.0182	0.0011
		Inhibition Curve Fit	2	4	0.0222	0.0013
			3	4	0.0202	0.0019
Negative Control	Battelle	Aromatase Activity	1	4	0.0433	0.0012
			2	4	0.0558	0.0008
	IVT	Aromatase Activity	1	4	0.0297	0.0011
			2	4	0.0380	0.0012
		Inhibition Curve Fit	2	4	0.0484	0.0010
			3	4	0.0417	0.0011

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	3.198×10^{-6} /df=18	3.3×10^{-5} /df=4 (p=0.0838)	p=0.1750
Background Activity Control	2.255×10^{-7} /df=18	0 /df=4 (p=NA)	p=1.0000
Positive Control	1.767×10^{-6} /df=18	1.5×10^{-5} /df=4 (p=0.0846)	p=0.1036
Negative Control	1.166×10^{-6} /df=18	6.5×10^{-5} /df=4 (p=0.0796)	p=0.2213

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 analysis of variance 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	22.478	4	24.545	5.014	0.0062
		2	19.379				
	Protein Determination	1	31.187				
		2	25.136				
IVT	Control Activity	1	8.951	6	7.815	0.927	
		2	8.086				
	Curve Fit	2	6.172				
		3	7.545				
	Protein Determination	2	8.202				
		3	7.934				

a. Two-sample t-test with unequal variances and Satterthwaite degrees of freedom, 3.14

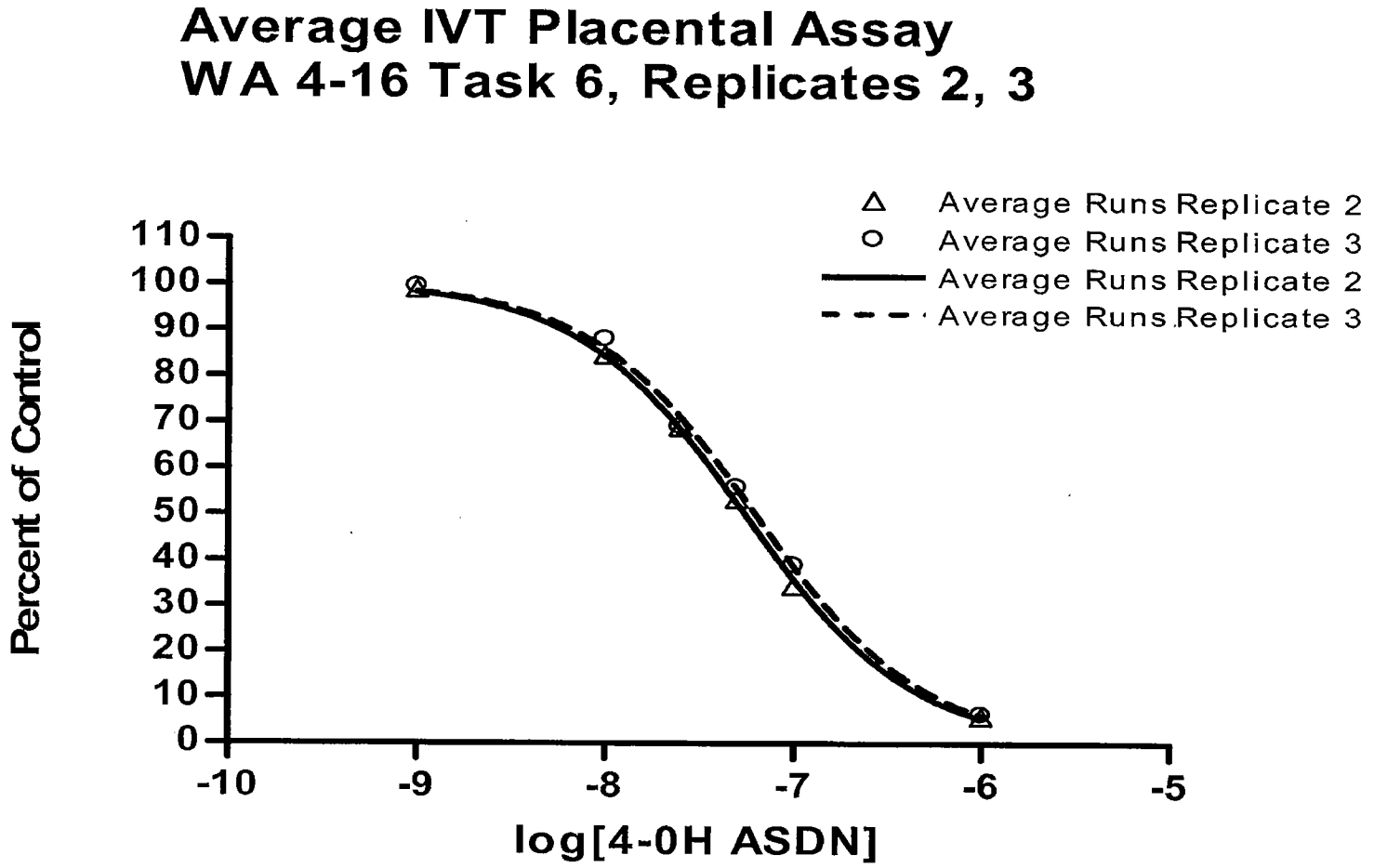

Figure 1. Concentration Response Curves and Averages of Repetitions Within 4-OH ASDN Concentrations. Placental Aromatase Assay. By Replicate. Microsomes Prepared by In Vitro Technologies.

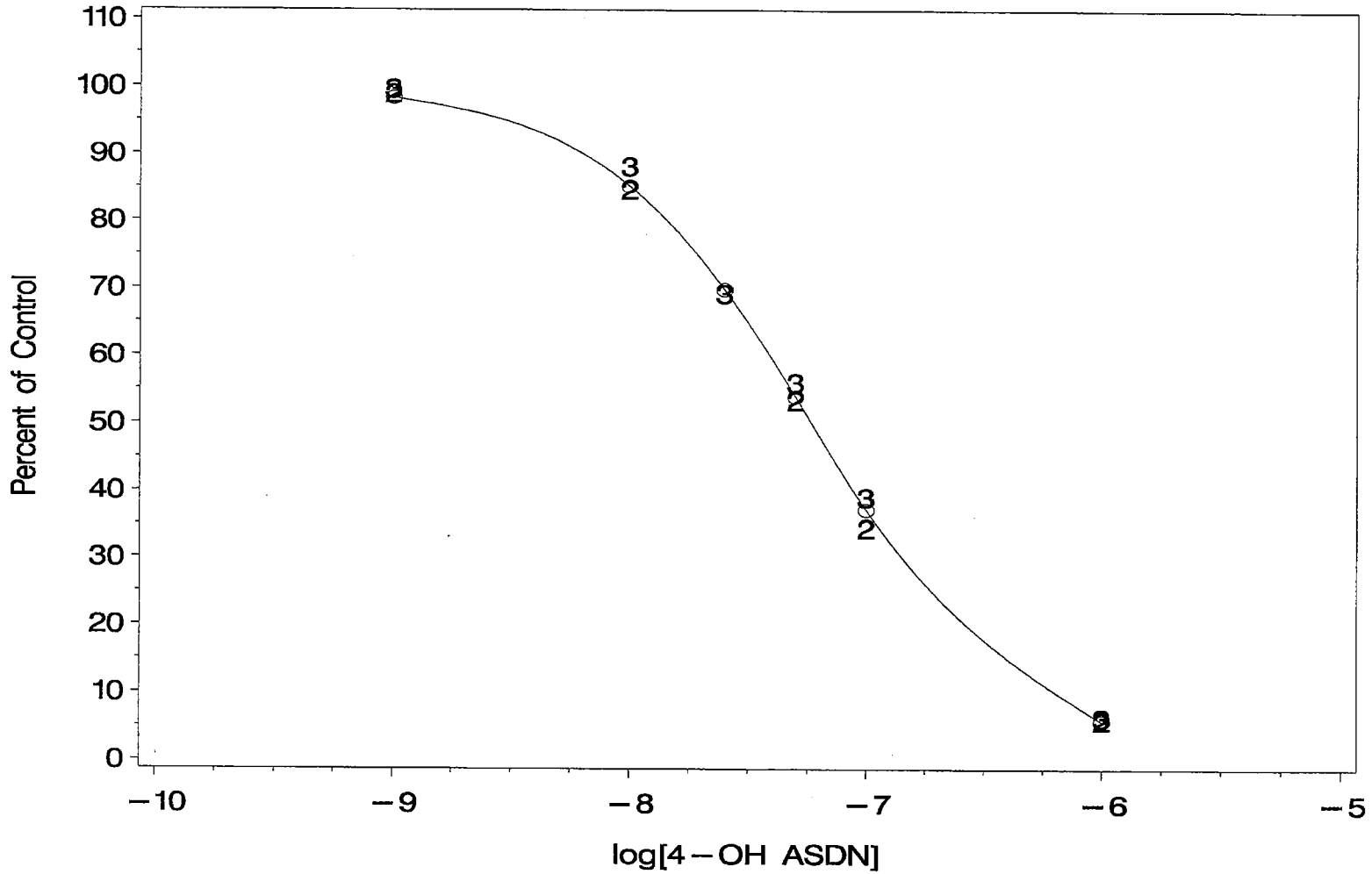

Figure 2. Overall Average Concentration Response Curve Across Replicates and Average Responses Across Repetitions Within 4-OH ASDN Concentrations. Placental Aromatase Assay. Parameters of Average Curve Based on One-Way Analysis of Variance Across Replicate Parameter Values. Microsomes Prepared by In Vitro Technologies.

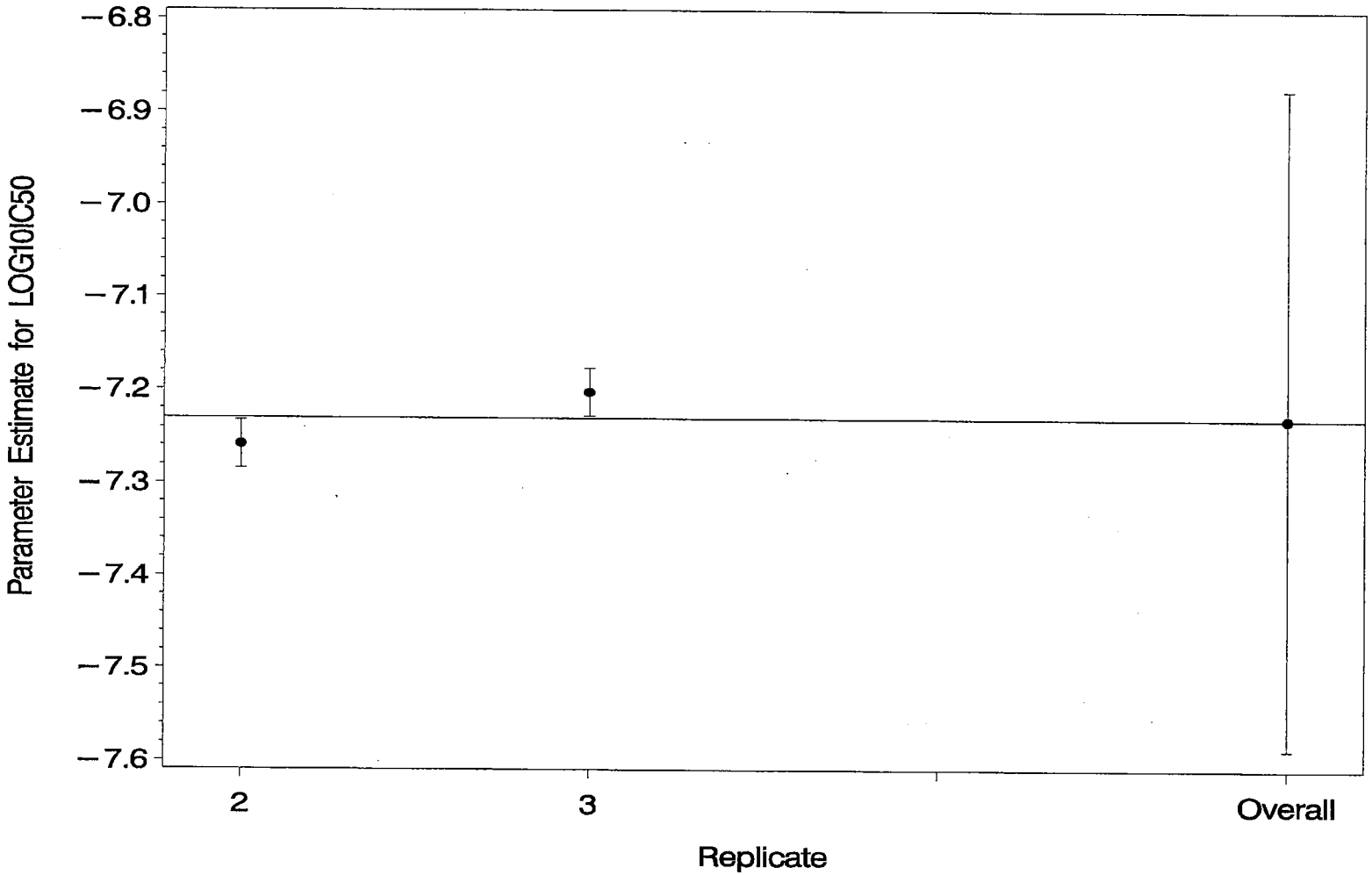

Figure 3. $Log_{10}IC_{50}$ Parameter Estimates and Their Associated 95% Confidence Intervals for Each Replicate and Averaged Across Replicates. Placental Aromatase Assay. The Solid Reference Line Corresponds to the Average Across Replicates. Microsomes Prepared by In Vitro Technologies.

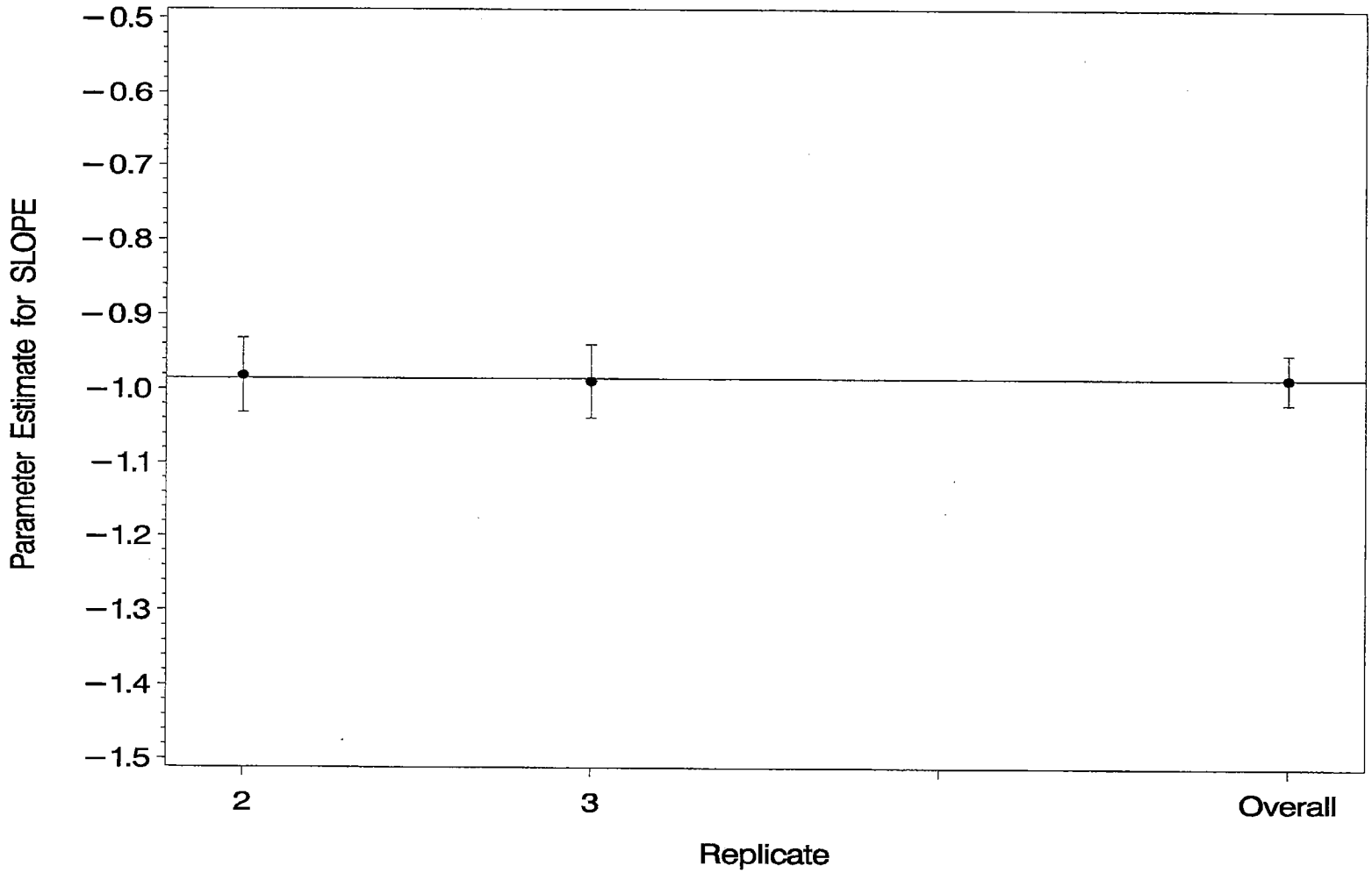

Figure 4. **Slope (β) Parameter Estimates and Their Associated 95% Confidence Intervals for Each Replicate and Averaged Across Replicates. Placental Aromatase Assay. The Solid Reference Line Corresponds to the Average Across Replicates. Microsomes Prepared by In Vitro Technologies.**

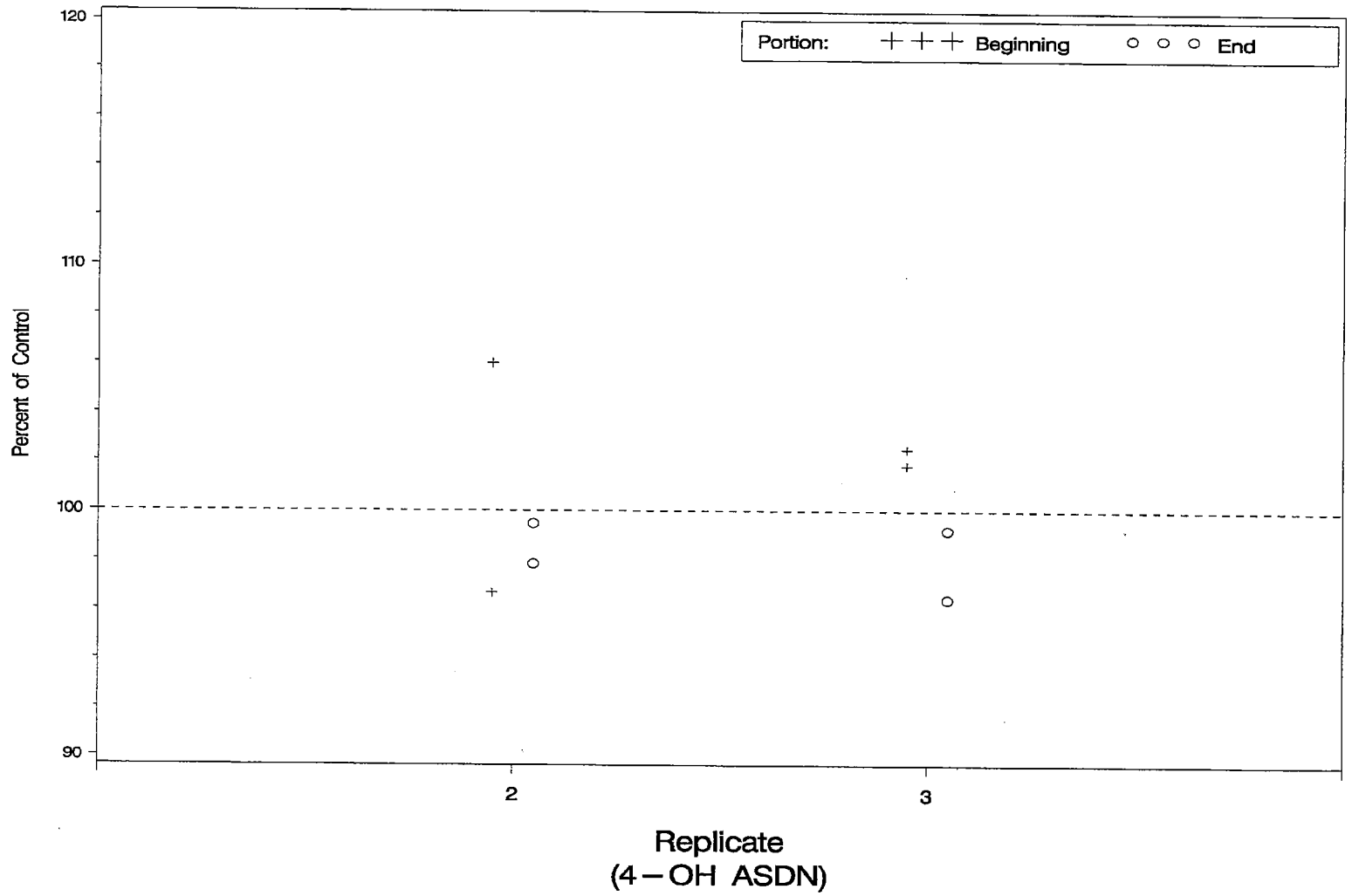

Figure 5. Full Enzyme Activity Control Data Associated with Inhibition Concentration Tests. Percent of Control by Replicate and Portion of Replicate (Beginning or End). Placental Aromatase Assay. Reference Line at 100%. Microsomes Prepared by In Vitro Technologies.

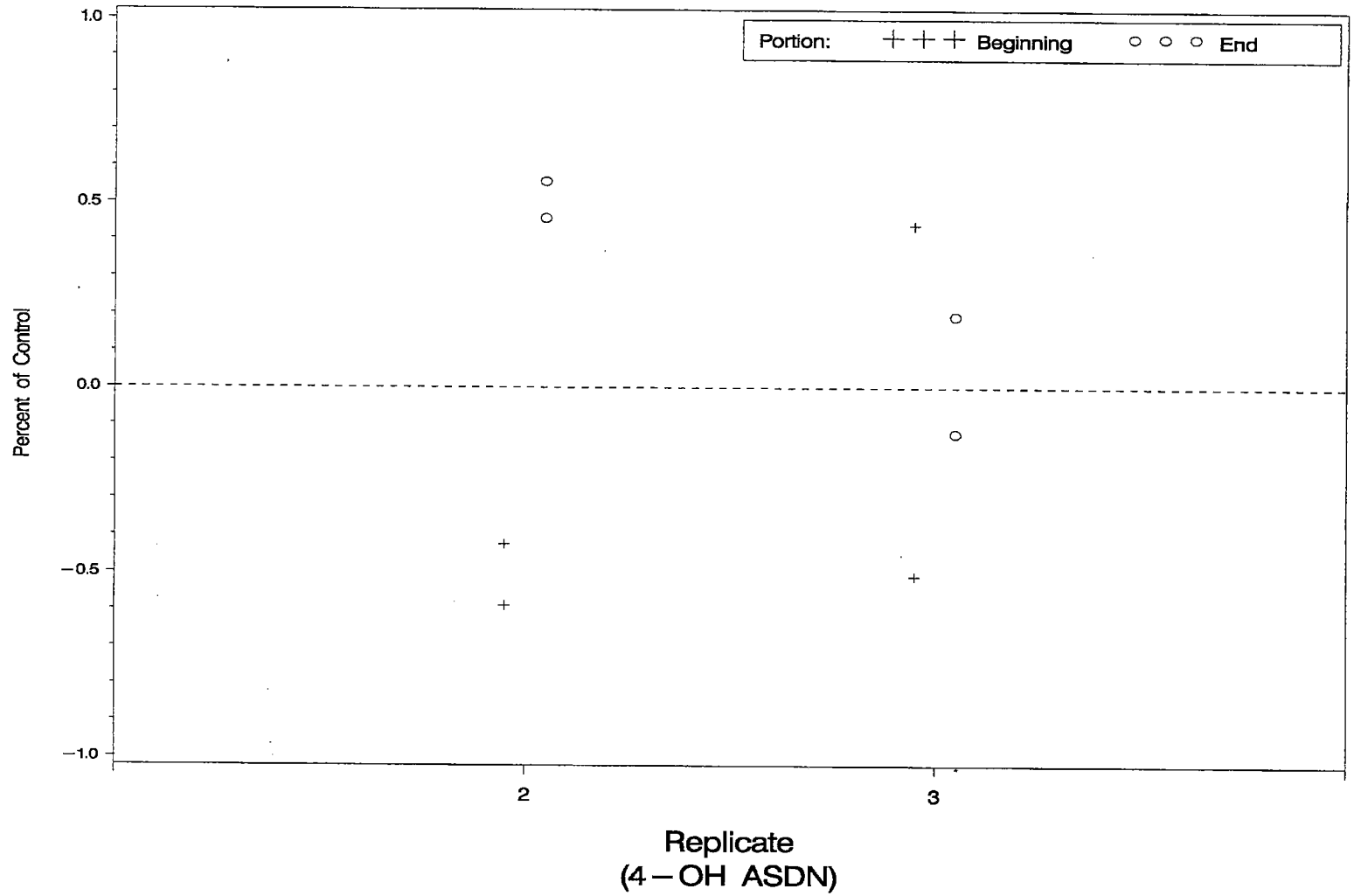

Figure 6. **Background Activity Control Data Associated with Inhibition Concentration Tests. Percent of Control by Replicate and Portion of Replicate (Beginning or End). Placental Aromatase Assay. Reference Line at 0%. Microsomes Prepared by In Vitro Technologies.**

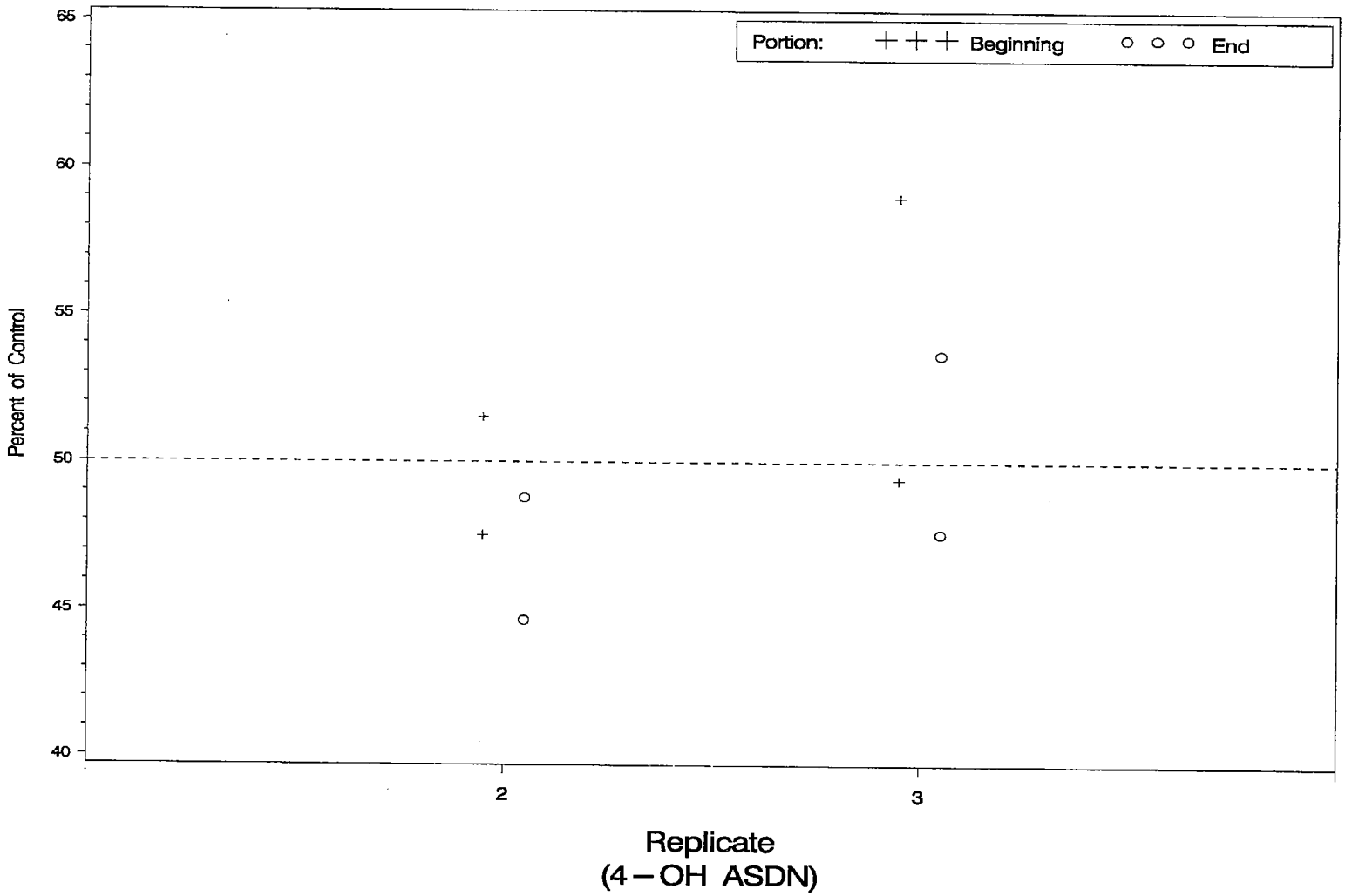

Figure 7. Positive Control Data Associated with Inhibition Concentration Tests. Percent of Control by Replicate and Portion of Replicate (Beginning or End). Placental Aromatase Assay. Reference Line at 50%. Microsomes Prepared by In Vitro Technologies.

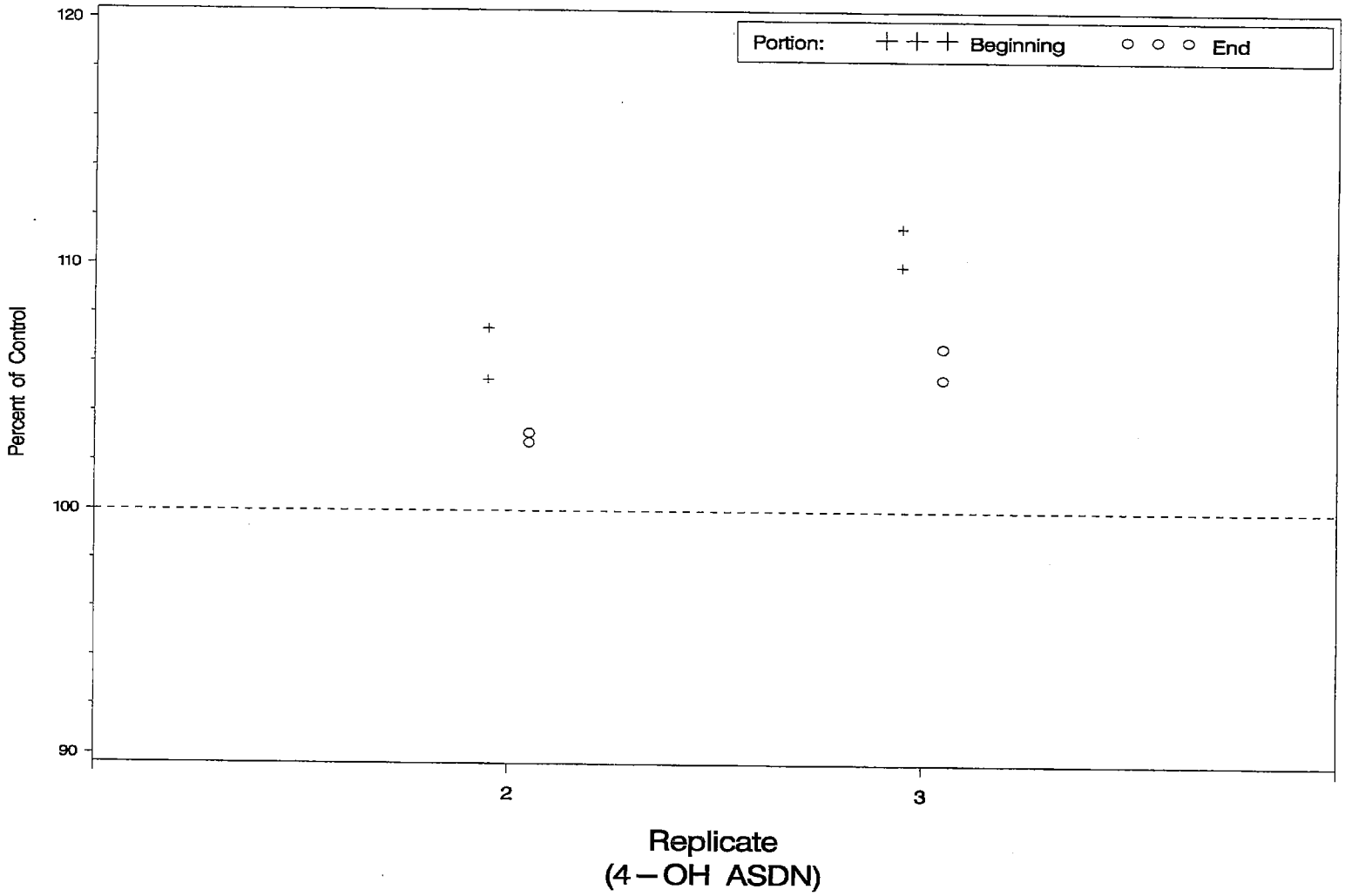

Figure 8. **Negative Activity Control Data Associated with Inhibition Concentration Tests. Percent of Control by Replicate and Portion of Replicate (Beginning or End). Placental Aromatase Assay. Reference Line at 100%. Microsomes Prepared by In Vitro Technologies.**

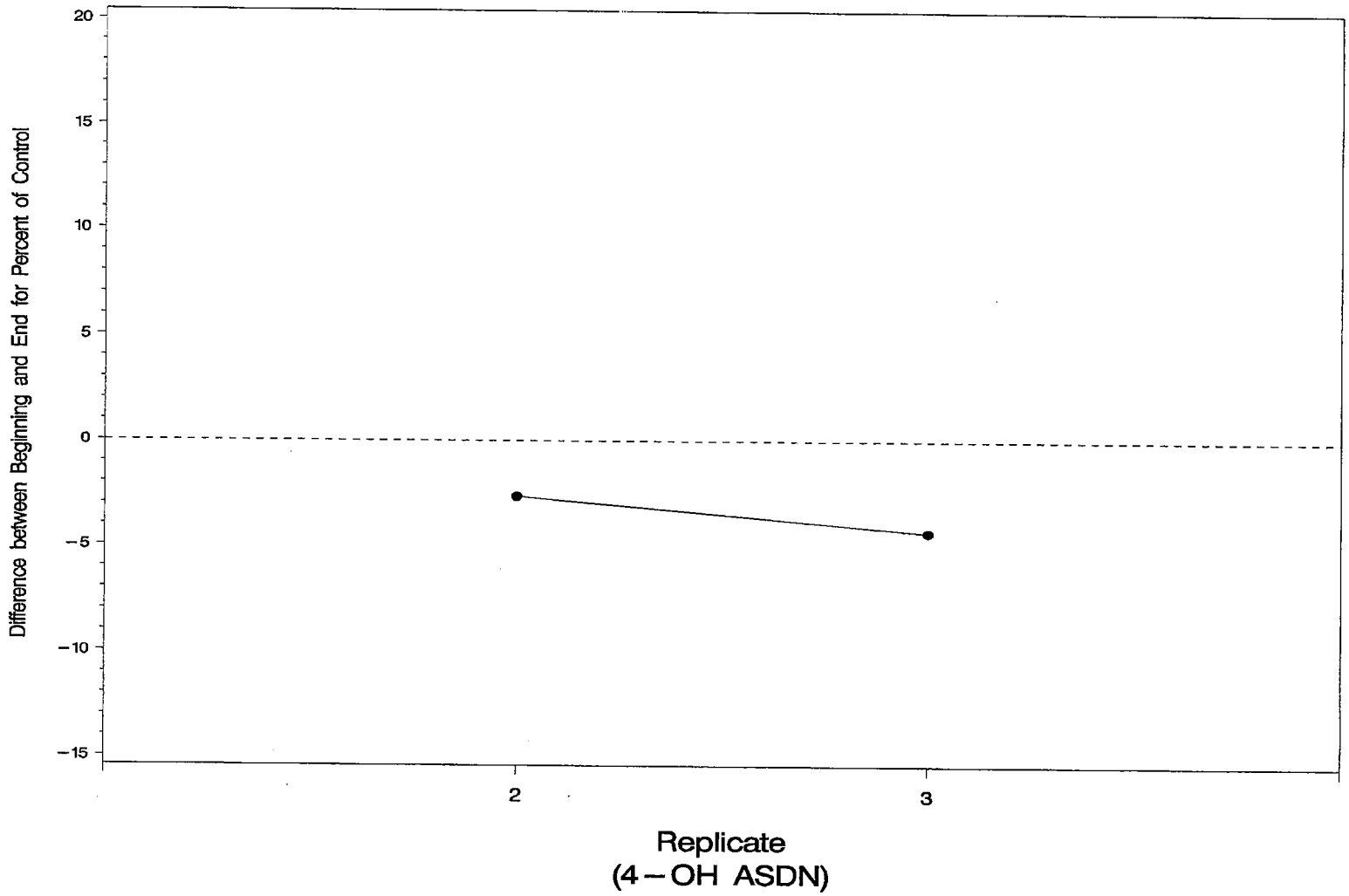

Figure 9. Full Enzyme Activity Control Data Associated with Inhibition Concentration Tests. Difference Between the Average of the Two End Percent of Control Responses and the Average of the Two Beginning Responses (End Minus Beginning). By Replicate. Reference Line at 0%. Microsomes Prepared by In Vitro Technologies.

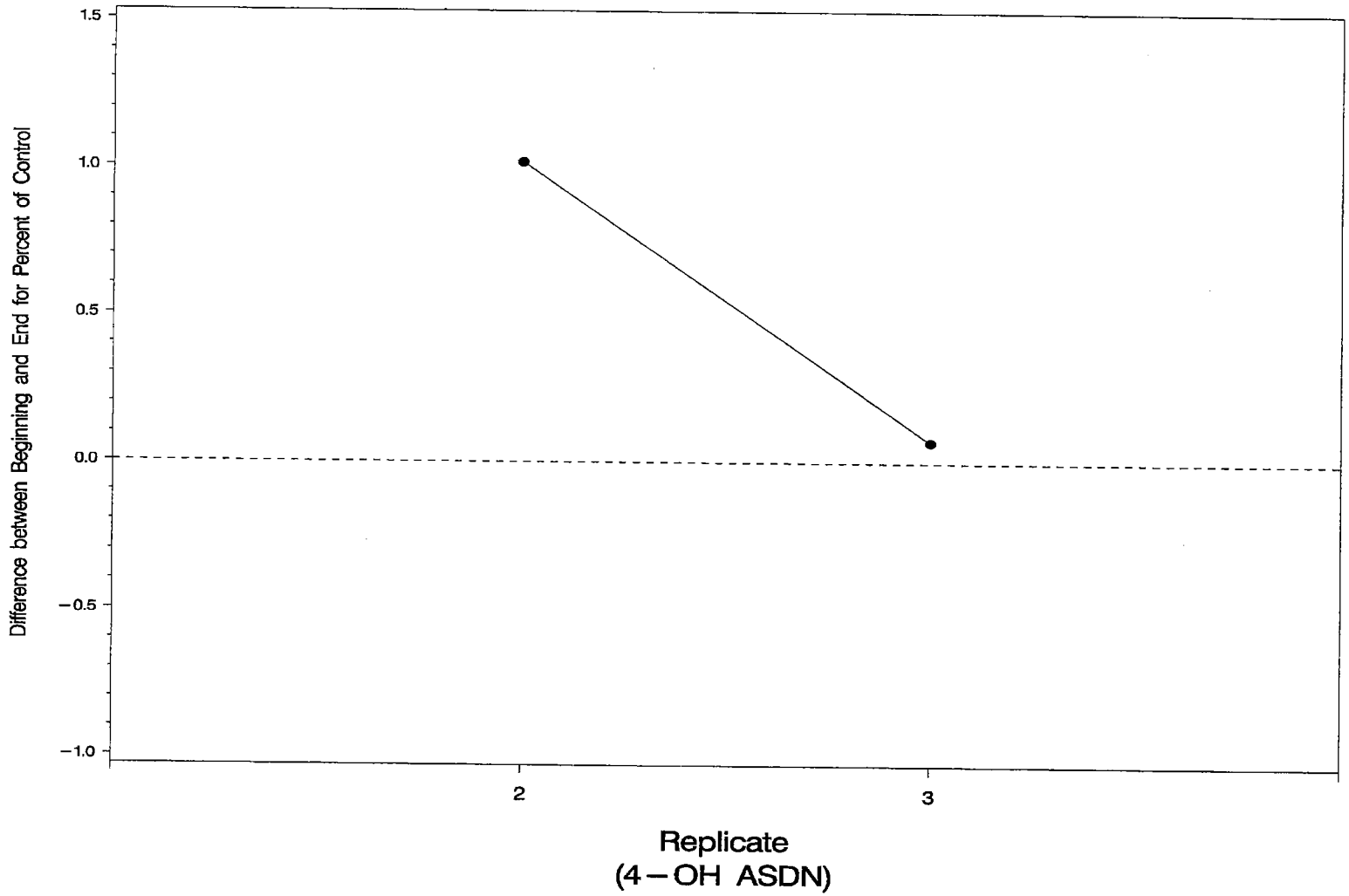

Figure 10. Background Activity Control Data Associated with Inhibition Concentration Tests. Difference Between the Average of the Two End Percent of Control Responses and the Average of the Two Beginning Responses (End Minus Beginning). By Replicate. Reference Line at 0%. Microsomes Prepared by In Vitro Technologies.

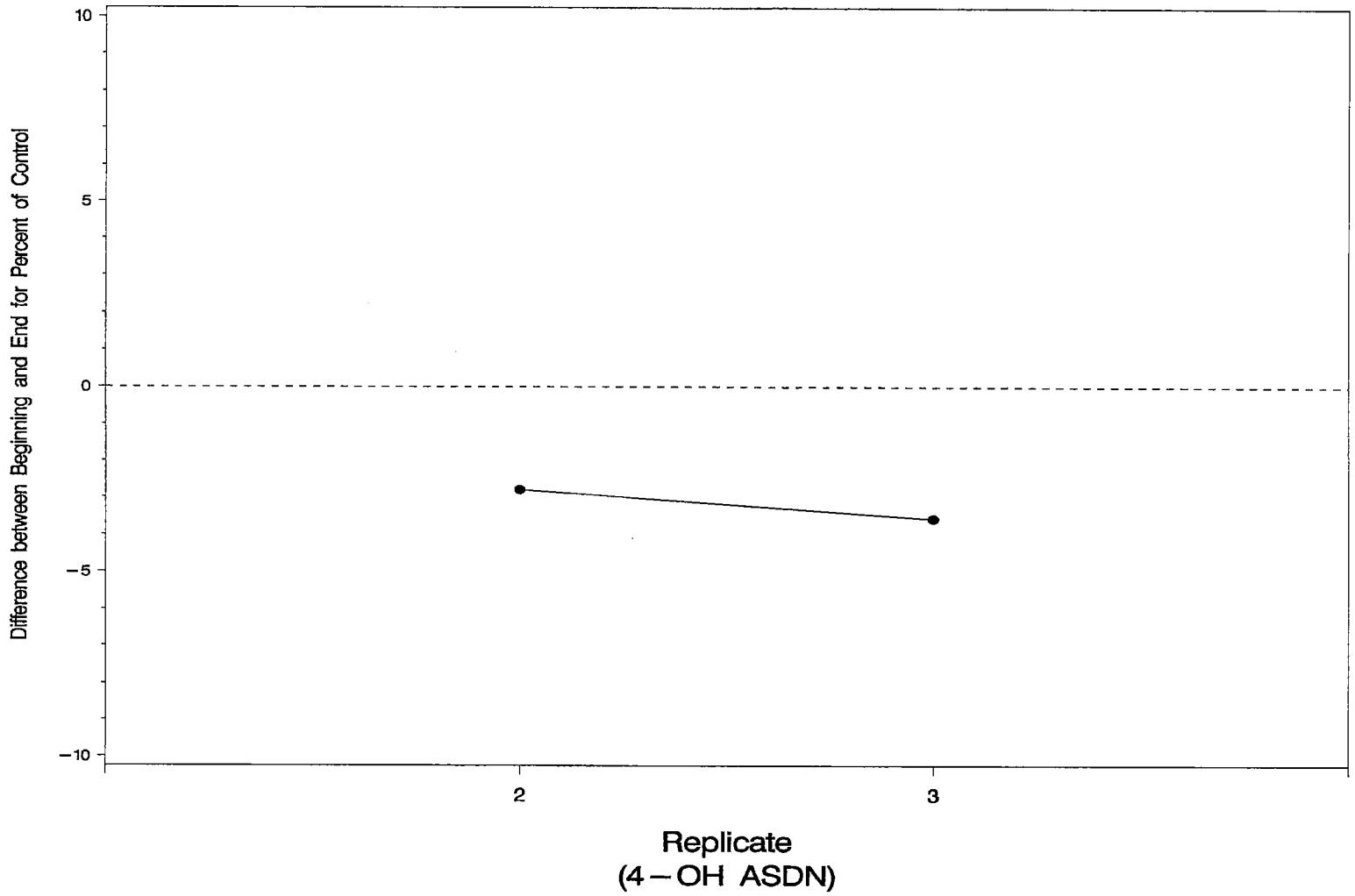

Figure 11. Positive Control Data Associated with Inhibition Concentration Tests. Difference Between the Average of the Two End Percent of Control Responses and the Average of the Two Beginning Responses (End Minus Beginning). By Replicate. Reference Line at 0%. Microsomes Prepared by In Vitro Technologies.

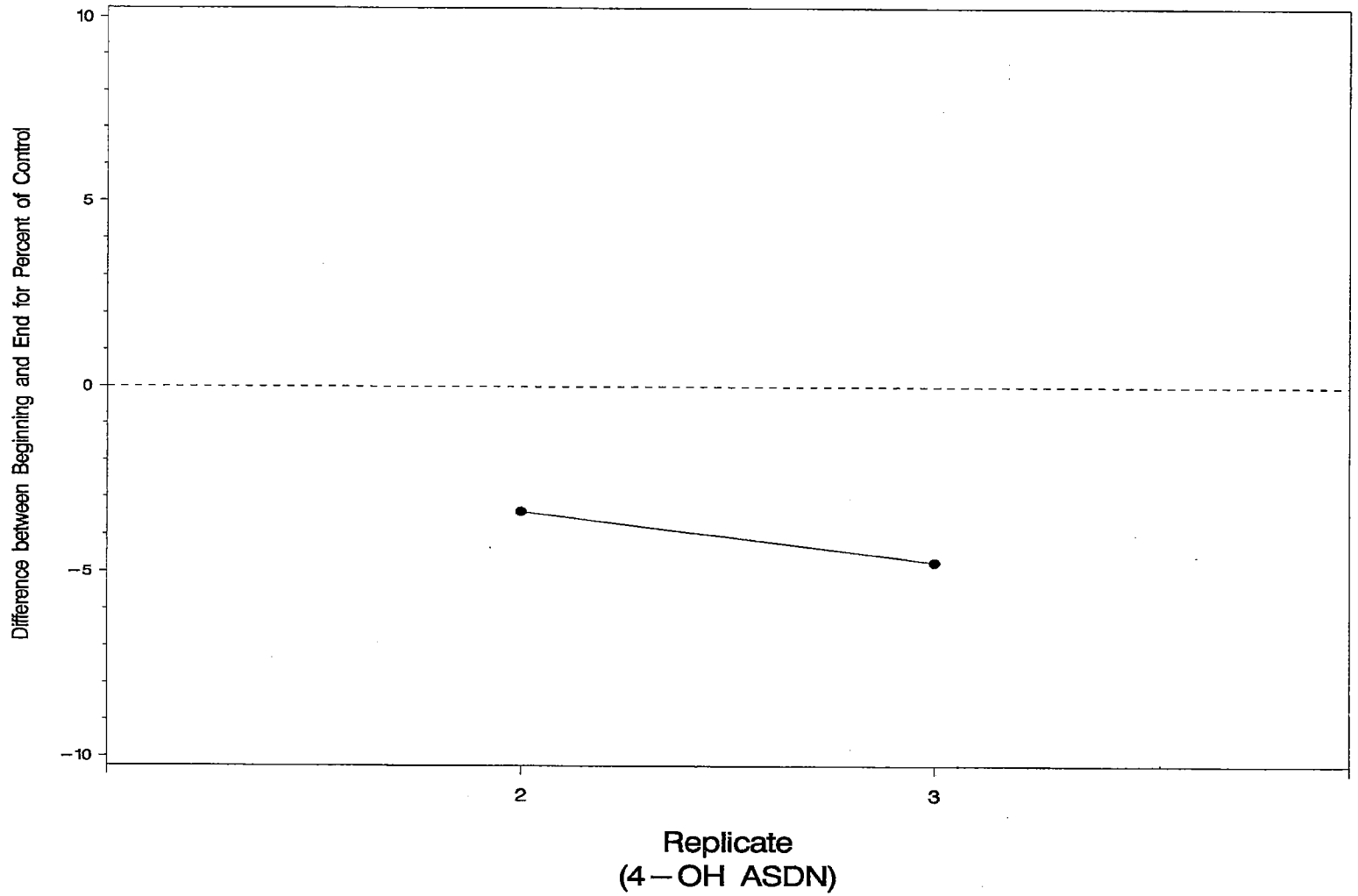

Figure 12. **Negative Control Data Associated with Inhibition Concentration Tests. Difference Between the Average of the Two End Percent of Control Responses and the Average of the Two Beginning Responses (End Minus Beginning). By Replicate. Reference Line at 0%. Microsomes Prepared by In Vitro Technologies.**

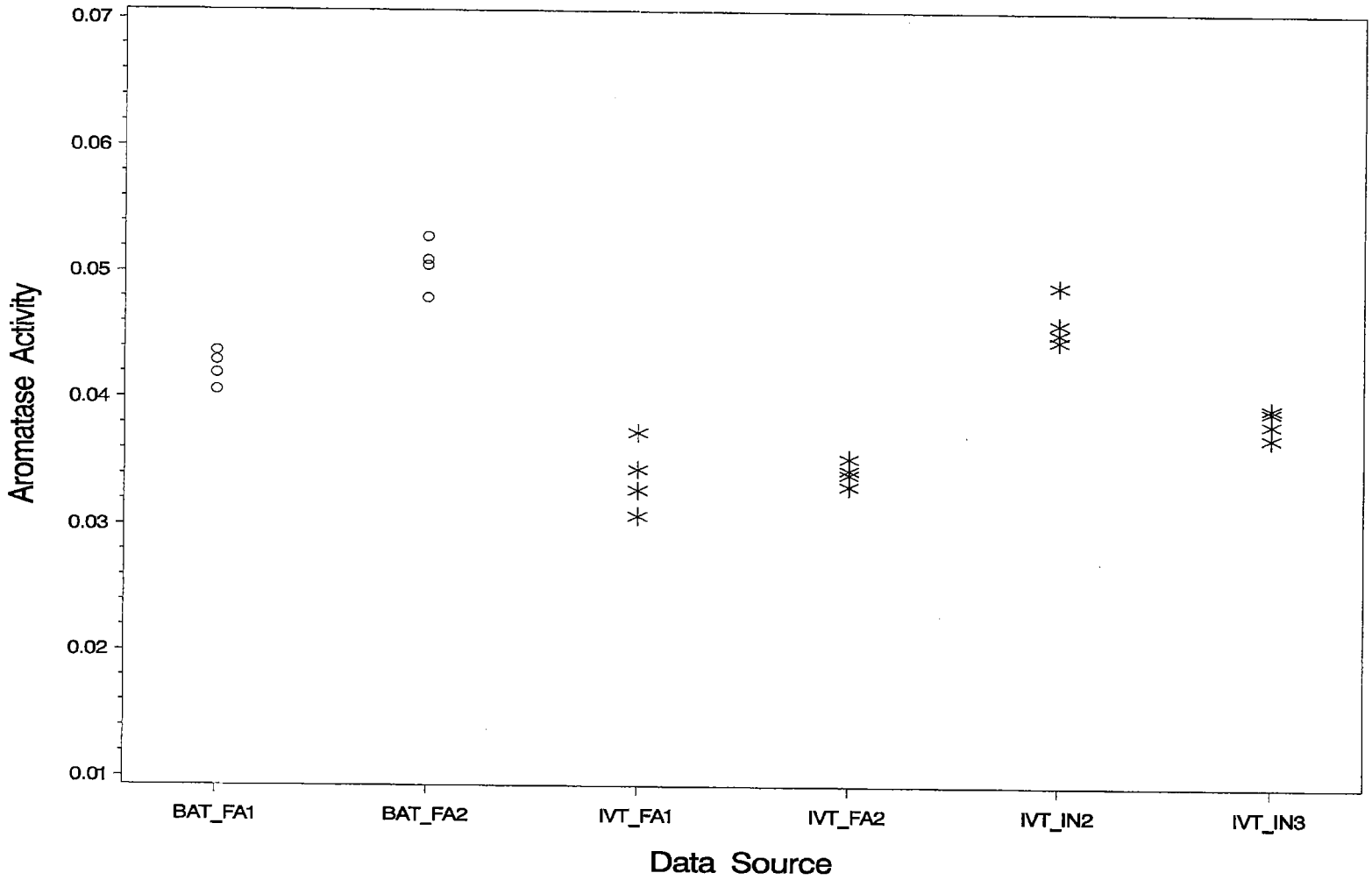

Figure 13. Aromatase Activity (nmol/mg protein/min). Full Enzyme Activity Controls. By Source and Replicate.

Note: **BAT-FA1 and BAT-FA2 – Battelle prepared microsomes, analyzed for aromatase activity. Replicates 1 and 2**
IVT-FA1 and IVT-FA2 – In Vitro prepared microsomes, analyzed for aromatase activity, Replicates 1 and 2
IVT-IN2 and IVT-IN3 – In Vitro prepared microsomes, analyzed for inhibition curve fits, Replicates 2 and 3

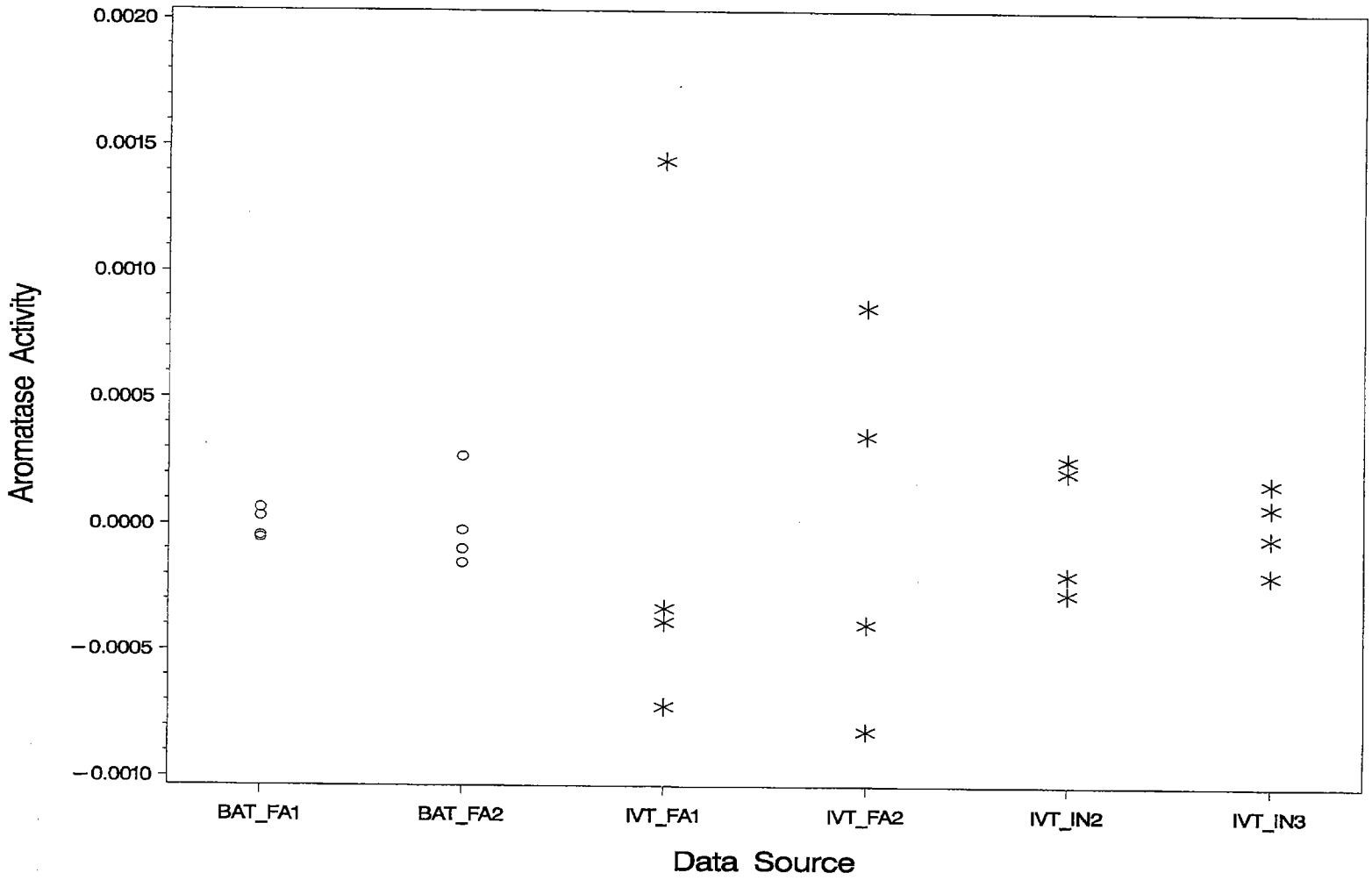

Figure 14. Aromatase Activity (nmol/mg protein/min). Background Activity Controls. By Source and Replicate.

Note: **BAT-FA1 and BAT-FA2 – Battelle prepared microsomes, analyzed for aromatase activity, replicates 1 and 2**
IVT-FA1 and IVT-FA2 – In Vitro prepared microsomes, analyzed for aromatase activity, replicates 1 and 2
IVT-IN2 and IVT-IN3 – In Vitro prepared microsomes, analyzed for inhibition curve fits, replicates 2 and 3

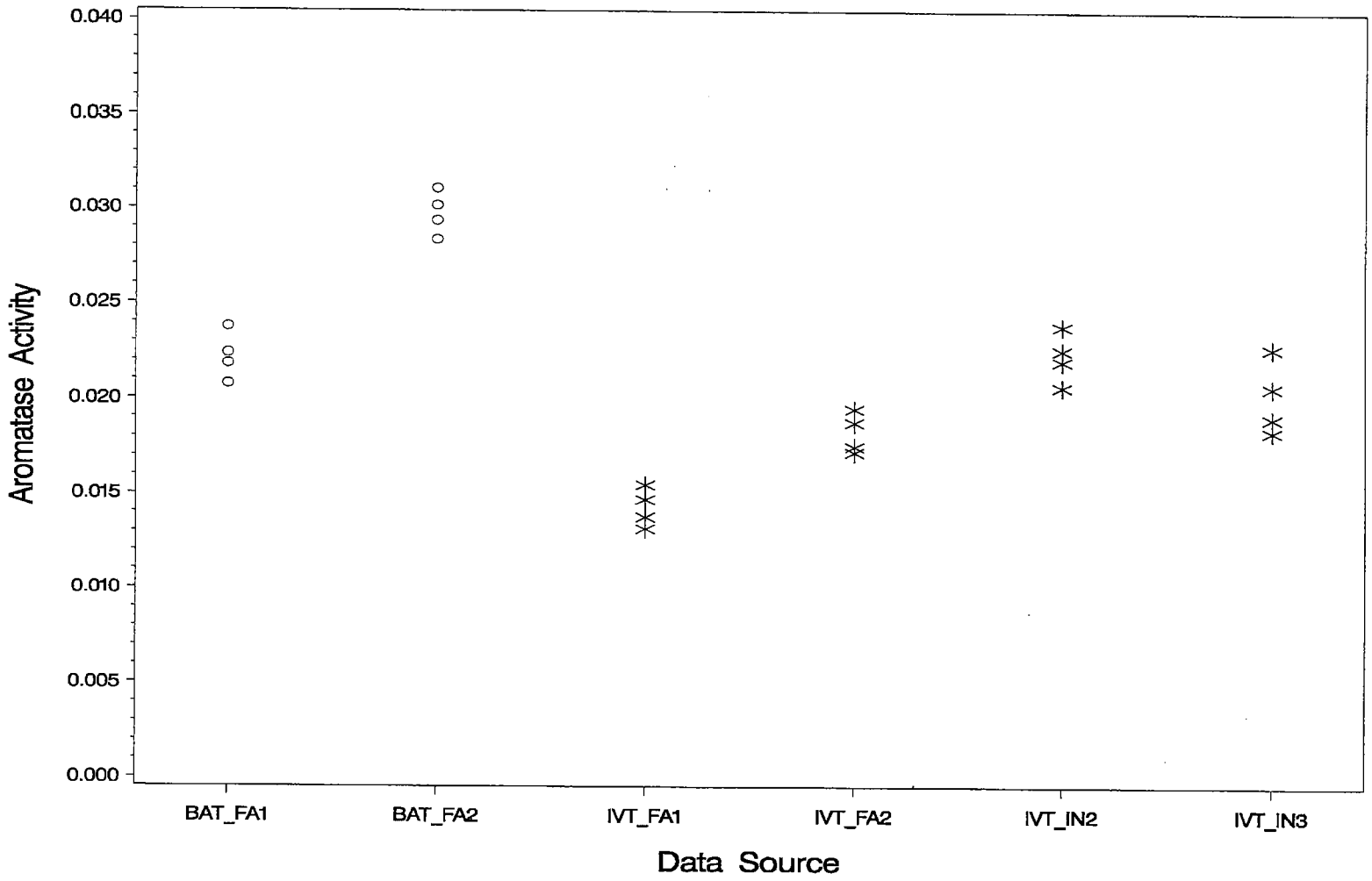

Figure 15. Aromatase Activity (nmol/mg protein/min). Positive Controls. By Source and Replicate.

Note: BAT-FA1 and BAT-FA2 – Battelle prepared microsomes, analyzed for aromatase activity, replicates 1 and 2
IVT-FA1 and IVT-FA2 – In Vitro prepared microsomes, analyzed for aromatase activity, replicates 1 and 2
IVT-IN2 and IVT-IN3 – In Vitro prepared microsomes, analyzed for inhibition curve fits, replicates 2 and 3

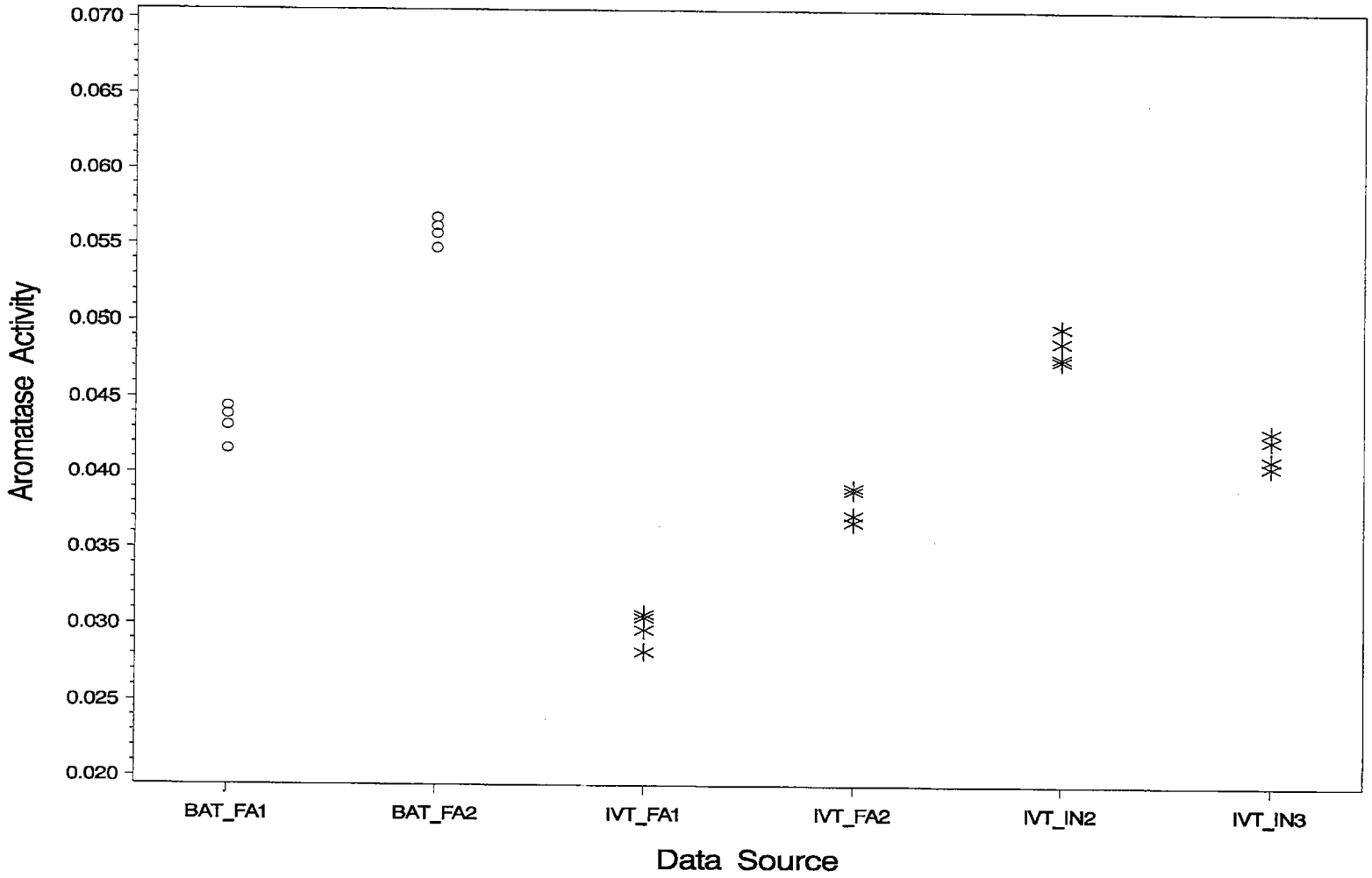

Figure 16. Aromatase Activity (nmol/mg protein/min). Negative Controls. By Source and Replicate.

Note: **BAT-FA1 and BAT-FA2 – Battelle prepared microsomes, analyzed for aromatase activity, replicates 1 and 2**
IVT-FA1 and IVT-FA2 – In Vitro prepared microsomes, analyzed for aromatase activity, replicates 1 and 2
IVT-IN2 and IVT-IN3 – In Vitro prepared microsomes, analyzed for inhibition curve fits, replicates 2 and 3

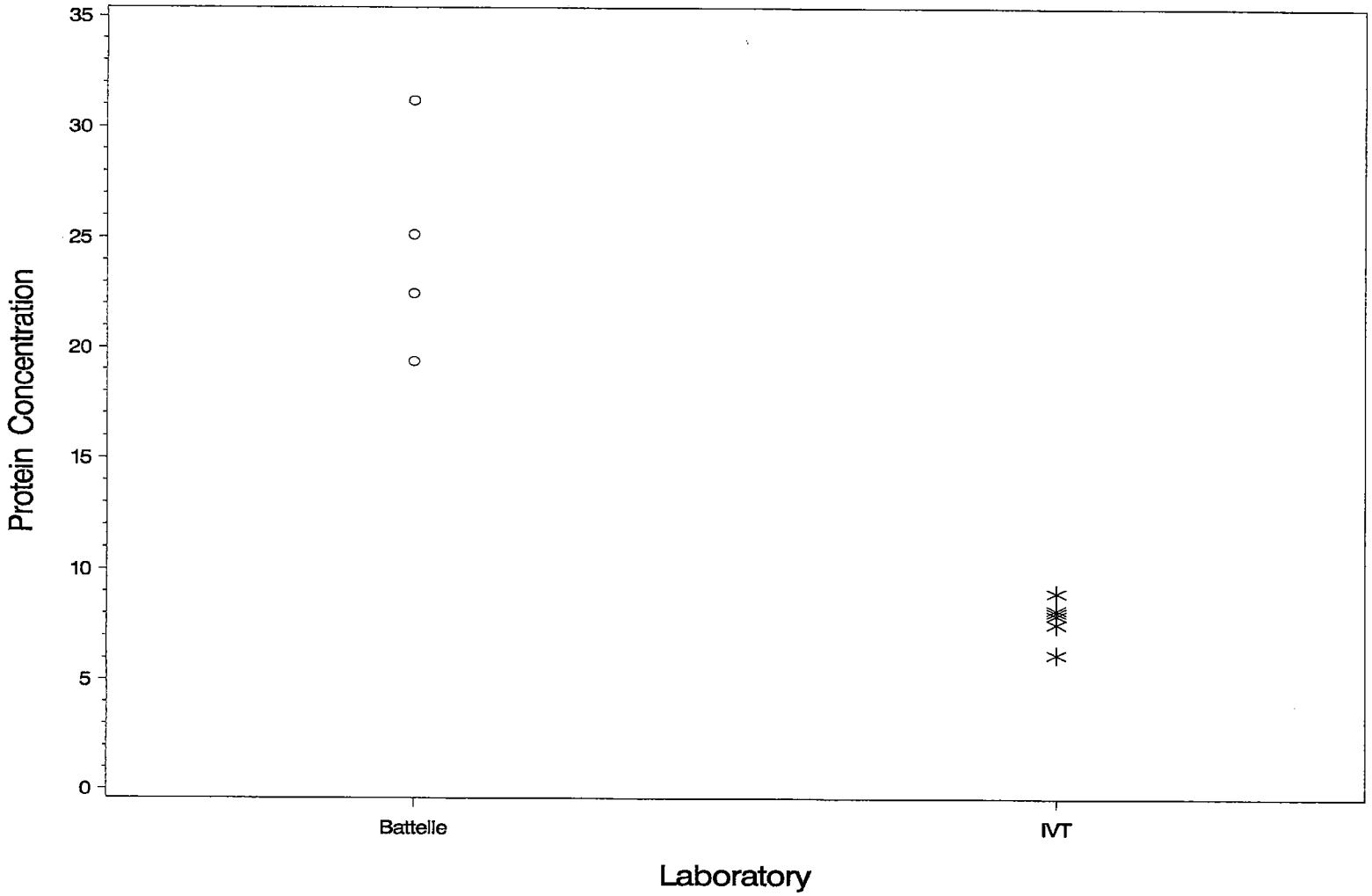

Figure 17. Protein Concentration (mg/mL). By Source.

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 In Vitro Technologies.

Replicate	Log [4-OH ASDN]	Percent of Control[a]		
		Repetition 1	Repetition 2	Repetition 3
2	-6.00	5.72	5.79	6.03
	-7.00	37.05	31.10	35.13
	-7.30	52.78	53.69	53.54
	-7.60	69.17	66.09	71.66
	-8.00	87.47	84.68	81.62
	-9.00	102.11	98.79	98.19
3	-6.00	7.64	5.14	5.88
	-7.00	41.08	38.14	37.72
	-7.30	54.34	55.39	57.99
	-7.60	66.81	69.70	70.91
	-8.00	89.96	86.95	87.18
	-9.00	102.90	99.46	104.33

[a] 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 In Vitro Technologies.

Aromatase Activity	Replicate	Portion	Corrected Aromatase Activity	% of Control[a]
Full Enzyme Activity Control	2	Beginning	0.04468	96.671
		Beginning	0.04899	106.005
		End	0.04522	97.845
		End	0.04598	99.480
	3	Beginning	0.03917	101.843
		Beginning	0.03943	102.517
		End	0.03709	96.425
		End	0.03816	99.215
Background Activity Control	2	Beginning	-0.00020	-0.425
		Beginning	-0.00027	-0.590
		End	0.00021	0.458
		End	0.00026	0.557
	3	Beginning	0.00017	0.440
		Beginning	-0.00020	-0.510
		End	-0.00005	-0.124
		End	0.00007	0.195
Positive Control	2	Beginning	0.02380	51.499
		Beginning	0.02196	47.514
		End	0.02255	48.784
		End	0.02063	44.642
	3	Beginning	0.02268	58.976
		Beginning	0.01900	49.406
		End	0.01830	47.589
		End	0.02063	53.644

Aromatase Activity	Replicate	Portion	Corrected Aromatase Activity	% of Control[a]
Negative Control	2	Beginning	0.04963	107.390
		Beginning	0.04868	105.320
		End	0.04768	103.161
		End	0.04750	102.767
	3	Beginning	0.04290	111.548
		Beginning	0.04230	109.966
		End	0.04053	105.373
		End	0.04102	106.644

a. 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 (nMol/mg protein/min)
Battelle	Aromatase Activity Test	1	1	0.0429
			2	0.0437
			3	0.0406
			4	0.0419
		2	1	0.0479
			2	0.0528
			3	0.0510
			4	0.0505
IVT	Aromatase Activity Test	1	1	0.0373
			2	0.0344
			3	0.0308
			4	0.0328
		2	1	0.0331
			2	0.0353
			3	0.0341
			4	0.0344
	Inhibition Curve Test	2	1	0.0447
			2	0.0490
			3	0.0452
			4	0.0460
		3	1	0.0392
			2	0.0394
			3	0.0371
			4	0.0382

Table A-4. Aromatase Activity of Background Activity Controls. By Microsome Source and Test Type.

Microsomes Prepared By	Data Source	Replicate	Repetition	Aromatase Activity (nMol/mg protein/min)
Battelle	Aromatase Activity Test	1	1	3.338E-05
			2	-4.485E-05
			3	-5.424E-05
			4	6.572E-05
		2	1	-2.144E-05
			2	-9.709E-05
			3	2.706E-04
			4	-1.521E-04
IVT	Aromatase Activity Test	1	1	-3.872E-04
			2	1.440E-03
			3	-7.213E-04
			4	-3.320E-04
		2	1	-3.945E-04
			2	3.532E-04
			3	-8.183E-04
			4	8.596E-04
	Inhibition Curve Test	2	1	-1.965E-04
			2	-2.726E-04
			3	2.116E-04
			4	2.575E-04
		3	1	1.691E-04
			2	-1.962E-04
			3	-4.779E-05
			4	7.495E-05

Table A-5. Aromatase Activity of Positive Controls. By Microsome Source and Test Type.

Microsomes Prepared By	Data Source	Replicate	Repetition	Aromatase Activity (nmol/mg protein/min)
Battelle	Aromatase Activity Test	1	1	0.0237
			2	0.0218
			3	0.0208
			4	0.0224
		2	1	0.0310
			2	0.0293
			3	0.0301
			4	0.0283
IVT	Aromatase Activity Test	1	1	0.0155
			2	0.0138
			3	0.0131
			4	0.0147
		2	1	0.0175
			2	0.0195
			3	0.0172
			4	0.0187
	Inhibition Curve Test	2	1	0.0238
			2	0.0220
			3	0.0225
			4	0.0206
		3	1	0.0227
			2	0.0190
			3	0.0183
			4	0.0206

Table A-6. Aromatase Activity of Negative Controls. By Microsome Source and Test Type.

Microsomes Prepared By	Data Source	Replicate	Repetition	Aromatase Activity (nMol/mg protein/min)
Battelle	Aromatase Activity Test	1	1	0.0432
			2	0.0444
			3	0.0439
			4	0.0416
		2	1	0.0568
			2	0.0562
			3	0.0548
			4	0.0557
IVT	Aromatase ActivityTest	1	1	0.0306
			2	0.0296
			3	0.0304
			4	0.0282
		2	1	0.0367
			2	0.0390
			3	0.0372
			4	0.0388
	Inhibition Curve Test	2	1	0.0496
			2	0.0487
			3	0.0477
			4	0.0475
		3	1	0.0429
			2	0.0423
			3	0.0405
			4	0.0410

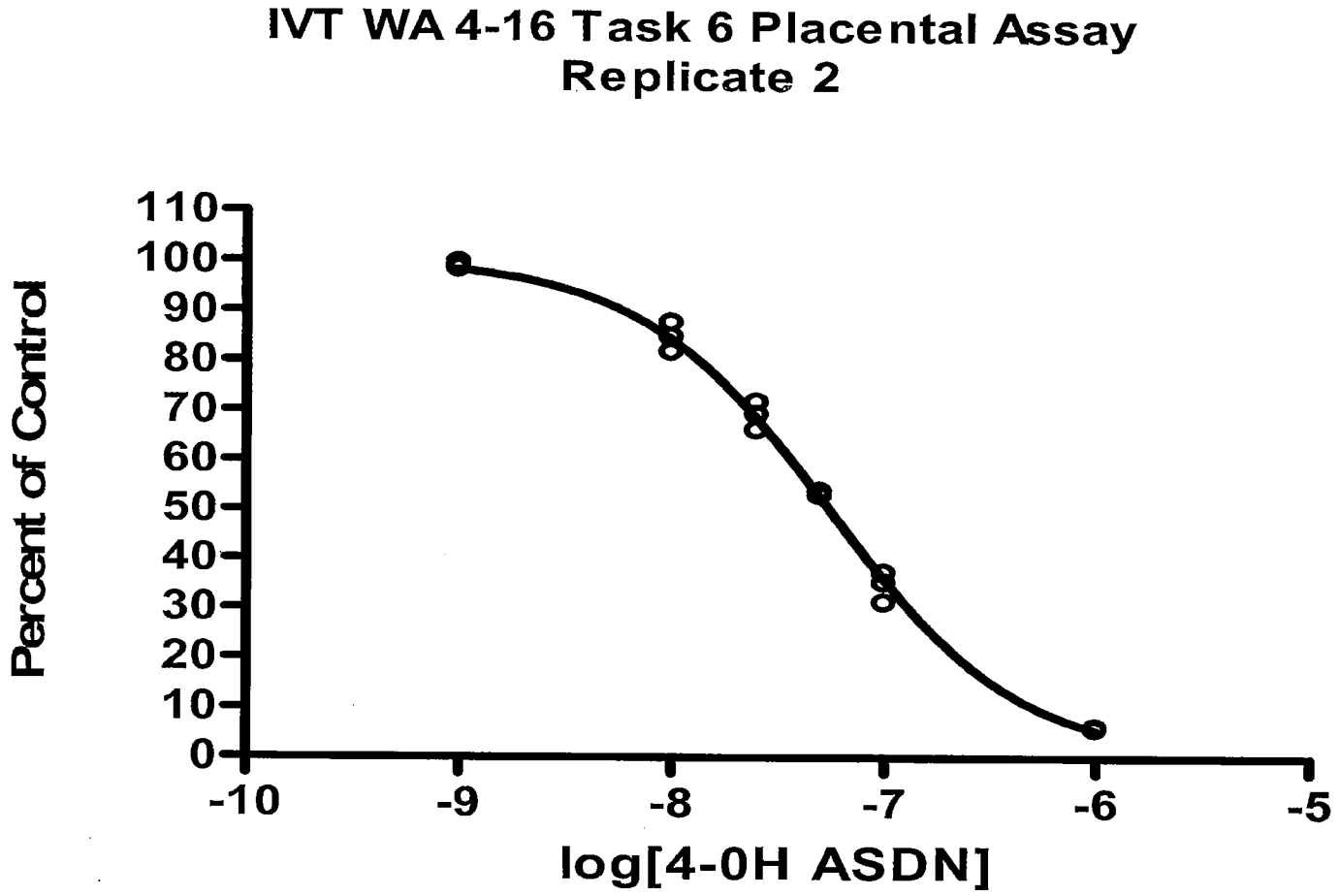

Figure A-1. **Replicate 2. Individual Percent of Control Values Vs. (Base 10) Logarithm of 4-OH ASDN Inhibitor Concentration. Concentration Response Curve Fitted to Average Responses Within Concentrations. Placental Aromatase Assay. Microsomes Prepared by In Vitro Technologies.**

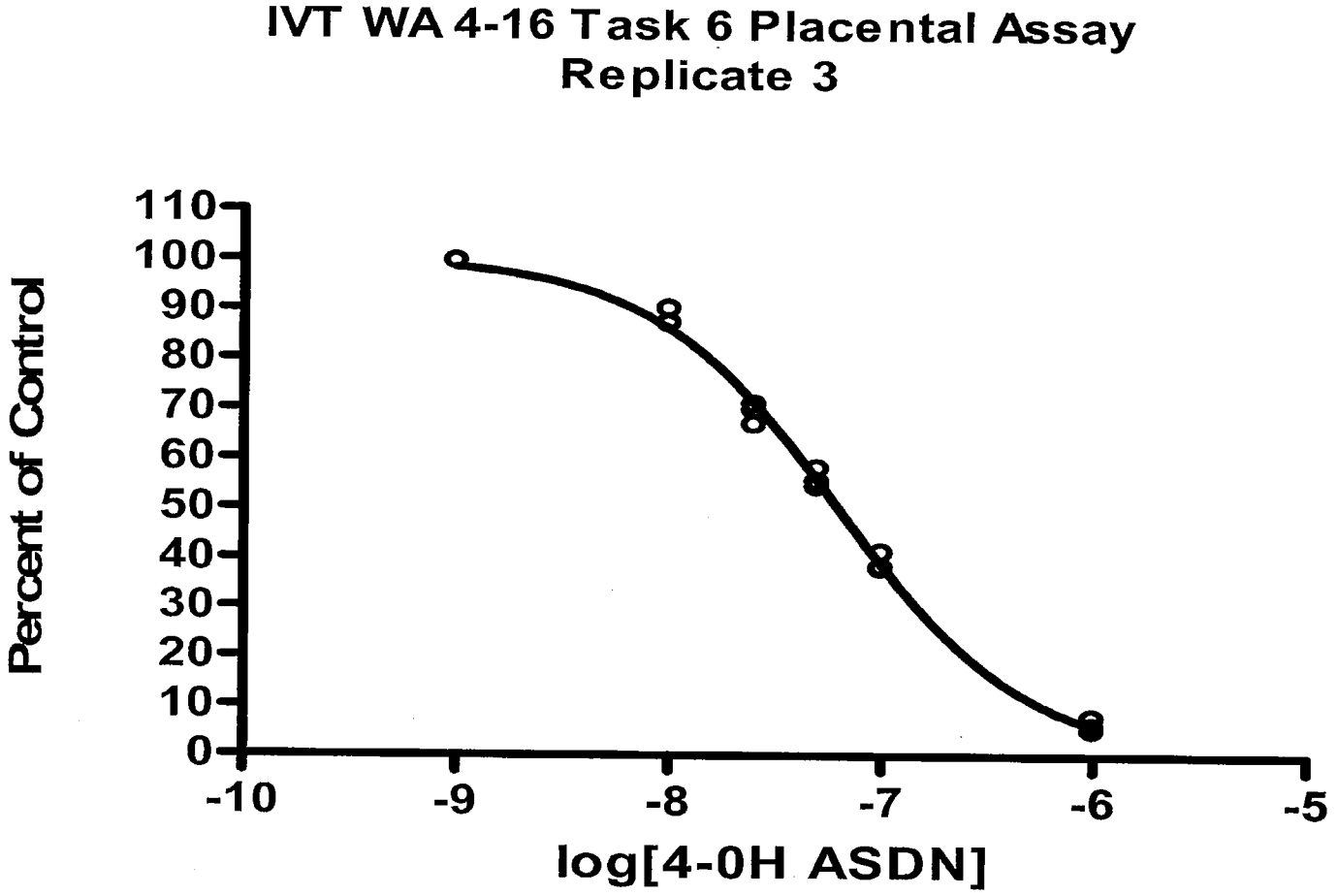

Figure A-2. Replicate 3. Individual Percent of Control Values Vs. (Base 10) Logarithm of 4-OH ASDN Inhibitor Concentration. Concentration Response Curve Fitted to Average Responses Within Concentrations. Placental Aromatase Assay. Microsomes Prepared by In Vitro Technologies.

APPENDIX C

RESEARCH TRIANGLE INSTITUTE REPORT

DRAFT TASK REPORT

PLACENTAL AROMATASE VALIDATION STUDY

WA 4-16 Task 6: Analyze Microsomes Prepared at Two Participating Laboratories

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

Sponsor:

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

Performing Laboratory:

RTI International
Drug Metabolism and Pharmacokinetics
Science and Engineering Group
Health Sciences Unit
P.O. Box 12194
Research Triangle Park, NC 27709

DRAFT REPORT

Title: **PLACENTAL AROMATASE VALIDATION STUDY WA 4-16 Task 6: Analyze Microsomes Prepared at Two Participating Laboratories**

Authors: Sherry Black, B.S.
James M. Mathews, Ph.D., DABT

Performing Laboratory: RTI International
Drug Metabolism and Pharmacokinetics
Science and Engineering Group
Health Sciences Unit
P.O. Box 12194
Research Triangle Park, NC 27709

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

Sponsor's Representatives: David P. Houchens, Ph.D.
EDSP Program Manager
Battelle Memorial Institute

Jerry D. Johnson, Ph.D., DABT
Work Assignment Leader
Battelle Memorial Institute

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Author:

James M. Mathews, Ph.D., DABT Date
Study Director
Director, Drug Metabolism and Pharmacokinetics
RTI International

Approved:

Alan H. Staple, M.Sc. Date
Vice President, Health Sciences
RTI International

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1.0 EXECUTIVE SUMMARY

In this task, RTI, as one of four participating laboratories, conducted studies with two human placental microsomal preparations in order produce data related to the aromatase activity and protein content of those preparations that could be compared with data from other participating laboratories. The microsomes tested were prepared by Battelle (Columbus, OH) and In Vitro Technologies (Baltimore, MD).

Each microsomal preparation was assayed for protein content in four independent replicates and for aromatase activity in two independent replicates.

Four types of control samples were included for each replicate of the aromatase assay. These included:

- full enzyme (aromatase) activity controls (substrate, β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH), propylene glycol, buffer, vehicle 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-hydroxyandrostenedione (4-OH ASDN) [5×10^{-8} M])
- negative controls (all components that are in the full aromatase activity controls, except vehicle, and with the addition of lindane [1×10^{-6} M]).

While they are different for the two microsomal preparations, both the protein content and aromatase activity are sufficient for use in the aromatase assay. The average measured full aromatase activity for the microsomal preparations was 0.0563 ± 0.0116 and 0.0365 ± 0.0007 nmol/mg/min for Battelle and In Vitro, respectively. Protein content and aromatase activity showed good assay-to-assay consistency. Each microsomal preparation 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 U.S. 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 EPA is implementing an Endocrine Disrupter 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 Method Validation 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 $P450_{arom}$ 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 10 exons, with the exact size of the gene exceeding 70 kilobases. Aromatase is also found in breast tissue, and the importance of intratumoral aromatase and local estrogen production is the subject of current research efforts. Aromatase inhibitors have been developed as efficacious therapeutic agents for estrogen-dependent breast cancer to reduce the stimulatory effects of estrogens in the progression of 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 as low as 0.04 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 contaminants 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 experiments 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.

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 inhibitors. This task was conducted in two stages as described below. RTI's role in this task is described in Section 2.2.3 below.

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

Battelle and In Vitro were assigned to obtained a human placenta and prepare microsomes. Protein concentration and aromatase activity (uninhibited) were determined by each of the laboratories using the microsomes that they prepared, i.e., Battelle and In Vitro. In addition, Battelle and In Vitro were assigned to conduct 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. The data from these studies were sent to Battelle's EDSP Program Office and, together with staff at RTI, the data was reviewed prior to submission to the EPA for approval.

2.2.2 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 microsomes to In Vitro, RTI, and WIL,

whereas In Vitro distributed microsomes to Battelle, RTI, and WIL. In this way, each laboratory used microsomes prepared by both laboratories in their tests. Protein concentration and estimated aromatase activity information was included with the shipped microsomes. Each laboratory was assigned to determine protein concentration and aromatase activity (uninhibited) of the microsomal preparations that they received. Thus, comparisons between microsome preparations can be carried out within laboratories and comparisons among laboratories can be carried out within microsome preparations. The preparation and analysis effects can be independently estimated.

2.2.3 Experiments Conducted at RTI

For each microsome preparation (one each from Battelle and In Vitro), the protein content was assayed in two independent replicates. Then, for each microsome preparation, the full aromatase activity, background activity, positive control activity and negative control activity (4 tubes each per replicate) was determined in two independent replicates. Protein content was necessarily determined with each replicate set of the aromatase activity controls. Therefore, for each microsomal preparation, the protein content was determined in a series of four independent replicates and the aromatase activity control set was conducted in two independent replicates. Only the results of these experiments are included in this report.

3.0 MATERIALS AND METHODS

3.1 Substrate

The substrate for the aromatase assay was androstenedione (ASDN). Non-radiolabeled and radiolabeled ASDN were used. The non-radiolabeled ASDN (Lot # 024K0809) was obtained from Sigma (St. Louis, MO) by the Sponsor's Chemical Repository and was then distributed to the participating laboratories. The radiolabeled androstenedione ([1-^{3}H]-androstenedione, [^{3}H]ASDN, Lot # 3538496), was obtained from Perkin Elmer Life Science (Boston) and had a reported specific activity of 25.3 Ci/mmol. Radiochemical purity was reported by the supplier to be > 97%. The results of the radiochemical purity analysis conducted at RTI in a previous task are presented in the report contained in Appendix E.

The substrate solution was prepared fresh each day of assay by combining solutions of [^{3}H]ASDN and non-radiolabeled ASDN in order to prepare a solution containing 2 μM ASDN with ca. 1 μCi/mL. A 1:100 dilution of the radiolabeled [^{3}H]ASDN stock in buffer was

prepared fresh each day of assay. A solution (1 mg/mL) of ASDN in ethanol was prepared (fresh each day) and then diluted in buffer to a final concentration of 1 µg/mL. The substrate solution was prepared by combining 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. 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 ca. 0.1 µCi/tube.

3.2 Control Substances

Battelle's Chemical Repository (CR) 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. These chemistry activities and results are described in the Sponsor's Chemistry report which is appended to this document (Appendix D)

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. Stock solutions (Table 1) were supplied by Battelle's CR and were stored refrigerated. Dilutions were made fresh each day of use in the same vehicle and lot number that was used to prepare the stock solutions (see Table 4, Section 3.4 for details). Tables 2 and 3 describe the dilution scheme for 4-OH ASDN and lindane, respectively.

Table 1. Control Chemicals for Task 6

Test Substance	Mfr Purity	CAS Number	Molecular Formula	Molecular Weight (g/mol)	Stock Solution ID	Stock Formulation Concentration (M)	Vehicle	Storage Conditions
4-OH ASDN	99	566-48-3	$C_{19}H_{26}O_3$	302.4	3-ASDN-1	0.01	95% ethanol	Refrigerate, Dark
Lindane	99.6	58-89-9	$C_6H_6Cl_6$	290.8	2-LIN-1	0.1	DMSO	Refrigerate, Dark

Table 2. Dilution Scheme for 4-OH ASDN Control

Dilution ID	µL EtOH	µL sample	Sample used	Solution concentration (M)	Final Concentration in assay (M)
1	9900	100	Stock	1.00E-04	-
2	9500	500	1	5.00E-06	5.00E-08

Table 3. Dilution Scheme for Lindane Control

Dilution ID	µL DMSO	µL sample	Sample used	Solution concentration (M)	Final Concentration in assay (M)
1	9900	100	Stock	1.00E-03	-
2	900	100	1	1.00E-04	1.00E-06

3.3 Microsomes

Human placental microsomes (Lot # 6-041305, 21 mg protein/mL, prepared at Battelle and Lot# BAA, 8 mg protein/mL, prepared at In Vitro) were used on this task. The microsomes were stored at ca. -70°C. Prior to assay, microsomes were thawed rapidly in a 37 ± 1°C water bath, rehomogenized by brief vortexing and then kept on ice until used. The microsome stock was diluted with buffer (1:800 and 1:320 overall for the Battelle and In Vitro microsomes, respectively) and maintained on ice until used. The time between thawing of the microsomes and their use in the assay was limited to less than 2 h and, in most cases was about 30 min.

3.4 Other Assay Components

In addition to substrate, control chemicals or vehicle, and microsomes, the aromatase assay contained NADPH, propylene glycol and phosphate buffer. Supplier and lot numbers for other aromatase assay components are presented in Table 4.

Table 4. Supplier and Lot Numbers for Aromatase Assay Components

Chemical	Supplier	Lot Number
NADPH	Sigma	103K7046
Propylene glycol	J.T.Baker	Y18600
Sodium phosphate dibasic	J.T.Baker	A08H50
Sodium phosphate monobasic	J.T.Baker	A12H20
Vehicle (DMSO) for Lindane (negative control chemical)	Battelle CR	2969A46428
Vehicle (95% EtOH) for 4-OH ASDN (positive control chemical)	Battelle CR	04H23QB

3.4.1 NADPH

A solution of NADPH (6 mM) in pH 7.4 sodium phosphate buffer (see Section 3.4.2) was prepared fresh each day of assay and was kept on ice until used.

3.4.2 Assay Buffer

The assay buffer was 0.1 M sodium phosphate buffer, pH 7.4. Sodium phosphate monobasic (JT Baker, cat # 4011-01, 137.99 g/mol) and sodium phosphate dibasic (JT Baker, Cat # 4062-01, 141.96 g/mol) were used in the preparation of the buffer. Solutions of each

reagent at 0.1 M were prepared in distilled, deionized water and then the solutions were combined to a final pH of 7.4. The assay buffer was stored for up to one month in the refrigerator (2-8 °C).

3.5 Protein Determination

The protein concentration of the microsome preparation was determined in four replicates for each microsomal preparation, two of which coincided with the use of the each batch of microsomes in the aromatase assay. A 6-point standard curve was prepared, ranging from 5 – 250 µg protein/mL. The protein standards were made from bovine serum albumin (BSA). Protein was determined by using a D_C Protein Assay kit purchased from BioRad (Hercules, CA). QC standards for use on this task were prepared by diluting a purchased protein standard to prepare samples containing 10 and 100 µg protein/mL. The same QC samples were used throughout the experiments on this task. Unknown and curve standards were run in triplicate and QC standards were run in duplicate. To a 200 µL aliquot of unknown, standard or QC sample, 100 µL of BioRad D_C Protein Kit Reagent A was added and mixed. Next, 0.8 mL of BioRad D_C Protein Kit Reagent B was added to each standard or unknown and the samples were vortex mixed. The samples were allowed to sit at room temperature for at least 15 min to allow for color development. Each sample (unknown and standards) was transferred to a disposable polystyrene cuvette and the absorbance (@ 750 nm) was measured using a spectrophotometer. The protein concentration of the microsomal sample was determined by interpolation of the absorbance value using the curve developed using the protein standards.

3.6 Cytochrome P450 Aromatase (CYP19) Activity

Two independent replicates were conducted (on separate days) for each microsomal preparation with all replicates conducted by the same technician. A single replicate study of an example microsomal preparation is described in Table 5.

Four types of control samples were included for each replicate. These included:

- full enzyme (aromatase) activity controls (FEAC) which contained: substrate, NADPH, propylene glycol, buffer, vehicle [DMSO] and microsomes
- background activity controls (BAC) which contained: all components that are in the full aromatase activity controls, except NADPH

- positive controls which contained: all components that are in the full aromatase activity controls, except vehicle, and with the addition of 4-OH ASDN at 5×10^{-8} M
- negative controls which contained: all components that are in the full aromatase activity controls, except vehicle, and with the addition of lindane at a 1×10^{-6} M.

Four test tubes of each type of control were included with each replicate and were treated the same as the other samples. 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.

The assays were performed in 13x100 mm test tubes maintained at 37 ± 1°C in a shaking water bath. Propylene glycol (100 µL), [^{3}H]ASDN, NADPH (as applicable) and control chemical or vehicle (DMSO), and buffer (0.1 M sodium phosphate buffer, pH 7.4) were combined in the test tubes (total volume 1 mL). The volume of the control chemical solutions or vehicle used was 20 µL (1% of total assay volume). The final concentrations for the assay components are presented in Table 6. The tubes and the microsomal suspension were placed in the 37 ± 1°C 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 was 2 mL, and the tubes were incubated for 15 min. The incubations were stopped by the addition of methylene chloride (2 mL); the tubes were vortex-mixed for ca. 5 s and placed on ice. The tubes were then vortex-mixed an additional 20-25 s. The tubes were then centrifuged using a Beckman GS-6R centrifuge with GH-3.8 rotor for 10 min at a setting of 1000 rpm to aid in the separation of the organic and aqueous phases. The methylene chloride layer was removed and discarded; the aqueous layers were extracted again with methylene chloride (2 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. The radiochemical content of each aliquot was determined by using liquid scintillation spectrometry (LSS). Radiolabel found in the aqueous fractions represented tritiated water formed. The aromatization of one mole of [^{3}H]ASDN resulted in the production of one mole of estrone (non-radiolabeled) and one mole of tritium associated with water.

Table 5. Aromatase Assay Study Design

Sample type	Repetitions (test tubes)	Description	Test Chemical concentration (M, final)
Full Enzyme Activity Control	4	Complete assay[a] with reference chemical vehicle control	N/A
Background Activity Control	4	Complete assay with reference chemical 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}

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

Table 6. 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

3.7 Data Analysis

3.7.1 Aromatase Activity and Percent of Control Calculations

Data were entered into an Excel spreadsheet for calculation of aromatase activity and percent of control. The master spreadsheet used was titled Aromatase_Master_Version1.3.xls.

For each repeat tube (FEAC, BAC, positive, and negative controls and each reference chemical concentration) the Excel spreadsheet included total observed (uncorrected) disintegrations per minute (DPMs) per tube and total aromatase activity per tube. The DPM and aromatase activity values were corrected for the background DPMs, as measured by the average of the BAC 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) DPMs and aromatase activity across the four BAC control repeat tubes were necessarily equal to 0 within each replicate.

3.7.2 Statistical Analyses

The protein concentrations and the aromatase activity was compared between the two microsome sources by two-sample *t*-tests, using the within laboratory-microsome preparation replicate determination variation as an error criterion.

3.7.2.1 Statistical Software. Statistical analyses and displays such as summary tables, graphical displays, analysis of variance, and multiple comparisons were carried out using the SAS statistical analysis system, Version 8.

4.0 RESULTS

4.1 Radiochemical Purity

The [^{3}H] ASDN was found to be 97% radiochemically pure by high performance liquid chromatography (HPLC) nalysis. The radiochemical purity report appears in Appendix E.

4.2 Stock Formulation Analysis

The formulation stability and formulation analysis results for the positive and negative control chemicals from the Battelle CR are included in the reports presented in Appendix D. Some of the formulation analysis data are summarized in Table 7.

Table 7. Formulation Analysis Data

Chemical ID	Stock Solution ID	Manufacturer's Purity (%)	Stock Solution Prep Date	Number of Days Known Stable	Date of Last Use on WA 4-16, Task 6
Lindane	2-LIN-1	99.6	3/21/2005	168	6/16/2005
4- OH ASDN	3-ASDN-1	99	3/21/2005	173	6/16/2005

4.3 Protein Analysis

Protein content of the human placental microsomes was measured each day of the aromatase assay and at two other times per microsome preparation as described in Section 3.5. The final dilution of the stock microsomes was used in the protein assay; protein concentration of the stock microsomes was calculated from the data. The measured protein concentration in the human placental microsomes for each day of use is presented in Table 8.

Table 8. Protein Concentration Data

Microsomes	Replicate	Protein concentration (mg/mL)	Mean	SE	Lower 95% CL	Upper 95% CL
Battelle	1	23.6	24.1	1.31	20.8	27.3
	2	24.7				
	3	19.8				
	4	28.2				
In Vitro	1	9.2	9.36	1.31	6.14	12.6
	2	7.8				
	3	9.5				
	4	11.0				

In order to better characterize the protein assay, QC standards were included on all runs (see Section 3.5). The QC results are presented in Table 9. The measured concentration of the 100 µg/mL QC standard was <10% different from the known value in 7 of 8 assays. The data indicate that there is more uncertainty in the protein values at the lower end of the curve, with the measured concentration of the 10 µg/mL QC measured at >20% different from known in 3 of the 8 assays, indicating a higher degree of uncertainty in this region of the standard curve.

Table 9. Protein QC Table

Known Concentration (µg/mL)	Measured Concentration (µg/mL)	Mean	SD	SEM	% CV	% Difference from Known
100	90.8	95.8	6.5	2.3	6.8	-9.2
	103.0					3.0
	93.3					-6.7
	92.0					-8.0
	96.0					-4.0
	105.5					5.5
	86.1					-13.9
	99.4					-0.6
10	7.4	8.3	2.3	0.8	28.2	-26.0
	9.1					-9.0
	8.9					-11.0
	11.8					18.0
	6.1					-39.0
	9.1					-9.0
	9.7					-3.0
	4.2					-58.0

4.4 Aromatase Activity

Aromatase activity was measured in four types of controls (see Section 3.6) in two independent replicates for each microsomal preparation. Information regarding assay dates, technicians, protein concentration and substrate specific activity is presented in Table 10. Radioactivity remaining in each assay tube after extraction of the unreacted substrate represented product estrone. 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.

Table 10. Aromatase Assay Summary

Microsome Source	Replicate	Assay Date	Technician	Protein Stock Concentration (measured) mg/mL	Substrate Soln SA µCi/µg ASDN
Battelle	1	5/19/2005	1	23.6	1.33
Battelle	2	5/26/2005	1	19.8	1.38
In Vitro	1	6/10/2005	1	9.2	1.19
In Vitro	2	6/16/2005	1	7.8	1.44

4.4.1 Control Results

Each replicate set for each chemical 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 a 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 the 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 activities for each type of control (except background) for the beginning and end groups and the overall means, SD, SEM and CVs across replicates and across microsome source are presented in Table 11.

Table 11. Control Activities[a]

Microsome Source	Replicate	Mean Beginning Control	Mean End Control	Overall Mean	SD[b]	SEM[b]	%CV[b]	Overall (by microsome source) Mean	SD	SEM	%CV
Full Aromatase Activity Controls											
Battelle	1	0.0662	0.0627	0.0645	0.0021	0.0010	3.24	0.0563	0.0116	0.0082	20.65
	2	0.0485	0.0476	0.0480	0.0012	0.0006	2.45				
In Vitro	1	0.0370	0.0370	0.0370	0.0008	0.0004	2.12	0.0365	0.0007	0.0005	1.82
	2	0.0365	0.0356	0.0360	0.0006	0.0003	1.67				
Positive Controls											
Battelle	1	0.0318	0.0292	0.0305	0.0018	0.0009	5.81	0.0268	0.0053	0.0037	19.74
	2	0.0237	0.0223	0.0230	0.0012	0.0006	5.41				
In Vitro	1	0.0177	0.0168	0.0173	0.0006	0.0003	3.67	0.0167	0.0007	0.0005	4.47
	2	0.0164	0.0160	0.0162	0.0005	0.0003	3.17				
Negative Controls											
Battelle	1	0.0649	0.0600	0.0624	0.0030	0.0015	4.82	0.0550	0.0105	0.0074	19.11
	2	0.0480	0.0471	0.0475	0.0011	0.0006	2.32				
In Vitro	1	0.0356	0.0326	0.0341	0.0021	0.0010	6.08	0.0353	0.0017	0.0012	4.75
	2	0.0363	0.0366	0.0365	0.0005	0.0003	1.37				

[a]Units are nmol/mg protein/min

[b]SD = Standard Deviation, SEM = Standard Error of the Mean, %CV = Coefficient of Variation.

The inherent aromatase activity varies between the two preparations, with the mean full aromatase activity for the Battelle microsomes at 0.0563 nmol/mg/min while that for the In Vitro microsomes is 0.0365 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 near 50% of control (full activity) and negative control activities were near 95% of control.

5.0 DISCUSSION

This WA 4-16, Task 6 involved assaying human placental microsomes from two sources for protein content and aromatase activity. Only the results obtained by RTI are presented here. For Task 6, the aromatase assay (controls only) was performed a total of 2 times per microsome preparation and the protein content of each microsomal preparation was measured a total of 4 times.

The microsomes used in this task were prepared at Battelle and In Vitro. The reported protein content for each preparation was 21 and 8 mg/mL for the Battelle and In Vitro preparations, respectively. The average measured protein content for the microsomal preparations was 24.1 ± 1.31 and 9.36 ± 1.31 for Battelle and In Vitro, respectively. These values are similar to the values reported by the supplying laboratories.

The microsomes were reported to have aromatase activity of 0.047 and 0.038 nmol/mg/min, respectively for the Battelle and In Vitro preparations. The average measured full aromatase activity for the microsomal preparations was 0.0563 ± 0.0116 and 0.0365 ± 0.0007 nmol/mg/min for Battelle and In Vitro, respectively. These values are similar to the values reported by the supplying laboratories..

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.

6.0 CONCLUSION

In this WA 4-16, Task 6 human placental microsomes from two sources were assayed for protein content and aromatase activity. The data reported here will be compared with data obtained from other participating laboratories using the same microsomal preparations.

While they are different for the two microsomal preparations, both the protein content and aromatase activity are sufficient for use in the aromatase assay. Protein content and aromatase activity showed good assay-to-assay consistency. Each microsomal preparation responded appropriately to the presence of both known aromatase inhibitors and non-inhibitors. Both preparations appear to be acceptable for use in the next task of this work assignment.

Appendix A

Study Protocol, Amendment and Deviations

EPA Contract No.: 68-W-01-023
EPA Work Assignment No.: 4-16
Task Number: 6
Study Code: **An05-940**

TITLE: Analyze Microsomes Prepared at Two Participating Laboratories

SPONSOR: Battelle
505 King Ave.
Columbus, OH 43201

TESTING FACILITY: RTI International*
DMPK
3040 Cornwallis Rd.
Research Triangle Park, NC 27709

PROPOSED EXPERIMENTAL START DATE: April 21, 2005

PROPOSED EXPERIMENTAL END DATE: May 20, 2005

AMENDMENTS:

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

RTI International is a trade name of Research Triangle Institute

Approved By:

James M. Mathews, Ph.D., DABT — Date 4-21-05
RTI Study Director

Jerry Johnson, Ph.D. DABT — Date 4-22-05
Work Assignment Leader, Battelle

Linda J. Phillips, Ph.D. — Date 4/26/05
EPA Project Officer

David P. Houchens, Ph.D. — Date 4/25/05
EDSP Program Manager, Battelle

Gary Timm, M.S., M.A. — Date 4/26/05
EPA Work Assignment Manager

Reviewed By:

Kim Collier, B.A. — Date 4-21-05
RTI, Quality Assurance Specialist

Terri L. Pollock, B.A. — Date 4-25-05
EDSP Quality Assurance Manager, Battelle

J. Thomas McClintock, Ph.D. — Date 4/26/05
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 objective of this protocol is 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-hydroxyandrostenedione (4-OH ASDN) to demonstrate the responsiveness of the assay to aromatase inhibitors. 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

Battelle's Endocrine Disruptor Screening Program (EDSP) Program Office selected two laboratories, Battelle and In Vitro, and gave them the assignment to obtain a human placenta for making new microsomal preparations. 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. Protein concentration and aromatase activity (uninhibited) will be determined by each of the laboratories using the microsomes that they prepare, i.e., Battelle and In Vitro. In addition, Battelle and In Vitro 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. 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 EPA for approval.

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

After receiving EPA's approval, Battelle and In Vitro will each ship their microsomes to each of the other 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. Information to be included with the shipped microsomes includes protein concentration and estimated aromatase activity. Each laboratory will determine protein concentration and aromatase activity (uninhibited) using the microsomal preparations that they received. The laboratories will conduct the analyses according to their protocols, which will be written to include the procedures described above. Thus, 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 can be independently estimated.

1.3 Experiments to be Conducted at RTI

For each microsome preparation (one each from Battelle and In Vitro), the protein content will be assayed in two independent replicates. Then, for each microsome preparation, the full aromatase activity, background activity, positive control activity and negative control activity (4 tubes each per replicate) will be determined in two independent replicates. Protein content will necessarily be determined with each replicate set of the aromatase activity controls. Therefore, for each microsomal preparation, the protein content will be determined in a series of four independent replicates and the aromatase activity control set will be run in two independent replicates.

1.4 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.

1.5 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 nonradiolabeled 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). Nonradiolabeled and radiolabeled ASDN will be used. The nonradiolabeled ASDN and the radiolabeled androstenedione ([1ß-^{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 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 Control Substances

The known aromatase inhibitor, 4-OH ASDN, is 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	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

2.2.1 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 buffer, 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 solutions will be provided by the CR.

2.3 Human Placental Microsomes

2.3.1 Source of Microsomes

RTI will receive human placental microsomes prepared by Battelle and In Vitro. The supplier will provide information regarding the protein content and aromatase activity of each microsome preparation.

2.3.2 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 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.

It is strongly recommended that stock microsomes to be refrozen be divided into aliquots appropriate for use prior to refreezing in liquid nitrogen in order to minimize the number of freeze/thaw cycles.

Diluted microsomes must be used only on the day of preparation. Under no conditions should diluted microsomes be refrozen for later use in the assay.

2.4 Other Assay Components

2.4.1 Buffer

The assay buffer is 0.1 M sodium phosphate buffer, pH 7.4. Sodium phosphate monobasic (JT Baker, cat # 4011-01, 137.99 g/mol) and sodium phosphate dibasic (JT Baker, cat # 4062-01, 141.96 g/mol) are used in the preparation of the buffer. Solutions of each reagent at 0.1 M are prepared in distilled, deionized water and then the solutions are combined to a final pH of 7.4. The assay buffer may be stored for up to one month in the refrigerator (2–8 °C).

2.4.2 Propylene Glycol

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

2.4.3 NADPH

NADPH (ß-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 will be 0.3 mM. Typically, a 6-mM stock solution will be 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

For this Task, the protein concentration of each microsome preparation prepared in this task is measured by all participating laboratories. The protein concentration of each microsome preparation will be determined a total of four times (See Section 1.3) in our laboratory. 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 (Rockford, Il) 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 used by each laboratory to measure the aromatase activity in the microsomal preparations. The procedure will be conducted in two independent replicates. Four types of control samples will be included for each replicate. These include:

- full enzyme (aromatase) activity controls (substrate, NADPH, propylene glycol, buffer, vehicle [DMSO, 20 µL] 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 5E-8M)
- negative controls (all components that are in the full aromatase activity controls, except vehicle, and with the addition of lindane at 1E-6M).

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 13 x 100 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 are then vortex-mixed an additional 20–25 s. The tubes will then be centrifuged using a Beckman GS-6R centrifuge with GH-3.8 rotor for 10 min 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 (three extractions total), 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
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 ^3H_2O formed.

5.0 DATA ANALYSIS

5.1 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.

5.2 *Statistical Analyses*

5.2.1 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 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.

5.2.2 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 (Battelle) 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
- 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.

5.2.3 Statistical Software

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.

5.2.4 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 5.2.2.

6.0 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.

7.0 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.

8.0 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, background corrected aromatase activity (for each control), percent of control activity.

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 5 of this document.

9.0 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.

EPA Contract No.: 68-W-01-023
EPA Work Assignment No.: 4-16
Task Number: 6
RTI Study Code: An05-940

Amendment 1

TITLE: Analyze Microsomes Prepared at Two Participating Laboratories

SPONSOR: Battelle
505 King Ave.
Columbus, OH 43201

TESTING FACILITY: RTI International*
DMPK
3040 Cornwallis Rd.
Research Triangle Park, NC 27709

PROPOSED EXPERIMENTAL START DATE: April 21, 2005
PROPOSED EXPERIMENTAL END DATE: May 20, 2005

RTI International is a trade name of Research Triangle Institute.

Approved By:

J. Matthews 5-18-05
James M. Mathews, Ph.D., DABT Date
RTI Study Director

Jerry D. Johnson 5-19-05
Jerry Johnson, Ph.D. DABT Date
Work Assignment Leader, Battelle

David P. Houchens 5/19/05
David P. Houchens, Ph.D. Date
EDSP Program Manager, Battelle

Reviewed By:

K. Collier 5-18-05
Kim Collier. B.A. Date
RTI, Quality Assurance Specialist

Terri L. Pollock 5-19-05
Terri L. Pollock, B.A. Date
EDSP Quality Assurance Manager, Battelle

Item 1

Section 2. 3.2 Use of Microsomes, which read:

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 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.
It is strongly recommended that stock microsomes to be refrozen be divided into aliquots appropriate for use prior to refreezing in liquid nitrogen in order to minimize the number of freeze/thaw cycles.
Diluted microsomes must be used only on the day of preparation. Under no conditions should diluted microsomes be refrozen for later use in the assay.

Is hereby amended as follows:

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 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.

Justification

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.

Item 2

Section 3.0 PROTEIN ASSAY which read:

For this Task, the protein concentration of each microsome preparation prepared in this task is measured by all participating laboratories. The protein concentration of each microsome preparation will be determined a total of four times (See Section 1.3) in our laboratory. 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 (Rockford, Il) 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.

Is hereby amended as follows:

The text above is replaced with:

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.

Justification

This change is made in order to improve the accuracy of the protein determination.

Protocol Deviation 1

ORIGINAL DOCUMENT SPECIFICATIONS:
Page 10, Section 5.2.1 Graphical and Analysis of Variance Comparisons of Full Enzyme Activity Control, Background Activity Control, and Positive and Negative Control Percent of Control Across Replicates

The section describes Statistical Analysis to be carried out by the laboratories that conducted the positive control studies (as described in Section 1.1 of the protocol).

DEVIATION:

Since RTI was not charged with conducting the positive control studies, the associated statistical analyses (as described in Section 5.2.1 of the protocol) were not carried out. See email in the record confirming that this is a correct interpretation of the intent of the study.

REASON/IMPACT OF CHANGE:
No impact.

J Matt
Study Director

1-25-06
Date

Appendix B

QAPP

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

JDJ 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.

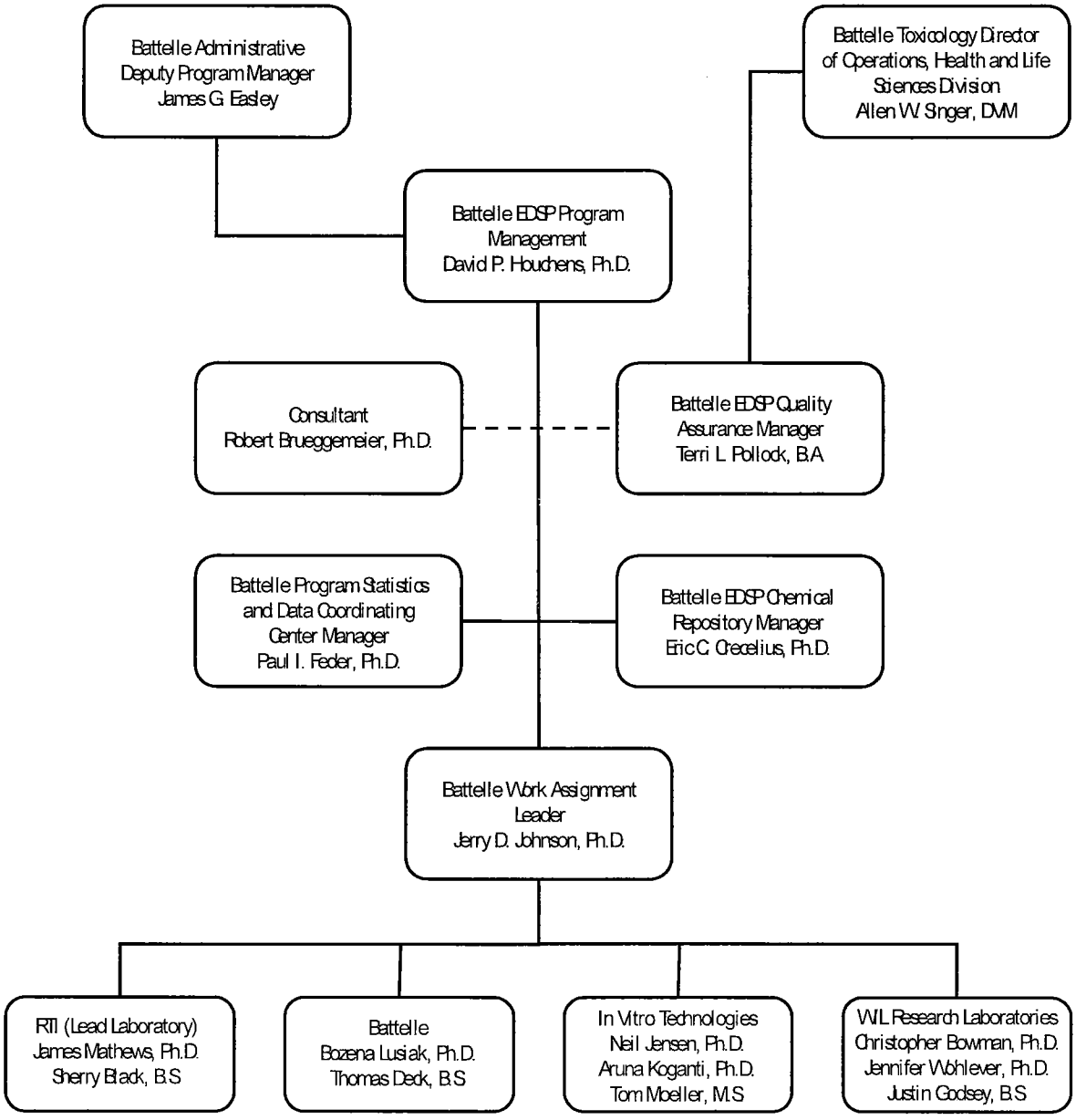

Figure 4-1. WA 4-16 Project Organization Overview

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

PROTOCOL		Page 1 of 18

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 □L 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.

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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.

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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
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%).

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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 $\log_{10}IC_{50}$ (□), the within replicate standard error of □, 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.

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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.

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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

Spreadsheets

Table C1. Aromatase Activity Calculation – Battelle Microsomes – 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 (initial)	% 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 (nmol estrogen formed/mg protein/min
Full activity control	1	2	0.5	1	6304	12608	12542	25084	0.1	185910	13.49	24945	0.0294	1	0.015	15	0.0665
			0.5	2	6238	12476											
	2	2	0.5	1	6209	12418	12416	24832	0.1	185910	13.36	24693	0.0291	1	0.015	15	0.0659
			0.5	2	6207	12414											
	3	2	0.5	1	5798	11596	11712	23424	0.1	185910	12.60	23285	0.0274	1	0.015	15	0.0621
			0.5	2	5914	11828											
	4	2	0.5	1	5873	11746	11940	23880	0.1	185910	12.84	23741	0.0280	1	0.015	15	0.0633
			0.5	2	6067	12134											
Background control	1	2	0.5	1	24	48	48	96	0.1	185910	0.05	-43	-0.0001	1	0.015	15	-0.0001
			0.5	2	24	48											
	2	2	0.5	1	31	62	57	114	0.1	185910	0.06	-25	0.0000	1	0.015	15	-0.0001
			0.5	2	26	52											
	3	2	0.5	1	59	118	128	256	0.1	185910	0.14	117	0.0001	1	0.015	15	0.0003
			0.5	2	69	138											
	4	2	0.5	1	21	42	45	90	0.1	185910	0.05	-49	-0.0001	1	0.015	15	-0.0001
			0.5	2	24	48											

Table C1. Aromatase Activity Calculation – Battelle Microsomes – Replicate 1 (continued)

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 (initial)	% 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 (nmol estrogen formed/mg protein/min
Positive control	1	2	0.5	1	3069	6138	6075	12150	0.1	185910	6.54	12011	0.0142	1	0.015	15	0.0320
			0.5	2	3006	6012											
	2	2	0.5	1	2971	5942	5978	11956	0.1	185910	6.43	11817	0.0139	1	0.015	15	0.0315
			0.5	2	3007	6014											
	3	2	0.5	1	2882	5764	5749	11498	0.1	185910	6.18	11359	0.0134	1	0.015	15	0.0303
			0.5	2	2867	5734											
	4	2	0.5	1	2660	5320	5329	10658	0.1	185910	5.73	10519	0.0124	1	0.015	15	0.0281
			0.5	2	2669	5338											
Negative Control	1	2	0.5	1	6185	12370	12115	24230	0.1	185910	13.03	24091	0.0284	1	0.015	15	0.0643
			0.5	2	5930	11860											
	2	2	0.5	1	6201	12402	12334	24668	0.1	185910	13.27	24529	0.0289	1	0.015	15	0.0654
			0.5	2	6133	12266											
	3	2	0.5	1	5743	11486	11515	23030	0.1	185910	12.39	22891	0.0270	1	0.015	15	0.0611
			0.5	2	5772	11544											
	4	2	0.5	1	5641	11282	11099	22198	0.1	185910	11.94	22059	0.0260	1	0.015	15	0.0588
			0.5	2	5458	10916											

Table C2. Aromatase Activity Calculation – Battelle Microsomes – 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)	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 (initial)	% 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 (nmol estrogen formed/mg protein/min
Full activity control	1	2	0.5	1	3888	7776	7829	15658	0.1	176422	8.88	15563	0.0177	1	0.012	15	0.0479
			0.5	2	3941	7882											
	2	2	0.5	1	3959	7918	8045	16090	0.1	176422	9.12	15995	0.0182	1	0.012	15	0.0492
			0.5	2	4086	8172											
	3	2	0.5	1	3955	7910	7952	15904	0.1	176422	9.01	15809	0.0180	1	0.012	15	0.0486
			0.5	2	3997	7994											
	4	2	0.5	1	3677	7354	7603	15206	0.1	176422	8.62	15111	0.0172	1	0.012	15	0.0465
			0.5	2	3926	7852											
Background control	1	2	0.5	1	27	54	52	104	0.1	176422	0.06	9	0.0000	1	0.012	15	0.0000
			0.5	2	25	50											
	2	2	0.5	1	20	40	57	114	0.1	176422	0.06	19	0.0000	1	0.012	15	0.0001
			0.5	2	37	74											
	3	2	0.5	1	18	36	41	82	0.1	176422	0.05	-14	0.0000	1	0.012	15	0.0000
			0.5	2	23	46											
	4	2	0.5	1	23	46	41	82	0.1	176422	0.05	-14	0.0000	1	0.012	15	0.0000
			0.5	2	18	36											

Table C2. Aromatase Activity Calculation – Battelle Microsomes – Replicate 2 (continued)

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 (initial)	% 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 (nmol estrogen formed/mg protein/min
Positive control	1	2	0.5	1	2009	4018	4044	8088	0.1	176422	4.58	7993	0.0091	1	0.012	15	0.0246
			0.5	2	2035	4070											
	2	2	0.5	1	1891	3782	3755	7510	0.1	176422	4.26	7415	0.0085	1	0.012	15	0.0228
			0.5	2	1864	3728											
	3	2	0.5	1	1758	3516	3551	7102	0.1	176422	4.03	7007	0.0080	1	0.012	15	0.0216
			0.5	2	1793	3586											
	4	2	0.5	1	1879	3758	3806	7612	0.1	176422	4.31	7517	0.0086	1	0.012	15	0.0231
			0.5	2	1927	3854											
Negative Control	1	2	0.5	1	3950	7900	7822	15644	0.1	176422	8.87	15549	0.0177	1	0.012	15	0.0478
			0.5	2	3872	7744											
	2	2	0.5	1	4045	8090	7881	15762	0.1	176422	8.93	15667	0.0179	1	0.012	15	0.0482
			0.5	2	3836	7672											
	3	2	0.5	1	3966	7932	7892	15784	0.1	176422	8.95	15689	0.0179	1	0.012	15	0.0483
			0.5	2	3926	7852											
	4	2	0.5	1	3785	7570	7511	15022	0.1	176422	8.51	14927	0.0170	1	0.012	15	0.0459

Table C3. Aromatase Activity Calculation – In Vitro Microsomes – 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 (initial)	% 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 (nmol estrogen formed/mg protein/min
Full activity control	1	2	0.5	1	2962	5924	5963	11926	0.1	176403	6.76	11807	0.0156	1	0.014	15	0.0363
			0.5	2	3001	6002											
	2	2	0.5	1	3065	6130	6189	12378	0.1	176403	7.02	12259	0.0162	1	0.014	15	0.0377
			0.5	2	3124	6248											
	3	2	0.5	1	2928	5856	5966	11932	0.1	176403	6.76	11813	0.0156	1	0.014	15	0.0363
			0.5	2	3038	6076											
	4	2	0.5	1	3078	6156	6182	12364	0.1	176403	7.01	12245	0.0162	1	0.014	15	0.0376
			0.5	2	3104	6208											
Background control	1	2	0.5	1	30	60	69	138	0.1	176403	0.08	19	0.0000	1	0.014	15	0.0001
			0.5	2	39	78											
	2	2	0.5	1	30	60	62	124	0.1	176403	0.07	5	0.0000	1	0.014	15	0.0000
			0.5	2	32	64											
	3	2	0.5	1	34	68	67	134	0.1	176403	0.08	15	0.0000	1	0.014	15	0.0000
			0.5	2	33	66											
	4	2	0.5	1	18	36	40	80	0.1	176403	0.05	-39	-0.0001	1	0.014	15	-0.0001
			0.5	2	22	44											

Table C3. Aromatase Activity Calculation – In Vitro Microsomes – Replicate 1 (continued)

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 (initial)	% 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 (nmol estrogen formed/mg protein/min
Positive control	1	2	0.5	1	1479	2958	2968	5936	0.1	176403	3.37	5817	0.0077	1	0.014	15	0.0179
			0.5	2	1489	2978											
	2	2	0.5	1	1466	2932	2913	5826	0.1	176403	3.30	5707	0.0075	1	0.014	15	0.0175
			0.5	2	1447	2894											
	3	2	0.5	1	1439	2878	2873	5746	0.1	176403	3.26	5627	0.0074	1	0.014	15	0.0173
			0.5	2	1434	2868											
	4	2	0.5	1	1321	2642	2727	5454	0.1	176403	3.09	5335	0.0071	1	0.014	15	0.0164
			0.5	2	1406	2812											
Negative Control	1	2	0.5	1	3017	6034	5890	11780	0.1	176403	6.68	11661	0.0154	1	0.014	15	0.0358
			0.5	2	2873	5746											
	2	2	0.5	1	2899	5798	5822	11644	0.1	176403	6.60	11525	0.0152	1	0.014	15	0.0354
			0.5	2	2923	5846											
	3	2	0.5	1	2782	5564	5584	11168	0.1	176403	6.33	11049	0.0146	1	0.014	15	0.0339
			0.5	2	2802	5604											
	4	2	0.5	1	2604	5208	5143	10286	0.1	176403	5.83	10167	0.0134	1	0.014	15	0.0312
			0.5	2	2539	5078											

Table C4. Aromatase Activity Calculation – In Vitro Microsomes – 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)	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 (initial)	% 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 (nmol estrogen formed/mg protein/min
Full activity control	1	2	0.5	1	3072	6144	6126	12252	0.1	181142	6.76	12109	0.0133	1	0.012	15	0.0364
			0.5	2	3054	6108											
	2	2	0.5	1	3081	6162	6152	12304	0.1	181142	6.79	12161	0.0133	1	0.012	15	0.0366
			0.5	2	3071	6142											
	3	2	0.5	1	3039	6078	6034	12068	0.1	181142	6.66	11925	0.0131	1	0.012	15	0.0359
			0.5	2	2995	5990											
	4	2	0.5	1	2972	5944	5932	11864	0.1	181142	6.55	11721	0.0129	1	0.012	15	0.0352
			0.5	2	2960	5920											
Background control	1	2	0.5	1	35	70	58	116	0.1	181142	0.06	-28	0.0000	1	0.012	15	-0.0001
			0.5	2	23	46											
	2	2	0.5	1	30	60	60	120	0.1	181142	0.07	-24	0.0000	1	0.012	15	-0.0001
			0.5	2	30	60											
	3	2	0.5	1	38	76	73	146	0.1	181142	0.08	3	0.0000	1	0.012	15	0.0000
			0.5	2	35	70											
	4	2	0.5	1	51	102	96	192	0.1	181142	0.11	49	0.0001	1	0.012	15	0.0001
			0.5	2	45	90											

Table C4. Aromatase Activity Calculation – In Vitro Microsomes – Replicate 2 (continued)

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 (initial)	% 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 (nmol estrogen formed/mg protein/min
Positive control	1	2	0.5	1	1434	2868	2788	5576	0.1	181142	3.08	5433	0.0060	1	0.012	15	0.0163
			0.5	2	1354	2708											
	2	2	0.5	1	1415	2830	2813	5626	0.1	181142	3.11	5483	0.0060	1	0.012	15	0.0165
			0.5	2	1398	2796											
	3	2	0.5	1	1309	2618	2641	5282	0.1	181142	2.92	5139	0.0056	1	0.012	15	0.0155
			0.5	2	1332	2664											
	4	2	0.5	1	1381	2762	2826	5652	0.1	181142	3.12	5509	0.0060	1	0.012	15	0.0166
			0.5	2	1445	2890											
Negative Control	1	2	0.5	1	3064	6128	6170	12340	0.1	181142	6.81	12197	0.0134	1	0.012	15	0.0367
			0.5	2	3106	6212											
	2	2	0.5	1	3060	6120	6045	12090	0.1	181142	6.67	11947	0.0131	1	0.012	15	0.0359
			0.5	2	2985	5970											
	3	2	0.5	1	3076	6152	6094	12188	0.1	181142	6.73	12045	0.0132	1	0.012	15	0.0362
			0.5	2	3018	6036											
	4	2	0.5	1	3172	6344	6234	12468	0.1	181142	6.88	12325	0.0135	1	0.012	15	0.0371
			0.5	2	3062	6124											

Appendix D

Chemistry Reports (from Battelle)

ANALYTICAL CHEMISTRY ACTIVITIES REPORT

4-HYDROXYANDROSTENEDIONE (4-OH ASDN)

CAS No.: 566-48-3

Lot No.: 063K4069 (Sigma Aldrich)

Receipt Date: 10/22/04

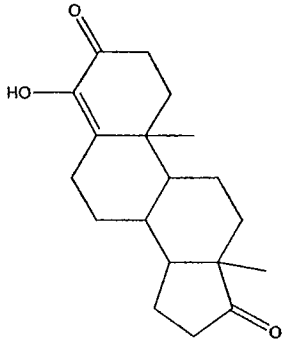
Amount Received: 3.1 g

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 12/9/05

Denise A. Contos, M.S.

Approved By:

Steven W. Graves

Steven W. Graves, B.S.

Manager, Chemistry Technical Center

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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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.

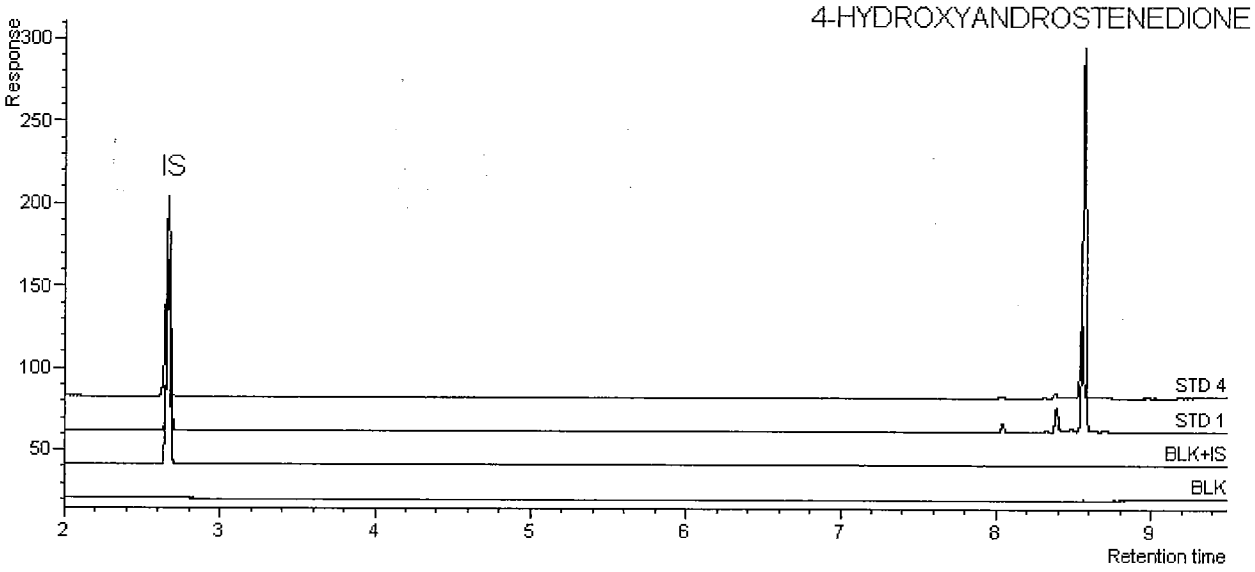


Figure 2 – Representative Overlaid Chromatograms from a Low and High Vehicle/Calibration Standard, Blank with IS, and Blank from the Validation (Shown Top to Bottom)

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
	496.8				-1.9	
506.4	494.5	509.6	24.2	4.7	-2.3	0.6
	537.5				6.1	
298.1	289.4	NA	NA	NA	-2.9	NA
202.6	198.8	NA	NA	NA	-1.9	NA
	100.7				1.3	
99.38	99.89	100.4	0.4	0.4	0.5	1.0
	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

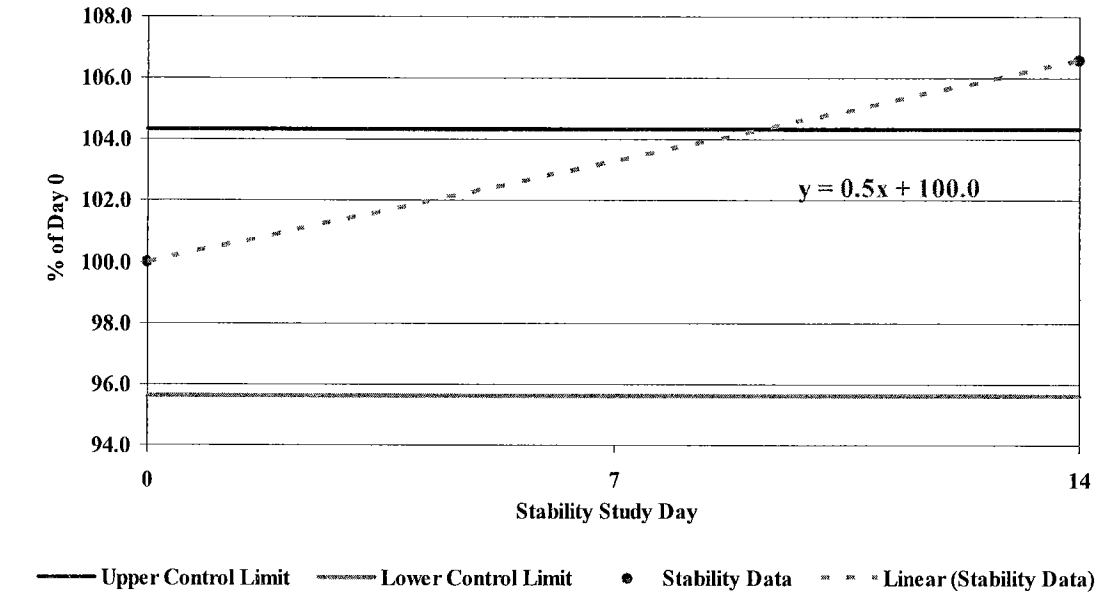
The results from the storage stability study are shown in Table 5 and presented in control chart format in Figure 3.

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.



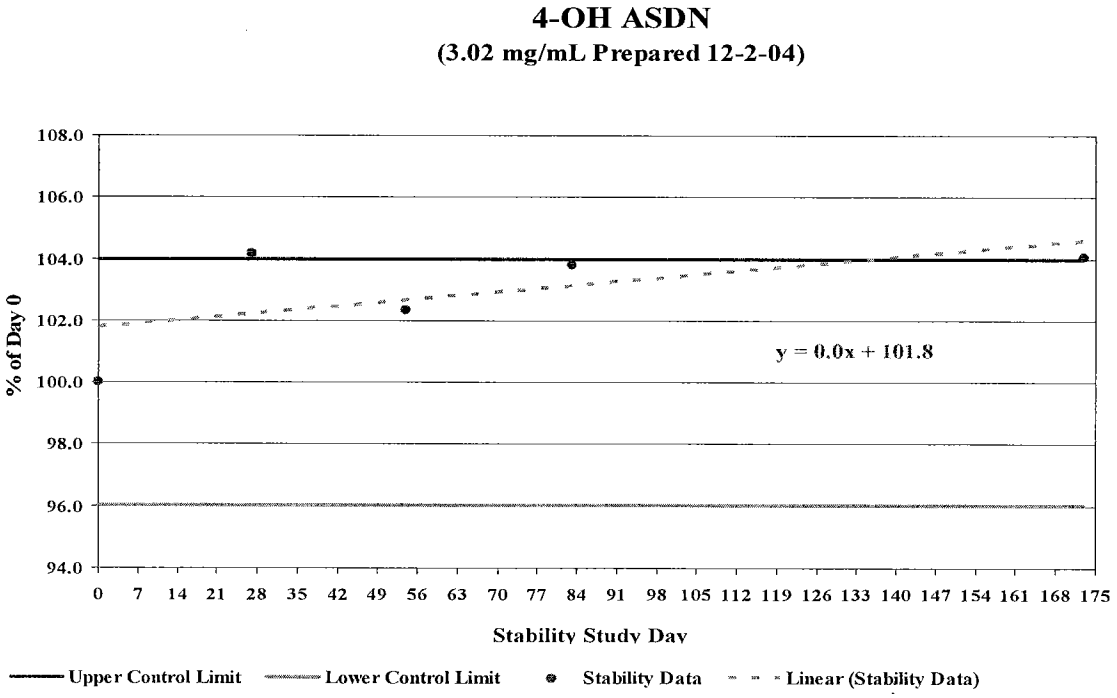


Figure 3 – Control Charts for the Storage Stability Studies

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.

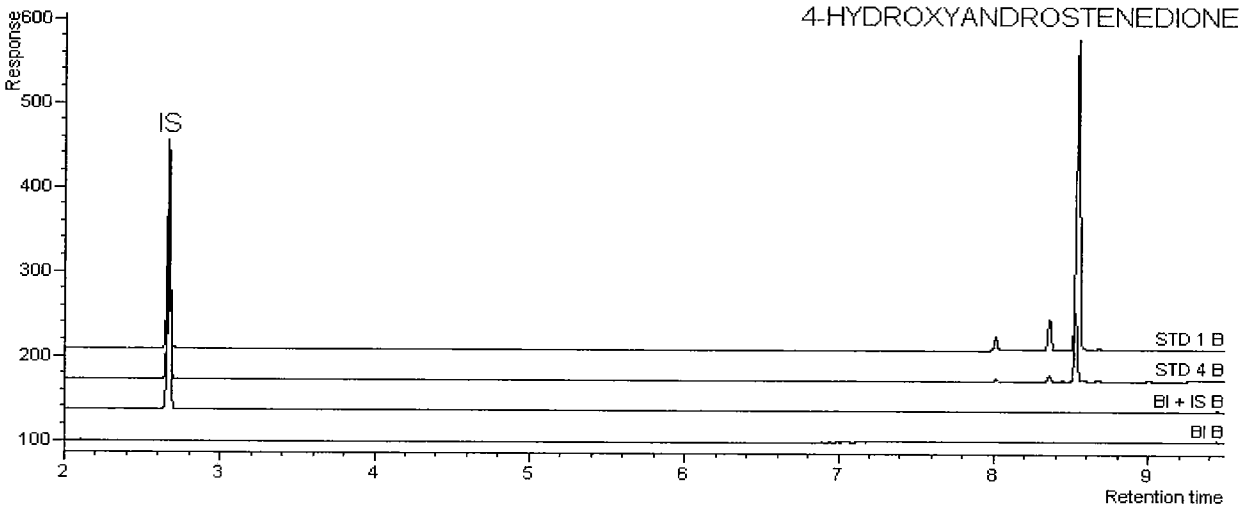


Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from Formulation Analysis

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 $\leq$ 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.

ANALYTICAL CHEMISTRY ACTIVITIES REPORT

LINDANE

CAS No.: 58-89-9

Lot No.: 14419EB (Sigma Aldrich)

Receipt Date: 1/6/05

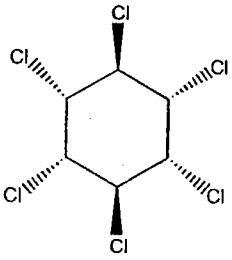
Amount Received: 10 g

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

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 C. 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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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.

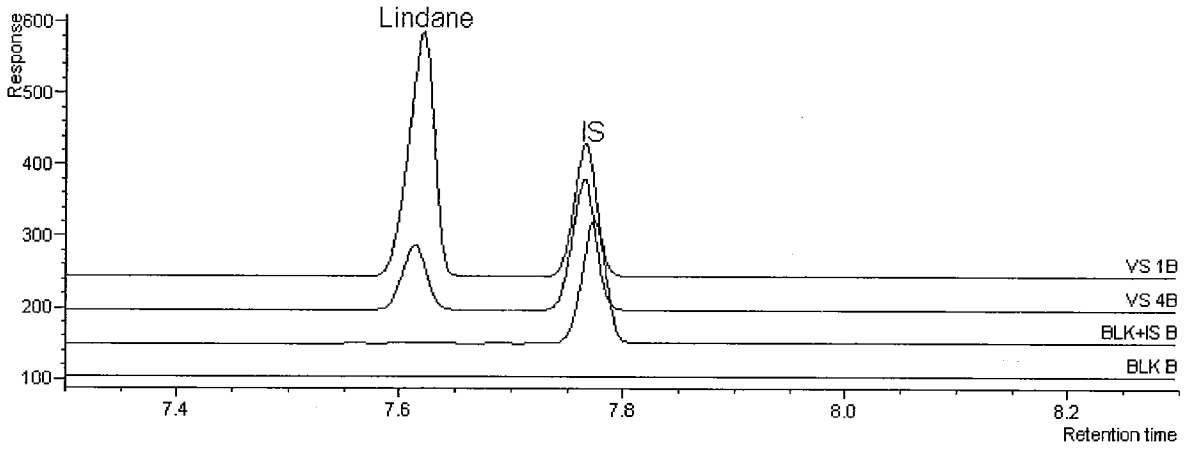


Figure 2 – Representative Overlaid Chromatograms from a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from the Validation (Shown Top to Bottom)

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
	777.3				0.1	
776.3	777.6	776.8	1.1	0.1	0.2	0.1
	775.6				-0.1	
600.2	598.4	NA	NA	NA	-0.3	NA
388.2	387.0	NA	NA	NA	-0.3	NA
	202.8				1.4	
200.1	200.1	200.5	2.1	1.1	0.0	0.2
	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.

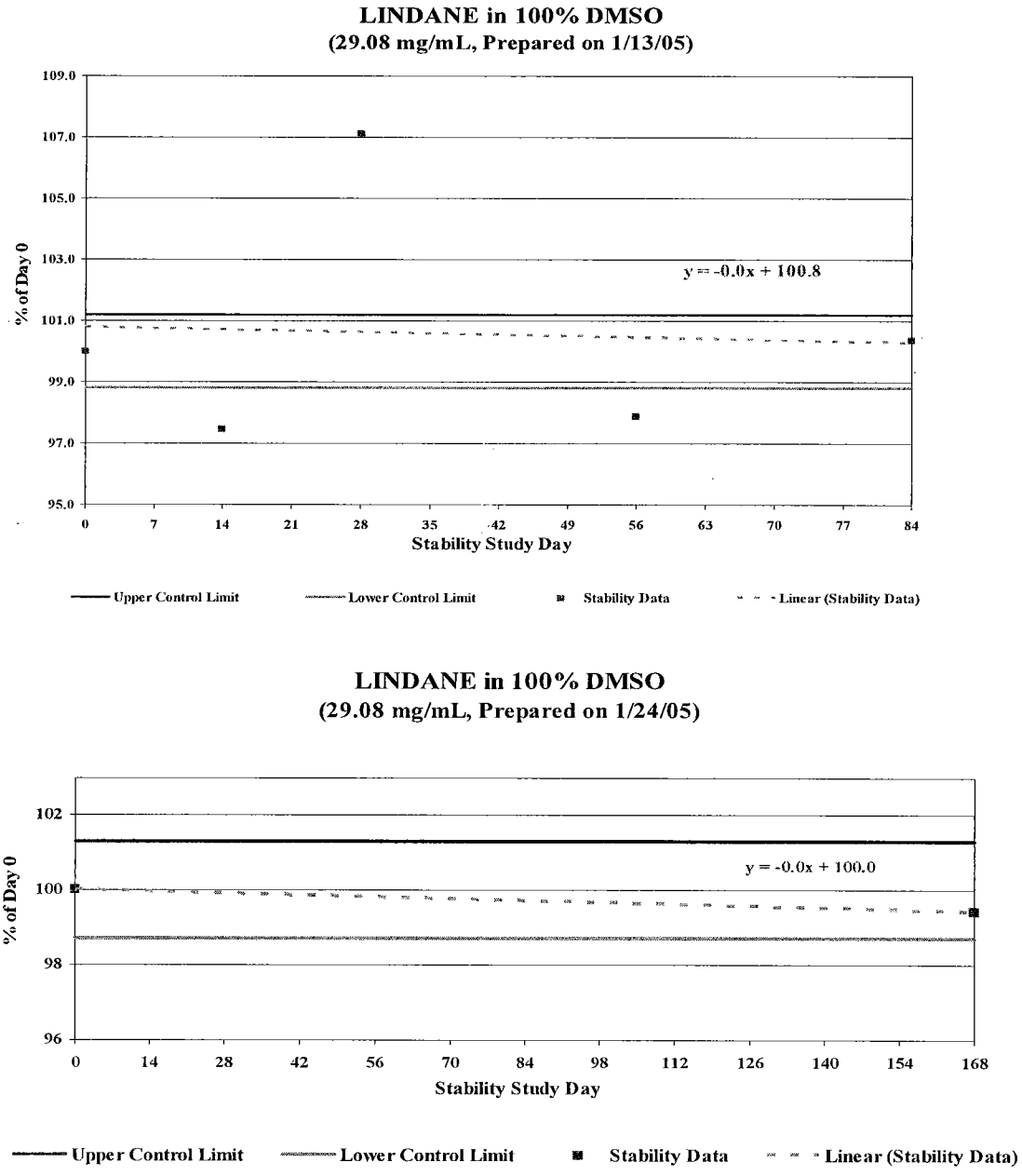


Figure 3 – Control Chart for the Storage Stability Study

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.

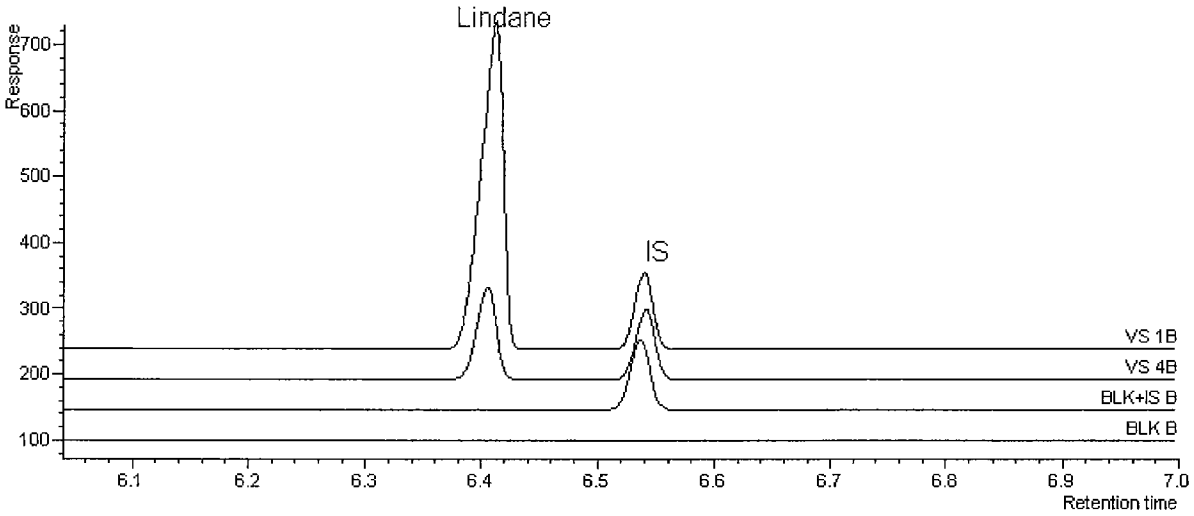


Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from a Formulation Analysis (Shown Top to Bottom)

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

[^{3}H]ASDN Purity Assessment Report

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

RTI INTERNATIONAL

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 J 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 — 9/28/2005

K. Collier
Quality Assurance Specialist

Date

Approval:

Carrie Ingalls — 09/28/2005

Carrie Ingalls
Quality Assurance Assistant Manager

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).

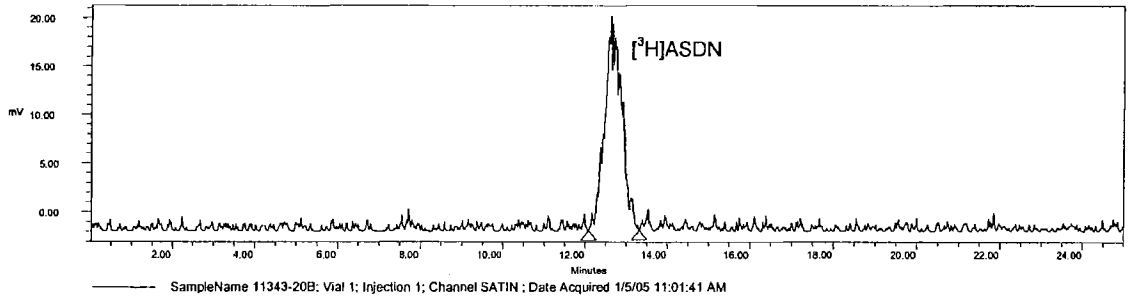
The radiochemical purity of the [^{3}H]ASDN (1:100 dilution in ethanol) was determined using high performance liquid chromatography (HPLC) and liquid scintillation counting. The HPLC system consists of a Waters 2690 Separations Module, a Waters 2487 Dual λ Absorbance Detector and a β-RAM Model 3 flow-through 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 F

Statistician's Report

Report on the Statistical Analysis for WA 4-16, Task 6

Microsomal Characterization within and between Laboratories (as described in Section 5.2.2 of the protocol)

Intralaboratory Analysis

In order to assess differences between the two microsome sources, two-sample t-tests were performed on the protein concentration results. Table 1 presents summary statistics of the protein concentrations by laboratory microsome source. Table 2 presents the test results from the two-sample t-tests.

Table 1. Summary Statistics of Protein Concentrations by Laboratory

Lab	Mean	Standard Error	Lower 95% CL	Upper 95% CL
Battelle	24.0557	1.3142	20.8399	27.2715
In Vitro	9.3570	1.3142	6.1412	12.5728

Table 2. T-Test Results Comparing Protein Concentration by Laboratory

Lab 1	Lab 2	Difference of Means	Standard Error	DF	T Statistic	P-Value
Battelle	In Vitro	14.6987	1.8586	6	7.91	0.0002

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

Two-sample t-tests were also conducted on the aromatase activity results for each control type. In order to appropriately account for the experimental design used in the assay, a mixed effects model was used in SAS to include the fixed and random effects. The following model was used in SAS PROC MIXED to account for the sources of variability within each of the four control types:

$$\text{Aromatase Activity}_{ijk} = \beta_0 + \beta_1 \times \text{Laboratory}_i + \alpha_{ij} \times \text{Replicate}_{ij} + \varepsilon_{ijk}$$

where laboratory is treated as a fixed effect, replicate is treated as a random effect and ε_{ijk} is the random variation associated with the repeated samples (residual). The covariance parameter estimates for the random effects components in the model, replicate and residual, by control type are provided in Table 3. The summary statistics of the aromatase activity by control type and laboratory microsome source are provided in Table 4. The results of the two-sample t-tests

comparing aromatase activity between the laboratory microsome sources within each control type are shown in Table 5.

Table 3. Covariance Parameter Estimates for Random Effects in Mixed Effects Model

Control Type	Covariance Parameter	Estimate	Standard Error
Background control	Replicate	1E-30	.
Background control	Residual	1.377E-8	0
Full activity control	Replicate	0.000035	0.000053
Full activity control	Residual	0.000020	7.847E-6
Negative Control	Replicate	0.000016	0.000028
Negative Control	Residual	0.000026	0.000010
Positive control	Replicate	8.538E-6	0.000013
Positive control	Residual	4.395E-6	1.724E-6

Table 4. Summary Statistics of Aromatase Activity by Control Type and Laboratory

Control Type	Lab	Mean	Standard Error	Lower 95% CL	Upper 95% CL
Background control	Battelle	-315E-23	0.000041	-0.00009	0.000090
Background control	In Vitro	-117E-24	0.000041	-0.00009	0.000090
Full activity control	Battelle	0.05626	0.004482	0.04657	0.06594
Full activity control	In Vitro	0.03649	0.004482	0.02681	0.04617
Negative Control	Battelle	0.05498	0.003375	0.04769	0.06227
Negative Control	In Vitro	0.03529	0.003375	0.02800	0.04258
Positive control	Battelle	0.02675	0.002195	0.02201	0.03149
Positive control	In Vitro	0.01674	0.002195	0.01200	0.02148

Table 5. T-Test Results Comparing Aromatase Activity by Laboratory Within Control Type

Control Type	Lab 1	Lab 2	Difference of Means	Standard Error	DF	T Statistic	P-Value
Background control	Battelle	In Vitro	-303E-23	0.000059	13	-0.00	1.0000
Full activity control	Battelle	In Vitro	0.01977	0.002236	13	8.84	<.0001
Negative Control	Battelle	In Vitro	0.01969	0.002562	13	7.69	<.0001
Positive control	Battelle	In Vitro	0.01001	0.001048	13	9.55	<.0001

The t-test results in Table 5 indicate that there were statistically very significant differences between the aromatase activity values from each of the laboratories for the full activity control, negative control and positive control. The background control aromatase activity values showed very little statistical significance in the difference of the means from each of the two laboratories.

APPENDIX D

WIL RESEARCH LABORATORIES REPORT

DRAFT TASK REPORT

VALIDATION OF THE PLACENTAL AROMATASE ASSAY USING PREPARED MICROSOMES (WA 4-16, TASK 6)

WA 4-16 Task 6: Prepare/Analyze Microsomes and Conduct Positive Control Study at Two Participating Laboratories; Analyze Microsomes at Each Laboratory

EPA Contract Number 68-W-01-023
Work Assignment 4-16
WIL-431008

Sponsor:

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

Performing Laboratory:

WIL Research Laboratories, LLC
1407 George Road
Ashland, OH 44805-9281

DRAFT REPORT

Title:	VALIDATION OF THE PLACENTAL AROMATASE ASSAY USING PREPARED MICROSOMES (WA 4-16, TASK 6)
Task 6:	Prepare/Analyze Microsomes and Conduct Positive Control Study at Two Participating Laboratories; Analyze Microsomes at Each Laboratory
Authors:	Jennifer A. Thomas-Wohlever, Ph.D. Justin Godsey, B.S., L.A.T.G.
Performing Laboratory:	WIL Research Laboratories, LLC 1407 George Road Ashland, OH 44805-9281
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:	April 25, 2005
Experimental Start Date:	June 1, 2005
Experimental Termination Date:	July 1, 2005
Draft Task Report Date	September 15, 2005

Quality Assurance Statements

The study was conducted in compliance and audited in accordance with the United States Environmental Protection Agency (EPA) Good Laboratory Practice Standards (40 CFR Part 160), October 16, 1989; the United States EPA Good Laboratory Practice Standards (40 CFR Part 792), September 18, 1989; the standard operating procedures of WIL Research Laboratories, LLC, and the protocol and protocol amendment as approved by the Sponsor, with the following exceptions. The Sponsor has assured GLP compliance of the initial chemical analyses (Appendix E) of the bulk chemicals for identity and purity and the preparation of stock formulations.

Intralaboratory data requiring statistical analysis were analyzed by BioSTAT Consultants, Inc., following the current procedural guidelines of BioSTAT Consultants, Inc. BioSTAT Consultants, Inc. provided a statistical analysis report, which is included as Appendix G. Quality Assurance auditing of the statistical report (for internal consistency with the study report) was conducted under the direction of the Quality Assurance Unit of WIL Research Laboratories, LLC.

Quality Assurance findings, derived from the inspections during the conduct of the study and from the inspections of the raw data and draft report are documented and have been reported to the Study Director. A status report is submitted to management monthly. This report accurately reflects the data generated during the study. The methods and procedures used in the study were those specified in the protocol, its amendments and the standard operating procedures of WIL Research Laboratories, LLC.

The raw data and draft report were audited by the WIL Quality Assurance Unit prior to submission to the Sponsor to assure that the Final Report accurately describes the conduct and the findings of the study. Quality control (QC) and quality assurance (QA) procedures followed those outlined in the Quality Assurance Project Plan (QAPP) that was prepared for this study (Appendix C).

Phases Inspected

Date of Inspection	Phases Inspected	Date(s) Findings Reported to Study Director	Date(s) Findings Reported to Management	Auditors
1-Jun-05	Protein and Aromatase Assays for Day 1	1-Jun-05	25-Jul-05	E.Crawford
24-Sep-2005, 25-Sep-2005	Draft Report	26-Sep-05	Oct-05	E.Crawford
13-Jul-2005, 14-Jul-2005, 16-Jul-2005, 17-Jul-2005, 18-Jul-2005, 19-Jul-2005	Study Records A-1 and Worksheet	19-Jul-05	25-Aug-05	E.Crawford
29-Mar-2005, 30-Mar-2005	Protocol Review	30-Mar-05	23-Apr-05	L.Goodrich
21-Apr-05	Protocol Review (Revised protocol)	21-Apr-05	28-May-05	L.Goodrich
20-May-05	Amendment I Review	20-May-05	29-Jun-05	L.Goodrich

Approval

This study was inspected according to the criteria discussed above.

Report Audited By:

Elizabeth S. Crawford, B.S. Date
Compliance Specialist

Report Released By:

Heather L. Osborn, B.S., RQAP-GLP Date
Manager, Quality Assurance

Compliance Statement

This study, designated WIL-431008, was conducted in compliance with the United States Environmental Protection Agency (EPA) Good Laboratory Practice Standards (40 CFR Part 160), October 16, 1989; the United States Environmental Protection Agency (EPA) Good Laboratory Practice Standards (40 CFR Part 792), September 18, 1989; the standard operating procedures of WIL Research Laboratories, LLC, and the protocol as approved by the sponsor.

_____ _____

Jennifer Thomas-Wohlever, Ph.D. Date
Research Scientist, Metabolism
Study Director

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1.0 Executive Summary

The placental microsomal aromatase assay combines microsomes, substrate, appropriate cofactors and test chemicals in a common reaction vessel under optimized conditions for the enzyme. The effect of the test chemicals on microsomal enzyme activity is evaluated by measuring the amount of product formed by the enzyme-catalyzed substrate oxidation. The general purpose of this assay is to screen chemicals for their ability to inhibit aromatase activity, an indication of the chemical's potential to disrupt endocrine function. This specific study was undertaken to demonstrate the conduct and responsiveness of the placental microsome aromatase assay at WIL Research Laboratories, LLC (a participating laboratory in the inter-laboratory validation) using microsomes prepared at Battelle Memorial Institute (Columbus, OH) or In Vitro Technologies, Inc. (Baltimore, MD).

The results of this control study using prepared placental microsomes were as expected based on the prevalidation work (WA 2-24 and 4-10), the training data (WA 4-16, Task 3) and previous validation work at WIL Research Laboratories, LLC (WA 4-16, Task 4, WIL-431006 and Task 5, WIL-431007). Generally, the Full Enzyme and Background Activity Controls demonstrated that the conditions were constant throughout each successful replicate test and that there was no background activity that might interfere with the interpretation of the results. Concentrations of 4-OH ASDN near the IC_{50} successfully inhibited approximately 50% of the enzymatic activity (positive control). The addition of lindane, known not to inhibit aromatase, had no effect on the enzymatic activity (negative control). The microsomal preparations from each laboratory had differing stock concentrations, which were adjusted by dilution for the assay. Microsomes from Battelle had an average activity of 0.0708 nmol/mg protein/min, while the average activity of the microsomes prepared at In Vitro Technologies was 0.0444 nmol/mg protein/min. Additional testing of these prepared placental microsomes using 10 potential aromatase inhibitors will be evaluated under WA 4-16, Task 7, WIL-431009.

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 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 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 or recombinant 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 recombinant 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 recombinant 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 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 placental aromatase (WA 2-24) were conducted to optimize the microsomal aromatase assay protocol for recombinant 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 objective of the current work assignment is to use the now optimized assay to obtain intra- and inter-laboratory assay variability estimates to further the validation of the human placental microsome aromatase assay using microsomes prepared at two laboratories.

2.2 Task Description and Objectives

WIL Research Laboratories, LLC (as one of the participating laboratories) will demonstrate the activity of placental microsomes prepared at two other laboratories, Battelle and In Vitro Technologies.

3.0 Materials and Methods

3.1 Substrate

The substrate for the aromatase assay was androstenedione (ASDN). Non-radiolabeled and radiolabeled ASDN were used. The non-radiolabeled ASDN (Lot # 024K0809) was obtained from Sigma (St. Louis, MO) by the Sponsor's Chemical Repository and was then distributed to the participating laboratories. It had a reported purity of 100%. The radiolabeled ASDN ([1β-^{3}H]-androstenedione, [^{3}H]ASDN, Lot # 3538-496), was obtained from Perkin Elmer Life Science (Boston, MA) and had a reported specific activity of 25.30 Ci/mmol. Radiochemical purity was reported by the supplier to be > 97%. Radiochemical purity was assessed by high pressure liquid chromatography (HPLC) by RTI International. The results of this analysis are presented in the report contained in Appendix D.

A mixture of ASDN and [^{3}H]ASDN was made such that the final concentration of ASDN in the assay was 100 nM, and each assay contained 0.1 µCi tritium. This was accomplished by preparing a 100-fold dilution of the radiolabeled stock in buffer. In addition, a 1 mg/mL solution of ASDN in ethanol was prepared, and then dilutions of stock ASDN were made in buffer to a final concentration of 1 µg/mL. To make 8 mL of substrate solution (enough for 80 tubes), 4.5 mL of the 1 µg/mL solution of ASDN, 800 µL of the [^{3}H]ASDN dilution and 2.7 mL of buffer were combined. For accuracy, the weight of each component added to the substrate solution was recorded. To determine the specific activity of the ASDN substrate, aliquots of substrate solution (approximately 20 µL, weighed) were combined with scintillation cocktail for radiochemical content analysis.

3.2 Test and 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. These chemistry activities and results are described in the Sponsor's Chemistry report which are appended to this document (Appendix E).

When the test chemicals arrived at WIL Research Laboratories, LLC, they were assigned a unique code number (MET-XXXXY, e.g., MET-0252A), which was recorded and dated on the log-in sheet as specified in WIL standard operating procedures. Also recorded on the log-in sheet was the label identification information, quantity received, storage conditions, storage location and a physical description of the material. Any documents accompanying the shipment were filed with the log-in sheet.

Test Chemical Information								
Chemical name	Chemical code	Mfr. Purity	CAS No.	Molecular formula	M.W.	Stock Solution (WIL ID)	Vehicle	Storage
4-Androsten-4-ol-3,17 dione	4-OH ASDN (Positive Control)	99%	566-48-3 Lot #: 063K4069	$C_{19}H_{26}O_3$	302.4	MET-0278A	95% Ethanol Lot #: 04H23QB	~5°C
Lindane	Negative Control	99.6%	58-89-9 Lot #: 14419EB	$C_6H_6Cl_6$	290.8	MET-0279A	DMSO Lot # 2969A46428	~5°C

3.3 Microsomes

Human placental microsomes were received as multiple frozen aliquots from participating laboratories. Upon receipt the sample code numbers MET-0296A and MET-0288A were assigned to the microsomes from In Vitro Technologies and Battelle, respectively. The microsomes were stored between approximately -70 to -80°C. On the day of the assay, microsomes were thawed rapidly in a 37 ± 1°C water bath, re-homogenized by briefly vortex mixing and then kept on ice until used. Separate aliquots of microsomes were used for each assay.

3.4 Other Assay Components

Assay Reagents - Information		
Chemical	Supplier	Lot Number
NADPH	Sigma-Aldrich	103K7046
Propylene glycol	J.T. Baker	Y41659
Sodium phosphate dibasic	J.T. Baker	A11H37
Sodium phosphate monobasic	J.T. Baker	A28H21
Test/control vehicle A – Ethanol, 95%	Sponsor	04H23QB

3.4.1 NADPH

Nicotinamide adenine dinucleotide phosphate (NADPH, reduced form tetrasodium salt) is a required cofactor for aromatase activity. As such, it was included in excess in the aromatase assay. First, 0.025 g NADPH was weighed and transferred into a 5-mL volumetric flask to make a 5 mg/mL solution in phosphate buffer (see below). Adding 100 µL of this NADPH solution to the reaction mixture resulted in a final assay concentration of 0.3 mM. NADPH was prepared fresh every assay day and was stored on ice until adding to the reaction mixture.

3.4.2 Assay Buffer

Sodium phosphate monobasic and sodium phosphate dibasic solutions (0.1 M each) were combined in an approximate 2:8 ratio to create a final 0.1 M, pH 7.4 solution. The assay buffer was stored refrigerated for up to one month.

3.5 Protein Determination

The protein concentration of the microsome preparation was determined on each day of use of the microsomes in the aromatase assay. The protein standards were made from bovine serum albumin (BSA). Protein was determined by using a DC Protein Assay kit purchased from Bio-Rad (Hercules, CA). To a 200-µL aliquot of unknown, standard, or quality control (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 to each standard or unknown and the samples were vortex mixed. The samples were allowed to sit at room temperature for at least 15 minutes to allow for color development. The absorbance is stable for about 1 hour. Each sample (unknown and standards) was transferred to appropriate cuvettes and the absorbance (750 nm) was measured using a spectrophotometer. Six protein standards ranging in concentration from 5 to 250 µg/mL BSA were used to generate the standard curve for the protein assay. The plot of concentration versus absorbance was slightly non-linear at the highest protein concentration, therefore the calibration curve was fitted to the data from 5 to 250 µg/mL BSA and to an abbreviated data set with concentrations of 5 to 125 µg/mL BSA. Two quality control (QC; 10 and 100 µg/mL BSA) standards were included in each protein assay and the experimental concentration of BSA was determined from both calibration curves. The calibration curve that resulted in the most accurate response for the QC standards was used to determine the protein concentration of the microsomal preparation.

3.6 Cytochrome P450 Aromatase (CYP19) Activity

Aromatase activity was determined via an *in vitro* screening assay. The assays were performed in 13 x 100 mm test tubes. Each test tube was uniquely identified with replicate, repetition and group information as necessary to differentiate the tubes. The assay design is summarized in the following table. Full Enzyme Activity Controls (includes vehicle but no inhibitor) and Background Activity Controls (tubes contain no NADPH cofactor) were utilized to determine 100% and 0% activity. Positive and negative controls confirmed that aromatase activity in the prepared microsomes was inhibited by 4-OH ASDN and not by lindane as expected.

Assay Design			
Sample Type/Group	Repetitions (test tubes)	Description	Test Chemical Concentration (M final)
Full Enzyme Activity Control-Beginning	2	Complete assay[a] with reference chemical vehicle control	N/A
Background Activity Control-Beginning	2	Complete assay with reference chemical vehicle control omitting NADPH	N/A
Positive Control – Beginning	2	Complete Assay with 4-OH ASDN added	5×10^{-8}
Negative Control – Beginning	2	Complete Assay with Lindane added	$1x\ 10^{-6}$
Full Enzyme Activity Control-End	2	Complete assay with reference chemical vehicle control	N/A
Background Activity Control-End	2	Complete assay with reference chemical vehicle control omitting NADPH	N/A
Positive Control – End	2	Complete Assay with 4-OH ASDN added	5×10^{-8}
Negative Control – End	2	Complete Assay with Lindane added	$1x\ 10^{-6}$

a = The complete assay contains buffer, propylene glycol, microsomal protein, substrate and NADPH
N/A = Not Applicable

Propylene glycol (100 µL), ASDN substrate solution (100 µL), NADPH (100 µL, excluded from background control) and vehicle or test chemical (20 µL) were added to the appropriate test tube with buffer for a total volume of 1 mL. Microsomes were diluted to the appropriate concentration and used within 2 hours of thawing. Both the reaction mixture and the microsomes were incubated at 37 ± 1°C independently for at least 5 minutes. After the addition of 1 mL microsomes to the first test tube containing the reaction mixture, the remaining assays were initiated at 15 second intervals. Each assay was incubated at 37 ± 1°C for 15 minutes. At the conclusion of the reaction time, tubes were quenched with 2.0 mL of methylene chloride in the order in which microsomes had been added, one every 15 seconds. The tubes were vortex-mixed for approximately 5 seconds and placed on ice until all tubes were quenched. The tubes were then vortex-mixed an additional 20-25 seconds. The tubes were centrifuged for 10 minutes at approximately 162 x g to facilitate separation of the organic and aqueous layers. The methylene chloride layer was removed and discarded; the aqueous layers were extracted again with methylene chloride (2 mL). This extraction procedure was performed one additional time. 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, approximately 10 mL) was added to each counting vial and shaken to mix the solution.

Analysis of the samples was performed using a liquid scintillation counter (LSC). Radiolabel found in the aqueous fractions represents ^{3}HOH formed. One ^{3}HOH molecule is released per molecule of radiolabeled ASDN converted to estrogen in a stereospecific reaction. Thus, the amount of estrogen product formed is determined by dividing the total amount of ^{3}HOH formed by the specific activity of the [^{3}H]ASDN substrate (expressed in DPM/nmol). Results were presented as the activity (velocity) of the enzyme reaction. The activity of the enzyme reaction is expressed in nmol/mg protein/min and was calculated by dividing the amount of estrogen formed by the product of mg microsomal protein used and incubation time, e.g. 15 minutes.

Each assay replicate was performed on the day shown in the table below. The same technician performed each replicate.

Assay Dates by Technician		
Replicate	Date	Technician
Battelle Day 1	6/1/2005	JG
Battelle Day 2	6/2/2005	JG
In Vitro Day 1	6/30/2005	JG
In Vitro Day 2	7/1/2005	JG

3.7 Data Analysis

Relevant data were entered into the latest version of the Microsoft® Excel spreadsheet Aromatase_Master_Version x.y.xls (where x and y denote version number designation) for calculation of aromatase activity and percent of control. Data recorded included assay date and run number, technician, chemical and log chemical concentration, total DPM-background DPM and percent activity. For each aqueous sample after extraction, the spreadsheet calculated DPM/mL for each aliquot counted, average DPM/mL per aliquot and total sample DPM. Total assay DPM was calculated by the multiplication of the volume of substrate solution added to the incubation by the substrate solution radiochemical content (DPM/mL) and yielded the total DPM present in the assay tube at initiation. Background DPM was calculated as the average of the DPM present in the aqueous portion for the background tubes, and was subtracted from total sample DPM for all samples to provide DPM for calculating aromatase activity. This corrected DPM was then converted to nmol product formed by dividing by the substrate specific activity (DPM/nmol). The activity of the enzyme reaction was expressed in nmol/mg protein/min and was calculated by dividing the amount of estrogen formed (nmol) by the product of mg microsomal protein used times the incubation time.

The average activity in the Full Enzyme Activity Control samples for a given study was calculated. Percent of control activity remaining in the presence of various inhibitor concentrations was calculated by dividing the aromatase activity at a given concentration by the average Full Enzyme Activity Control and multiplying by 100. Thus, the average percent activity across the four Background Activity repeat tubes must necessarily equal 0 within each replicate and the average percent activity across the four full enzyme activity repeat tubes must necessarily equal 100 within each replicate.

3.8 Data Retention

The Sponsor has title to all documentation records, raw data, specimens or other work product generated during the performance of the study. All work product generated by WIL Research Laboratories, LLC, including raw paper data and pertinent electronic storage media, are retained in the Archives at WIL Research Laboratories, LLC, as specified in the study protocol. Data generated by BioSTAT Consultants, Inc. will be maintained in the archives at WIL Research Laboratories, LLC. Data generated by the Sponsor will be maintained as defined in the Sponsor's applicable standard operating procedures. Pertinent electronic storage media and the original final report are retained in the Archives at WIL Research Laboratories, LLC, in compliance with regulatory requirements.

4.0 Results

4.1 Radiochemical Purity

Purity Report: Appendix D

The radiochemical purity of the [^{3}H]ASDN was determined by RTI International to be 97%. The specific activity of the substrate for each replicate and the final concentration of the substrate (ASDN + [^{3}H]ASDN) are shown in the table below. The concentration and specific activity of the substrate was used to calculate the aromatase activity in the assay.

Assay Substrate Analysis Results					
Replicate	*Radiochemical Code*	*Radiochemical ID*	*Radiochemical Stock Concentration (µCi/g)*	*Assay Substrate Final Concentration* (µg/g) (ASDN + [^{3}H]ASDN)	*Substrate solution Specific Activity* (dpm/nmol)
Battelle Day 1	[^{3}H]ASDN	MET-0251A	0.699	0.578	768856
Battelle Day 2	[^{3}H]ASDN	MET-0251A	0.702	0.574	777386
In Vitro Day 1	[^{3}H]ASDN	MET-0251A	0.598	0.585	649842
In Vitro Day 2	[^{3}H]ASDN	MET-0251A	0.679	0.579	745123

4.2 Stock Formulation Analysis

Chemistry Report: Appendix E

Stock formulation analysis was performed by the sponsor, as reported in Appendix E. Briefly, solubility and formulation analyses showed that the 3.02 mg/mL 4-OH ASDN stock formulation in 95% ethanol provided to the laboratories for this study was within the acceptance criteria for both average concentration and percent relative standard deviation between analyses. The 29.08 mg/mL lindane in DMSO also met the acceptance criteria. Both formulations were stable for the duration of the study.

Test Chemical Stock Solution Results				
Test Chemical Code	Test Chemical ID	Target Stock Solution Concentration	Actual Stock Solution Concentration	Stock Solution Expiration Date
4-OH ASDN	MET-0278A	3.02 mg/mL	3.076 mg/mL	*To be included in final chem. report*
Lindane	MET-0279A	29.08 mg/mL	29.37 mg/mL	*To be included in final chem. report*

4.3 Protein Analysis (Microsomes)

There were two independent determinations of protein concentration of each microsomal preparation on each day that the microsomes were used in the assay. Analysis of the protein concentration determinations indicated that the concentrations were statistically different between laboratory preparations and would require different dilution factors for use in the microsomal assay.

Replicate	Assay Date	Used in Aromatase Assay?	Protein stock concentration (mg/mL, measured)
Battelle Day 1	6/1/2005	Y	19.479
Battelle Day 1	6/1/2005	N	19.687
Battelle Day 2	6/2/2005	Y	23.582
Battelle Day 2	6/2/2005	N	25.629
In Vitro Day 1	6/30/2005	Y	6.978
In Vitro Day 1	6/30/2005	N	8.689
In Vitro Day 2	7/1/2005	Y	8.229
In Vitro Day 2	7/1/2005	N	8.345

4.4 Control Aromatase Activity

Appendix F –Replicate Spreadsheets

Controls were conducted in two groups in duplicate on each of 2 days for a total of 8 replicates. The following table (Summary of Full Enzyme Activity Results) presents the individual full enzyme aromatase activity values for each replicate and the mean activities (nmol/mg protein/min). For microsomes prepared at Battelle, the mean value was 0.0708 nmol/mg protein/min. For microsomes prepared at In Vitro Technologies, the mean value was 0.0444 nmol/mg protein/min.

Full Enzyme Activity Control Aromatase Activity								
Replicate	nmol/mg protein/min				Within Replicate Mean (±sd, sem)	% CV	Overall Mean (±sd, sem)	Overall % CV
	Rep 1	Rep 2	Rep 3	Rep 4				
Battelle Day 1	0.0738	0.0729	0.0693	0.0724	0.0721 (0.0019, 0.0010)	2.68	0.0708 (0.0094, 0.0033)	13.29
Battelle Day 2	0.0907	0.0626	0.0627	0.0622	0.0695 (0.0141, 0.0070)	20.26		
In Vitro Day 1	0.0488	0.0490	0.0482	0.0463	0.0481 (0.0012, 0.0006)	2.51	0.0444 (0.0048, 0.0017)	10.81
In Vitro Day 2	0.0444	0.0414	0.0351	0.0415	0.0406 (0.0039, 0.0020)	9.64		

Background Activity Controls were conducted in duplicate at the beginning and end of each assay. The aromatase activity in these control samples was negligible, indicating that there was no background activity (potentially caused by nonspecific turnover of reactant to product, or unintentional NADPH contamination) that might interfere with the interpretation of the results.

The positive control was the known aromatase inhibitor 4-OH ASDN at a concentration (5×10^{-8} M) near its IC_{50} value. The microsomes from Battelle and from In Vitro Technologies were inhibited by approximately 50% in the presence of 4-OH ASDN (range of 40.9% to 47.0% of the Full Enzyme Activity Control). This is clearly illustrated in the following table. Lindane, which is known not to inhibit aromatase, was the negative control. As expected, the negative control activity is similar to the activity of the Full Enzyme Activity Controls. Therefore, the negative

control activity shown in the following table has a calculated percent of Full Enzyme Activity Control of 88.6% to 101.4%.

Positive Control Aromatase Activity									
Replicate	nmol/mg protein/min				Within Replicate Mean (±sd, sem)	% of Full Enzyme Activity Control	% CV	Overall Mean (±sd, sem)	Overall %CV
	Rep 1	Rep 2	Rep 3	Rep4					
Battelle Day 1	0.0342	0.0339	0.0327	0.0329	0.0334 (0.0007, 0.0004)	46.4	2.12	0.0309 (0.0027, 0.0010)	8.78
Battelle Day 2	0.0290	0.0284	0.0281	0.0283	0.0285 (0.0004, 0.0002)	40.9	1.42		
In Vitro Day 1	0.0230	0.0226	0.0221	0.0227	0.0226 (0.0004, 0.0002)	47.0	1.61	0.0208 (0.0019, 0.0007)	9.21
In Vitro Day 2	0.0194	0.0187	0.0191	0.0191	0.0190 (0.0003, 0.0001)	46.9	1.56		

Negative Control Aromatase Activity									
Replicate	nmol/mg protein/min				Within Replicate Mean (±sd, sem)	% of Full Enzyme Activity Control	% CV	Overall Mean (±sd, sem)	Overall %CV
	Rep 1	Rep 2	Rep 3	Rep4					
Battelle Day 1	0.0751	0.0733	0.0724	0.0716	0.0731 (0.0015, 0.0007)	101.4	2.04	0.0674 (0.0063, 0.0022)	9.41
Battelle Day 2	0.0634	0.0631	0.0591	0.0609	0.0616 (0.0020, 0.0010)	88.6	3.26		
In Vitro Day 1	0.0473	0.0469	0.0477	0.0462	0.0470 (0.0006, 0.0003)	97.8	1.34	0.0440 (0.0033, 0.0012)	7.56
In Vitro Day 2	0.0420	0.0397	0.0414	0.0407	0.0410 (0.0010, 0.0005)	100.8	2.38		

4.5 Statistical Analysis

Appendix G – Statistical Analysis Summary
Figure 1 Mean Aromatase Control Activity

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 from Battelle or from In Vitro Technologies.

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 Technologies based on the experimentally determined concentration and the number of samples 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 from In Vitro Technologies. Average activity of the microsomes from Battelle and In Vitro Technologies were compared by ANOVA with a fixed term for microsome source and a 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 Technologies. 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.

5.0 Discussion

Microsomes prepared at In Vitro Technologies and at Battelle were examined at WIL Research Laboratories, LLC for protein concentration, activity and response to test chemicals. The protein concentration of the microsome preparations were significantly different based on the site of preparation, as determined by a two-sample T-test. Therefore, different dilution factors had to be employed in order to use the microsomes in the aromatase assay. Once the protein concentrations were normalized to 0.0125 mg/mL in each assay tube, the Full Enzyme Activity of the In Vitro Technologies microsomes (in nmol/mg protein/min) was approximately 1/3 of the Full Enzyme Activity of the Battelle microsomes. This lesser activity may have been a result of using different placenta as the source of the enzyme, or the preparation technique. The difference in activity may also be attributed to the presence of inactive enzyme, which is included as part of the total concentration in the protein assay.

Despite the difference in Full Enzyme Activity, both microsome preparations responded in the expected manner to the presence of 4-OH ASDN and lindane. 4-OH ASDN decreased the activity of the microsomes by approximately 50% when included in the reaction mixture at 5×10^{-8} M, a concentration near the IC_{50}. The inclusion of lindane, a known non-inhibitor of aromatase, had no effect on the enzyme's activity. Background Control tubes had negligible enzyme activity, indicating that there was neither inadvertent contamination of control tubes by NADPH, nor substrate oxidation occurring non-enzymatically.

6.0 Conclusion

The response of the microsomes prepared at Battelle and In Vitro Technologies to the known inhibitor 4-OH ASDN and the known non-inhibitor lindane were successfully demonstrated, despite the difference in microsomal stock protein concentration and overall Full Enzyme Activity which may have been a result of the enzyme source and preparation technique. Thus, the results of this positive control study using microsomes prepared at participating laboratories in the presence of control chemicals were as expected based on the prevalidation work (WA 2-24 and 4-10) and the results of WA 4-16 Task 4 (WIL-431006) and Task 5 (WIL-431007). Further testing of the prepared placental microsomes using 10 additional inhibitors will be evaluated under WA 4-16 Task 7 (WIL-431009).

7.0 References

Bowman, C.J. WA 4-16, Task 4. Validation Of The Placental Aromatase Assay: Positive Control Study. (Study No. WIL 431006). WIL Research Laboratories, LLC, Ashland, OH, Draft

Thomas-Wohlever, J.A. WA 4-16, Task 5. Validation Of The Placental Aromatase Assay For Endocrine Disruptor Screening. (Study No. WIL 431007). WIL Research Laboratories, LLC, Ashland, OH, Draft

Thomas-Wohlever, J.A. WA 4-16, Task 7: Conduct Multiple Chemical Studies With Microsomes Prepared In Participating Laboratories. (Study No. WIL-431009). WIL Research Laboratories, LLC, Ashland, OH, Draft

8.0 Key Personnel and Report Submission

Report Submitted By:

Jennifer A. Thomas-Wohlever, Ph.D. Research Scientist, Metabolism Study Director	Date

Report Reviewed By:

Christopher J. Bowman, Ph.D. Staff Toxicologist, Developmental and Reproductive Toxicology	Date
Terry L. Johnson, Ph.D. Associate Director, Metabolism	Date
Mark D. Nemec, B.S., D.A.B.T. Director, Developmental and Reproductive Toxicology	Date
Daniel W. Sved, Ph.D. Director, Metabolism and Analytical Chemistry	Date
Robert A. Wally, B.S., RAC Acting Manager, Reporting and Regulatory Technical Services	Date

FIGURE 1

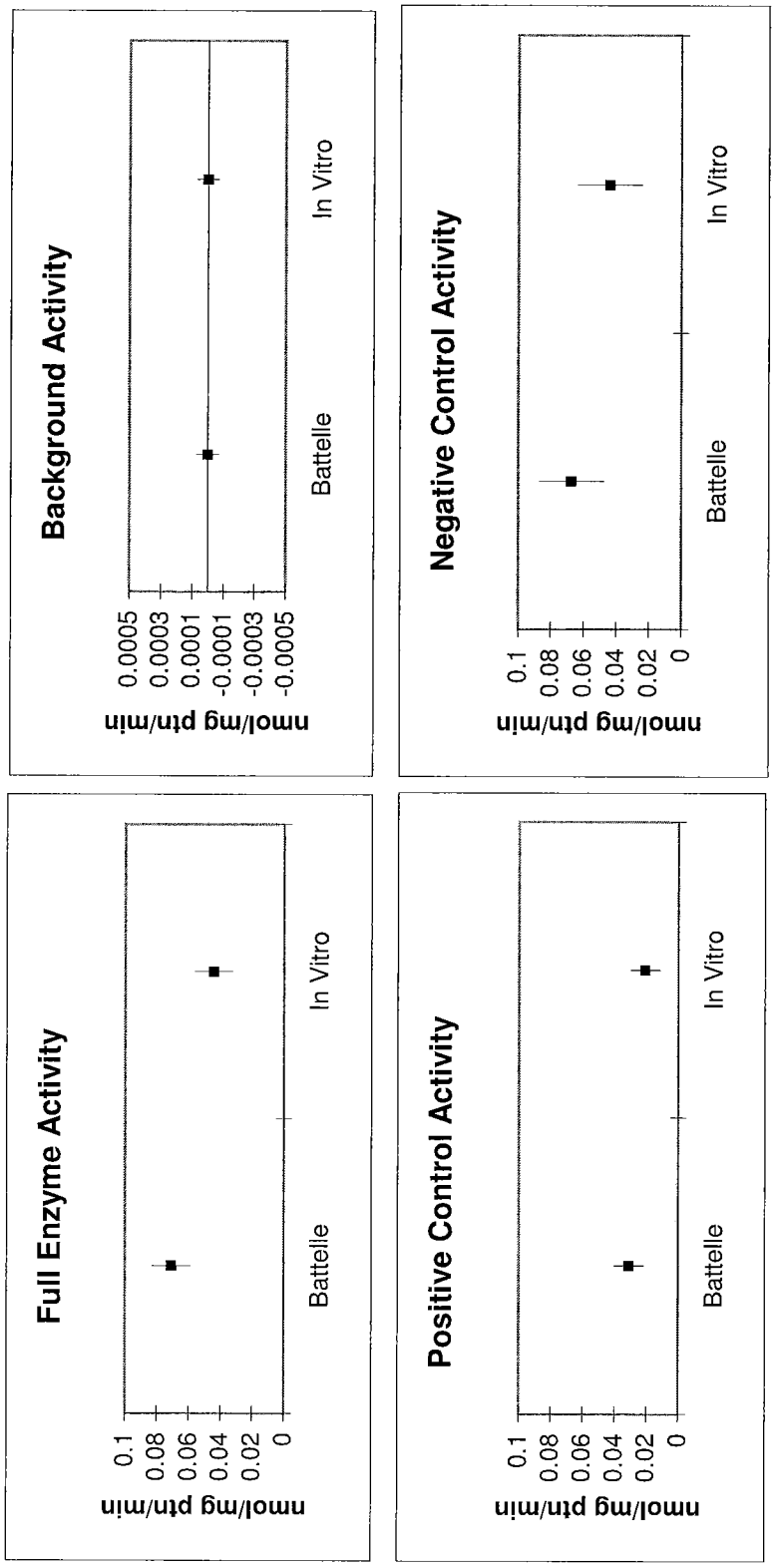

Figure 1. Mean aromatase control activity.

Appendix A

Study Protocol (with Amendment)

Study Number: WIL-431008

PROTOCOL AMENDMENT I

Sponsor: Battelle Memorial Institute

EPA Contract No.: 68-W-01-023

A. Title of Study:

Validation of the Placental Aromatase Assay using Prepared Microsomes (WA 4-16, Task 6).

B. Protocol Modifications:

1) **5.3** **Use of Human Placental Microsomes:**

The final paragraph of this section is changed to the following:

1. On the day of use, microsomes are thawed quickly in a 37 ± 1 °C water bath and then are immediately transferred to an ice bath. The microsomes will be rehomogenized using a Potter-Elvejhem homogenizer (chilled in ice bath, about 5-10 passes) or vortexed to mix (ca. 5 seconds) prior to use.

2. Prepare an appropriate dilution (ca. 0.025 mg/mL) in buffer for use in the aromatase assay and its associated protein assays. 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. For a microsome stock that contains ca. 8 mg/mL protein, a 1:320 dilution is recommended. Stock microsomes that contain 20 mg/mL protein should be diluted 1:800. In the event that the stock concentration differs from the examples given, the dilution factor will be adjusted as necessary to achieve a final concentration of approximately 0.025 mg/mL.

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 independent protein assay.

4. Be sure to prepare completely independent protein standards for each protein assay. Run all assays (aromatase plus two protein assays) required for a given tube of microsomes on the same day.

5. 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. Microsomes will not be left on ice for longer than approximately 1 hour before proceeding with the assay or microsomal enzyme activity may be decreased. The time of thaw and assay initiation time are documented in the study records. Under no conditions will microsomes that have been thawed or diluted be refrozen for later use in the assay.

2) **6 PROTEIN ASSAY:**

This section is changed to the following:

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 at a minimum from two independent assays on each aromatase assay day. Each lab should prepare a single batch of QC standards and prepare a sufficiently large batch such that aliquots can be taken from that single batch each time the assay is run during the conduct of Task 6. This will better help determine assay to assay variability within a lab.

Standard curve range: 5-250 µg/mL

Protein Assay Kit: BioRad DC Protein Assay Kit (Hercules, CA).

Prepare standards.

1. Prepare a 1:10 dilution of a 2.5 mg/mL stock solution of bovine serum albumin (BSA) – 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 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

Prepare QC samples using standards from Pierce (Woburn, MA).

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.

Assay procedure

1. Pipet 200 µL unknown or QC sample into each tube. (Standards 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.

6. The protein concentration of the microsomal samples will be determined by extrapolation of the absorbance value using the curve developed using the protein standards.

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

C. Reasons for Protocol Modifications

1. This change is made to allow all required experiments (four protein assays and two aromatase assays) to be conducted using a total of two tubes of microsomes.

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Protocol Amendment I
Page 4

2. These changes in the protein assay are made to improve the accuracy of the protein determination following the manufacturer's microassay instructions.

Approved By:

Jennifer Thomas-Wohlever, Ph.D. Date
Study Director
WIL Research Laboratories, LLC

5/31/05

Daniel W. Sved, Ph.D. Date
Director, Metabolism and
Analytical Chemistry
WIL Research Laboratories, LLC

5/31/04

Jerry D. Johnson, Ph.D., D.A.B.T. Date
Work Assignment Leader/Study Monitor
Endocrine Disruptor Screening Program
Battelle Memorial Institute

6-2-05

PROTOCOL

VALIDATION OF THE PLACENTAL AROMATASE ASSAY USING PREPARED MICROSOMES (WA 4-16, TASK 6)

EPA Contract No.: 68-W-01-023

Submitted To:

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

WIL Research Laboratories, LLC
1407 George Road
Ashland, OH 44805-9281

1 OBJECTIVE:

Task 6: The objectives of this protocol are to describe procedures for the preparation of human placental microsomes, the analysis of the 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. The primary responsibility of WIL Research Laboratories, LLC is to determine protein concentration and uninhibited aromatase activity of the microsomal preparation. Additional duties are described in the event that WIL is called upon to participate in these activities at a later date.

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.

There is no applicable route of administration in the sense of a dose administration route for this in vitro test. This in vitro test method involves combining microsomes, substrate, appropriate co-factors and test substances in a common reaction vessel. The effect of the control chemicals on microsomal enzyme activity is evaluated by measuring the amount of the product of the enzyme-catalyzed substrate oxidation that is formed.

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

In Stage 1, only two of the four laboratories participating in WA 4-16 Task 6 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. As one of the two remaining laboratories, 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 the event that WIL does not find it feasible to obtain a human placenta, WIL will only be responsible for performing stage 2 as described below.

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.

2 PERSONNEL INVOLVED IN THE STUDY:

2.1 Sponsor Representatives:

Jerry D. Johnson, Ph.D., D.A.B.T.
Work Assignment Leader/Study Monitor
Endocrine Disruptor Screening Program
Battelle Memorial Institute
Tel: (614) 424-4499
Fax: (614) 424-5221
Email: johnsojd@battelle.org

David P. Houchens, Ph.D.
Program Manager
Endocrine Disruptor Screening Program
Battelle Memorial Institute

2.2 U.S. EPA Representatives:

Gary E. Timm, M.S., M.A.
Work Assignment Manager
Endocrine Disruptor Screening Program
U.S. EPA

Linda Phillips, Ph.D.
Project Officer
U.S. EPA

2.3 WIL Study Director:

Jennifer Thomas-Wohlever, Ph.D.
Research Scientist
Tel: (419) 289-8700
Fax: (419) 289-3650
Email: jwohlever@wilresearch.com

2.4 WIL Deputy Director:

Justin Godsey, B.S., L.A.T.G.
Biologist, Metabolism

2.5 WIL Staff Involved with Study:

Joseph F. Holson, Ph.D.
President, Director

Daniel W. Sved, Ph.D.
Director, Metabolism and Analytical Chemistry

Terry L. Johnson, Ph.D.
Associate Director, Metabolism

Christopher J. Bowman, Ph.D.
Staff Toxicologist, Developmental
and Reproductive Toxicology

Heather L. Osborn, B.S., RQAP-GLP
Manager, Quality Assurance

Jay G. Henson, B.S.
Group Manager, Study Analysis and Reports

Pete Resnis, B.S.
Senior Research Chemist, Metabolism

Aimee Mahoney, B.S.
Group Supervisor, Metabolism

2.6 Statistical Analysis:

Les Freshwater, M.S.
BioSTAT Consultants, Inc.

2.7 Institutional Review Board (if required):

Western Institutional Review Board

3 STUDY SCHEDULE:

Proposed Experimental Start Date:	April 25, 2005
Proposed Experimental Termination Date:	May 2, 2005
Proposed Unaudited Data Submission Date:	May 9, 2005
Proposed Audited Report Date:	June13, 2005

4 TEST SUBSTANCE & CONTROL CHEMICAL DATA:

Reserve samples of the reference and control chemicals used in this study will be collected by the Sponsor and will be stored at the Sponsor's facility. Therefore, no reserve samples for this study will be collected by WIL Research Laboratories, LLC.

4.1 Control Chemicals For Determination of Uninhibited Enzyme Activity:

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

Control Chemical	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

Control substance stock solutions will be prepared and analyzed by Battelle's Chemical Repository (CR) and distributed to the laboratories. Lindane will be formulated in dimethylsulfoxide (DMSO) and 4-OH ASDN will be formulated in 95% ethanol. 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 solutions will be provided by the CR.

4.2 Test Substance For Determination of Aromatase Activity Response:

4-Hydroxyandrostenedione (4-OH ASDN) is a known aromatase inhibitor. 4-OH ASDN will be purchased from Sigma by the CR, formulated in 95% ethanol and stored at 2-8 °C. Other known or potential inhibitors may be tested. Test substance stock solutions will be prepared and analyzed by the CR and distributed to the laboratories with lot numbers and purity provided in an analysis report.

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 95% 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.

5 ASSAY MATERIALS RECEIPT AND/OR PREPARATION:

A sufficient supply of chemical reagents, radiolabeled androstenedione, and 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. The detailed procedures

for preparation of the assay substrate, assay buffer, microsomes and β-nicotinamide adenine dinucleotide phosphate, reduced form, tetrasodium salt (NADPH) solution will be documented in the study records.

The procedure for identification of the test system will be that each test tube used in the conduct of the aromatase assay will be uniquely identified by applying a label or writing directly on the test tube.

5.1 Assay Substrate, [^{3}H]ASDN + ASDN:

5.1.1 Substrate Name/Supplier:

The substrate for the aromatase assay is androstenedione (ASDN). Non-radiolabeled and radiolabeled ASDN will be used. The non-radiolabeled ASDN will be provided to the laboratories by the CR. The radiolabeled androstenedione ([1β-^{3}H]-ASDN, [^{3}H]ASDN) will be provided to the laboratories by Perkin Elmer. 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 (WIL Study Number WIL-431006) and was found to be 97%.

5.1.2 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 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 (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.

5.2 Preparation of Human Placental Microsomes:

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.

For consent and privacy issues, research involving human subjects, including tissues, is subject to review by an Institutional Review Board (IRB) as defined in 21 CFR Part 50 and 21 CFR Part 56. Western Institutional Review Board (WIRB), an independent review board (IRB) has been selected to perform this function for WIL. If WIL is not called upon to complete microsome preparation from placentas, the Review will be cancelled.

5.2.1 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.

5.2.2 Microsome Preparation Buffers:

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

First prepare the 0.05 M 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 1 L 0.05 M sodium phosphate buffer, pH 7.0. The buffer may be stored for up to one month in the refrigerator (2-8°C).

5.2.2.2 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).

5.2.2.3 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.

5.2.3 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 or equivalent 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 x 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 x g for 30 minutes in an appropriate centrifuge (such as an IEC B-22M or equivalent). 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 x g) in an appropriate ultracentrifuge (such as a Beckman L5-50B Ultracentrifuge or equivalent) 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 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.

5.3 Use of Human Placental Microsomes:

Human placental microsomes will be supplied by the two separate laboratories. These samples should be treated as potentially infectious and appropriate precautions must be employed. The microsomes must be stored at -70 to -80°C. The approximate protein content of the microsomes will be included in the shipping documents.

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.

On the day of use, microsomes are thawed quickly in a 37 ± 1 °C water bath and then are immediately transferred to an ice bath. The microsomes will be rehomogenized using a Potter-Elvejhem homogenizer (chilled in ice bath, about 5-10 passes) or vortexed to mix (ca. 5 seconds) prior to use. The microsomes are 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. Microsomes will not be left on ice for longer than approximately 1 hour before proceeding with the assay or microsomal enzyme activity may be decreased. The time of thaw and assay initiation time are documented in the study records. Under no conditions will microsomes that have been thawed or diluted be refrozen for later use in the assay.

5.4 Other Assay Components:

5.4.1 Buffer:

The assay buffer is 0.1 M sodium phosphate buffer, pH 7.4. It is prepared as described in Section 5.2.2.2 above for Buffer B. The assay buffer may be stored for up to one month in the refrigerator (2-8 °C).

5.4.2 Propylene Glycol:

Propylene glycol is added directly to the assay as described in Section 7.

5.4.3 NADPH:

NADPH is the required co-factor for CYP19 (aromatase enzyme). The Sponsor will provide the NADPH to be used in the assay. The final concentration in the assay will be 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.

6 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 at a minimum from two independent assays, and again on each day of use of the microsomes in the aromatase assay. 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 hour. Each sample (unknown and standards) will be transferred to appropriate 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.

7 AROMATASE ASSAY (SEE APPENDIX A):

This procedure will be used to measure the aromatase activity in the microsomal preparations by all the labs, as described in Section 1.2 (Stage 2). Two independent replicates will be run, each replicate will consist of four repetitions (four test tubes) of each of the control samples. The control sets are split so that two repetitions (tubes) of each control type will be run at the beginning and at the end of each replicate. Four types of control samples will be included. These include:

- Full enzyme (aromatase) activity controls (substrate, NADPH, propylene glycol, buffer, vehicle [ethanol, used for preparation of test substance solutions for determination of aromatase response in Section 8] 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 of 5 x 10^{-8} M).

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

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 + ASDN substrate, 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 below.

Table 2. Human Placental Microsomal Aromatase Assay-Optimized Conditions

Microsomal Protein	0.0125 mg/mL[a]
NADPH	0.3 mM[a]
[^{3}H]ASDN + ASDN	100 nM[a]
Incubation Time	15 minutes

[a] Final concentrations

The tubes and the microsomal suspension will be placed in a 37 ± 1°C water bath for at least five minutes prior to initiation of the assay by the addition of 1 mL of the diluted microsomal suspension to the reaction mixture in the labeled test tube. The total assay volume will be 2 mL, and the tubes will be incubated for 15 minutes. The incubations will be stopped by the addition of methylene chloride (2 mL); the tubes will be vortex-mixed for approximately 5 seconds and placed on ice. The tubes are then vortex-mixed an additional 20-25 seconds. The tubes will then be centrifuged for 10 minutes at approximately 162 x g. 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, approximately 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.

Analysis of the samples will be performed using a liquid scintillation counter (LSC). Radiolabel found in the aqueous fractions represents ^3H_2O formed. One ^3H_2O molecule is released per molecule of ASDN converted to estrogen in a stereospecific reaction. Thus, the amount of estrogen product formed is determined by dividing the total amount of ^3H_2O formed by the specific activity of the [^{3}H]ASDN substrate (expressed in disintegrations per minute (DPM)/nmol). Results will be presented as the activity (velocity) of the enzyme reaction. The activity of the enzyme reaction is

expressed in nmol(mg protein)$^{-1}$min^{-1} and is calculated by dividing the amount of estrogen formed by the product of mg microsomal protein used times the incubation time, e.g. 15 minutes.

8 DETERMINATION OF THE RESPONSE OF AROMATASE ACTIVITY TO TEST SUBSTANCE (4-OH ASDN):

Only the laboratories that procured the placenta and prepared the microsomes will perform the experiments described in this section (Stage 1, Section 1.1), which is similar to the Positive Control experiment conducted in WA 4-16, Task 4 (WIL Study Number WIL-431006). 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 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 7 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. 4- OH ASDN Study Design

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

		substance (4-OH ASDN) added	

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

9 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.

9.1 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 (WIL Study Number WIL-431007).

9.2 Statistical Analysis:

9.2.1 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 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) 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)
μ = $\log_{10}IC_{50}$ (IC_{50} is the concentration of inhibitor 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. 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 (i.e., 20% inhibition).

9.2.2 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.

9.2.3 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 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.

9.2.4 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 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
- 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.

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

9.2.6 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 9.2.4.

10 QUALITY ASSURANCE:

The study will be audited by the WIL Quality Assurance Unit with in-phase inspections to assure compliance with the study protocol and protocol amendments, WIL standard operating procedures and the appropriate provisions of the EPA TSCA and FIFRA Good Laboratory Practice Standards published in the Federal Register (40 CFR Part 792 and 40 CFR Part 160). The raw data and draft report will be audited by the WIL Quality Assurance Unit prior to submission to the Sponsor to assure that the Final Report accurately describes the conduct and the findings of the study. 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.

Data requiring statistical analysis will be analyzed by BioSTAT Consultants, Inc. following the current procedural guidelines of BioSTAT Consultants, Inc. BioSTAT Consultants, Inc. will provide a statistical analysis report, which will be included as an appendix to the final report. Quality Assurance auditing of the statistical report

(for internal consistency with the study report) will be conducted under the direction of the Quality Assurance Unit of WIL Research Laboratories, LLC.

Formulation of the control chemical stock solutions will be conducted by the Sponsor following the standard operating procedures of the Sponsor and in accordance with GLPs. Quality assurance monitoring of these activities for SOP and GLP compliance is the responsibility of the Sponsor. Upon completion of the prescribed activities the Sponsor will provide a signed Quality Assurance statement that will be included in the Battelle Chemical Repository Chemistry Report and included in the final report as an appendix.

This study will be included on the WIL master list of regulated studies.

11 RECORDS TO BE MAINTAINED:

All specimens and original raw data records, as defined by WIL SOPs and the applicable GLPs, will be stored as described in Section 12 in the Archives at WIL Research Laboratories, LLC.

Raw data records generated by the Sponsor will be stored as defined by the Sponsor's applicable standard operating procedures.

12 WORK PRODUCT:

The Sponsor will have title to all documentation records, raw data, specimens and other work product generated during the performance of the study. All work product, including raw paper data, pertinent electronic storage media and specimens, will be retained at no charge for a period of six months following issuance of the final report in the Archives at WIL Research Laboratories, LLC. Thereafter, WIL Research Laboratories, LLC will charge a monthly archiving fee for retention of all work product. Appropriate supporting documentation for statistical analyses conducted and reported by BioSTAT Consultants, Inc. will be maintained in the Archives at WIL Research Laboratories, LLC. All work product will be stored in compliance with regulatory requirements.

Any work product, including documents, specimens, and samples, that are required by this protocol, its amendments, or other written instructions of the Sponsor, to be shipped by WIL Research Laboratories, LLC to another location will be appropriately packaged and labeled as defined by WIL's SOPs and delivered to a common carrier for shipment. WIL Research Laboratories, LLC will not be responsible for shipment following delivery to the common carrier.

13 REPORTS:

An interim data set, in the form of a spreadsheet and data summary, will be submitted to the Sponsor. The spreadsheets will be submitted within 14 calendar days of

completing the incubations/analyses. This interim data submission will not be audited by the Quality Assurance Unit and will be identified as "unaudited preliminary data." The data will be checked for accuracy by the technical staff.

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 9 of this document.

Draft final and final reports will be written. The format for the draft final report will be provided by the Sponsor. The draft final report will be submitted to the Sponsor. One revision of the full report will be permitted as part of the cost of the study, from which the Sponsor's reasonable revisions and suggestions will be incorporated into the final report, as appropriate. Additional changes or revisions, requiring a new report, may be made, at extra cost. It is expected that the Sponsor will review the draft report and provide comments to WIL within a two-month time frame following submission. WIL will submit the final report in a timely manner following receipt of comments. If the Sponsor's comments and/or authorization to finalize the report have not been received by WIL within one year following submission of the draft report, WIL may elect to finalize the report following appropriate written notification to the Sponsor. One electronic copy (PDF format) will be provided; requests for paper copies of the final report may result in additional charges.

14 PROTOCOL MODIFICATION:

Modification of the protocol may be accomplished during the course of this investigation. However, no changes will be made in the study design without the written permission (electronic email or paper document) of the Sponsor. In the event that the Sponsor requests or approves a change in the protocol, such changes will be made by appropriate documentation in the form of a protocol amendment. All alterations of the protocol and reasons for the modification(s) will be signed by the Study Director and the Sponsor Representative.

15 REFERENCES

WIL-431006, Validation of the Placental Aromatase Assay: Positive Control Study (WA 4-16, Task 4), WIL Research Laboratories, LLC, **2004**.

WIL-431007, Validation of the Placental Aromatase Assay for Endocrine Disruptor Screening (WA 4-16, Task 5), WIL Research Laboratories, LLC, **2005**.

16 PROTOCOL APPROVAL:

Sponsor approval received via N/A on 5/3/05
Date

Jennifer Thomas-Wohlever 4/25/05
Jennifer Thomas-Wohlever, Ph.D. Date
Study Director
WIL Research Laboratories, LLC

Daniel W. Sved 4/25/05
Daniel W. Sved, Ph.D. Date
Director, Metabolism and
Analytical Chemistry
WIL Research Laboratories, LLC

Jerry D. Johnson 5-2-05
Jerry D. Johnson, Ph.D., D.A.B.T. Date
Work Assignment Leader/Study Monitor
Endocrine Disruptor Screening Program
Battelle Memorial Institute

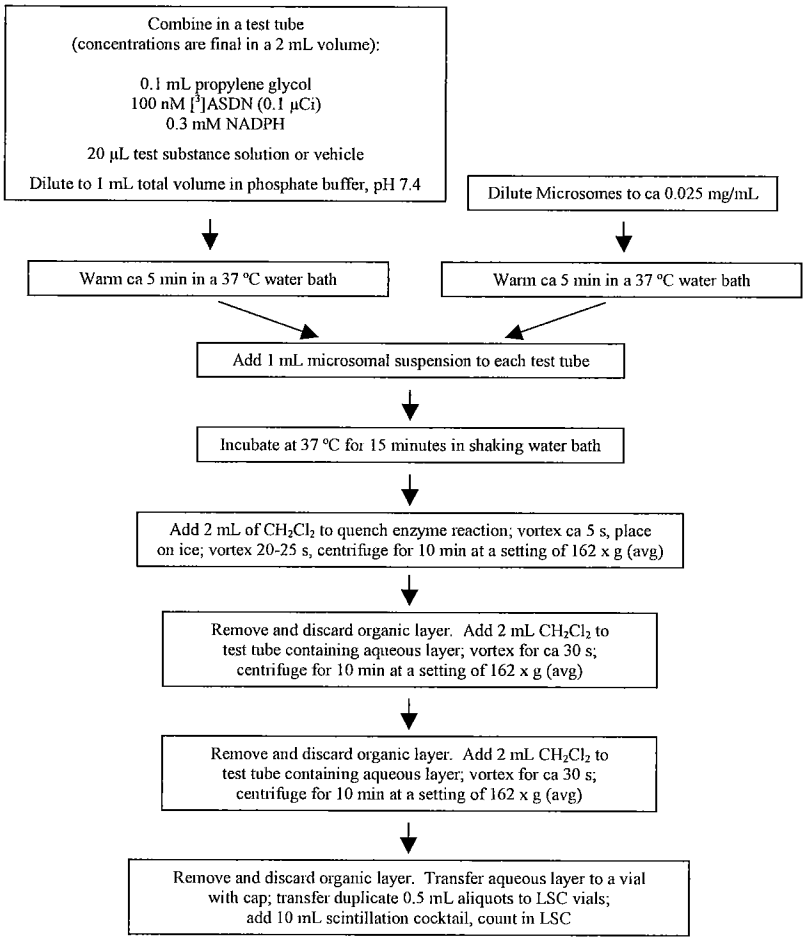
David P. Houchens 5/2/05
David P. Houchens, Ph.D. Date
Program Manager
Endocrine Disruptor Screening Program
Battelle Memorial Institute

17 PROTOCOL REVIEW:

Heather L Osborn 4/25/05
Heather L. Osborn, B.S., RQAP-GLP Date
Manager, Quality Assurance
WIL Research Laboratories, LLC

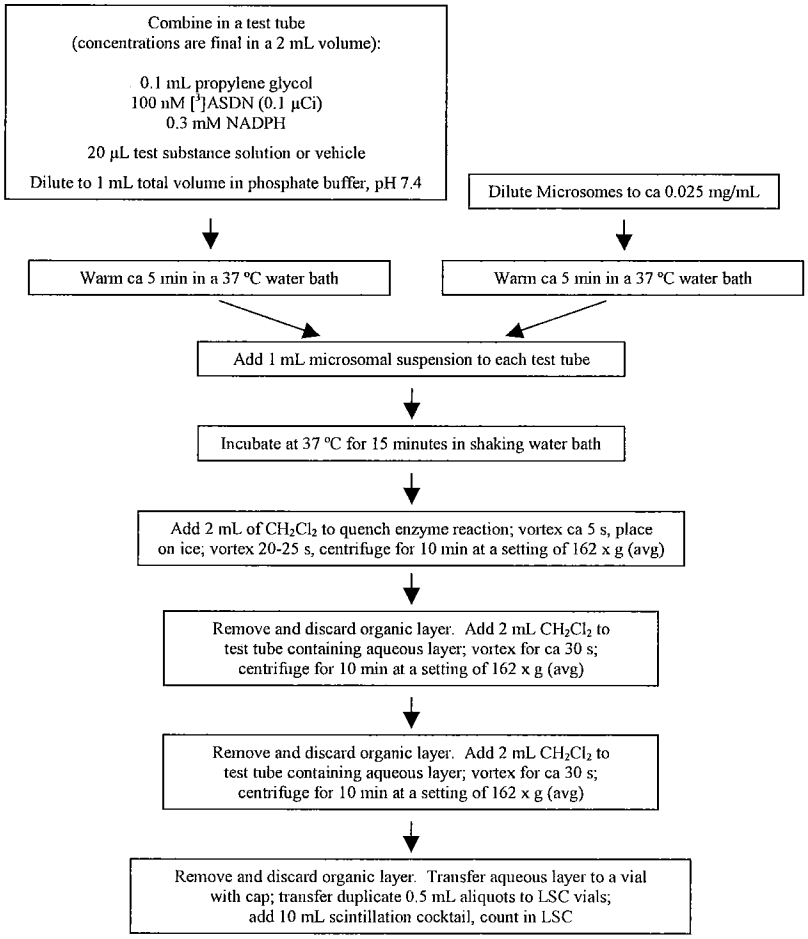
Terri L Pollock 5-2-05
Terri L. Pollock, B.A. Date
Quality Assurance Manager
Endocrine Disruptor Screening Program
Battelle Memorial Institute

Appendix A


Combine in a test tube
(concentrations are final in a 2 mL volume):
0.1 mL propylene glycol
100 nM [3]ASDN (0.1 µCi)
0.3 mM NADPH
20 µL test substance solution or vehicle
Dilute to 1 mL total volume in phosphate buffer, pH 7.4
Dilute Microsomes to ca 0.025 mg/mL
Warm ca 5 min in a 37 °C water bath
Warm ca 5 min in a 37 °C water bath
Add 1 mL microsomal suspension to each test tube
Incubate at 37 °C for 15 minutes in shaking water bath
Add 2 mL of CH_2Cl_2 to quench enzyme reaction; vortex ca 5 s, place on ice; vortex 20-25 s, centrifuge for 10 min at a setting of 162 x g (avg)
Remove and discard organic layer. Add 2 mL CH_2Cl_2 to test tube containing aqueous layer; vortex for ca 30 s; centrifuge for 10 min at a setting of 162 x g (avg)
Remove and discard organic layer. Add 2 mL CH_2Cl_2 to test tube containing aqueous layer; vortex for ca 30 s; centrifuge for 10 min at a setting of 162 x g (avg)
Remove and discard organic layer. Transfer aqueous layer to a vial with cap; transfer duplicate 0.5 mL aliquots to LSC vials; add 10 mL scintillation cocktail, count in LSC

Appendix B

Assay Procedure


Combine in a test tube
(concentrations are final in a 2 mL volume):
0.1 mL propylene glycol
100 nM [3]JASDN (0.1 µCi)
0.3 mM NADPH
20 µL test substance solution or vehicle
Dilute to 1 mL total volume in phosphate buffer, pH 7.4
Dilute Microsomes to ca 0.025 mg/mL
Warm ca 5 min in a 37 °C water bath
Warm ca 5 min in a 37 °C water bath
Add 1 mL microsomal suspension to each test tube
Incubate at 37 °C for 15 minutes in shaking water bath
Add 2 mL of CH_2Cl_2 to quench enzyme reaction; vortex ca 5 s, place on ice; vortex 20-25 s, centrifuge for 10 min at a setting of 162 x g (avg)
Remove and discard organic layer. Add 2 mL CH_2Cl_2 to test tube containing aqueous layer; vortex for ca 30 s; centrifuge for 10 min at a setting of 162 x g (avg)
Remove and discard organic layer. Add 2 mL CH_2Cl_2 to test tube containing aqueous layer; vortex for ca 30 s; centrifuge for 10 min at a setting of 162 x g (avg)
Remove and discard organic layer. Transfer aqueous layer to a vial with cap; transfer duplicate 0.5 mL aliquots to LSC vials; add 10 mL scintillation cocktail, count in LSC

Appendix C

Quality Assurance Project Plan

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

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 5/21/05
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.

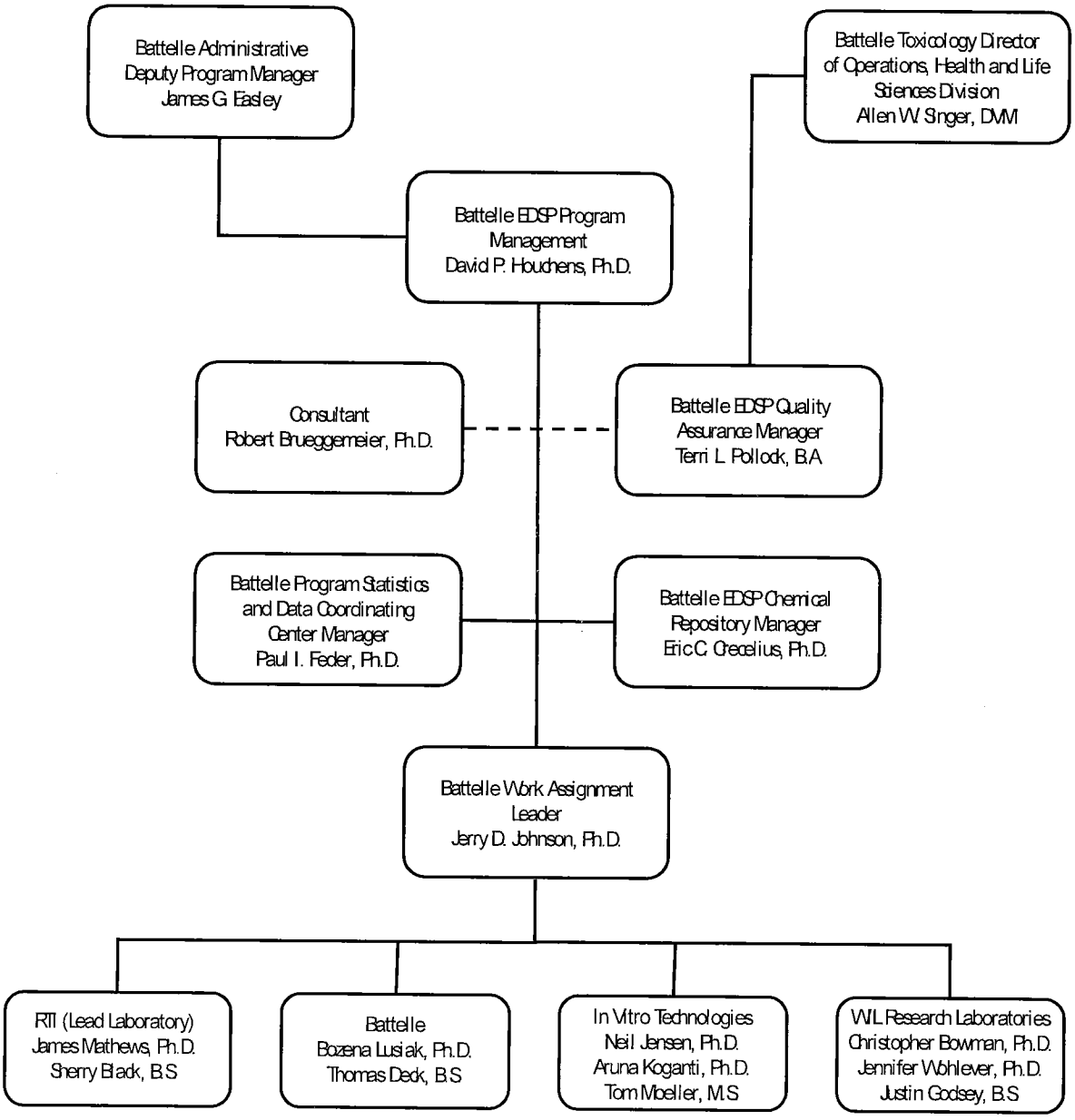

Figure 4-1. WA 4-16 Project Organization Overview

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

PROTOCOL		Page 1 of 18

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 □L 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
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 $\log_{10}IC_{50}$ (□), the within replicate standard error of □, 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 D

[^{3}H]ASDN Purity Assessment Report (RTI International)

DRAFT 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

DRAFT 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
Study Initiation Date:	
Experimental Dates:	January 5, 2005
Draft Task Report Date	February 3, 2005

Author:		Approved:	
Sherry Black Research Chemist	Date	James Mathews, Ph. D, DABT Study Director	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).

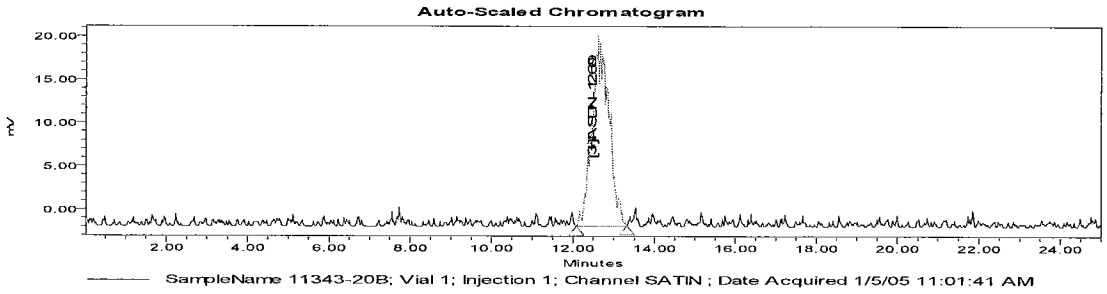
The radiochemical purity of the [^{3}H]ASDN (1:100 dilution in ethanol) was determined using high performance liquid chromatography (HPLC) and liquid scintillation counting. The HPLC system consists of a Waters 2690 Separations Module, a Waters 2487 Dual λ Absorbance Detector and a β-RAM Model 3 flow-through 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 [3H]ASDN



Conclusion

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

Appendix E

Chemistry Reports (Sponsor-Provided Data)

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ANALYTICAL CHEMISTRY ACTIVITIES REPORT

4-HYDROXYANDROSTENEDIONE (4-OH ASDN)

CAS No.: 566-48-3

Lot No.: 063K4069 (Sigma Aldrich)

Receipt Date: 10/22/04

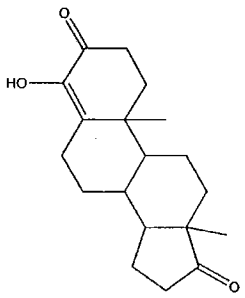
Amount Received: 3.1 g

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

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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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.

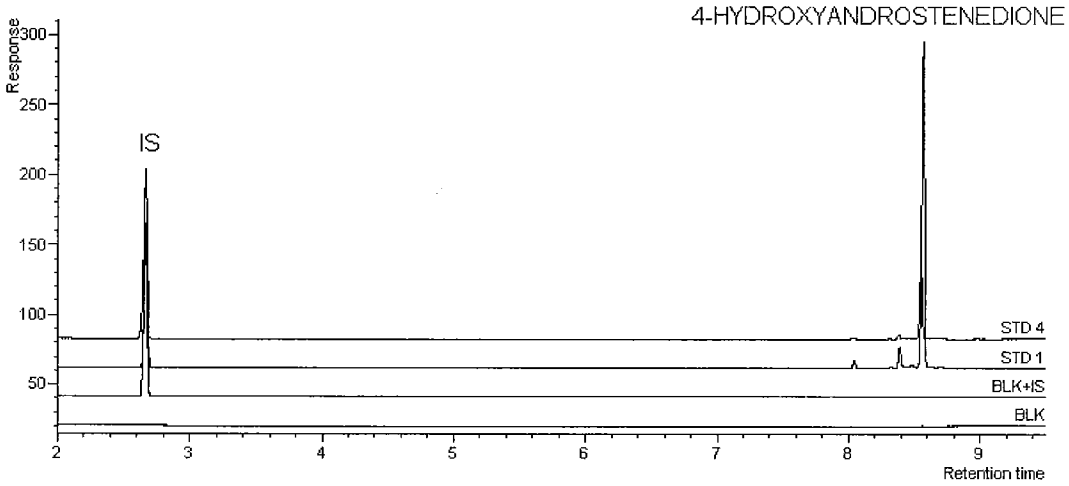


Figure 2 – Representative Overlaid Chromatograms from a Low and High Vehicle/Calibration Standard, Blank with IS, and Blank from the Validation (Shown Top to Bottom)

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
	496.8				-1.9	
506.4	494.5	509.6	24.2	4.7	-2.3	0.6
	537.5				6.1	
298.1	289.4	NA	NA	NA	-2.9	NA
202.6	198.8	NA	NA	NA	-1.9	NA
	100.7				1.3	
99.38	99.89	100.4	0.4	0.4	0.5	1.0
	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.

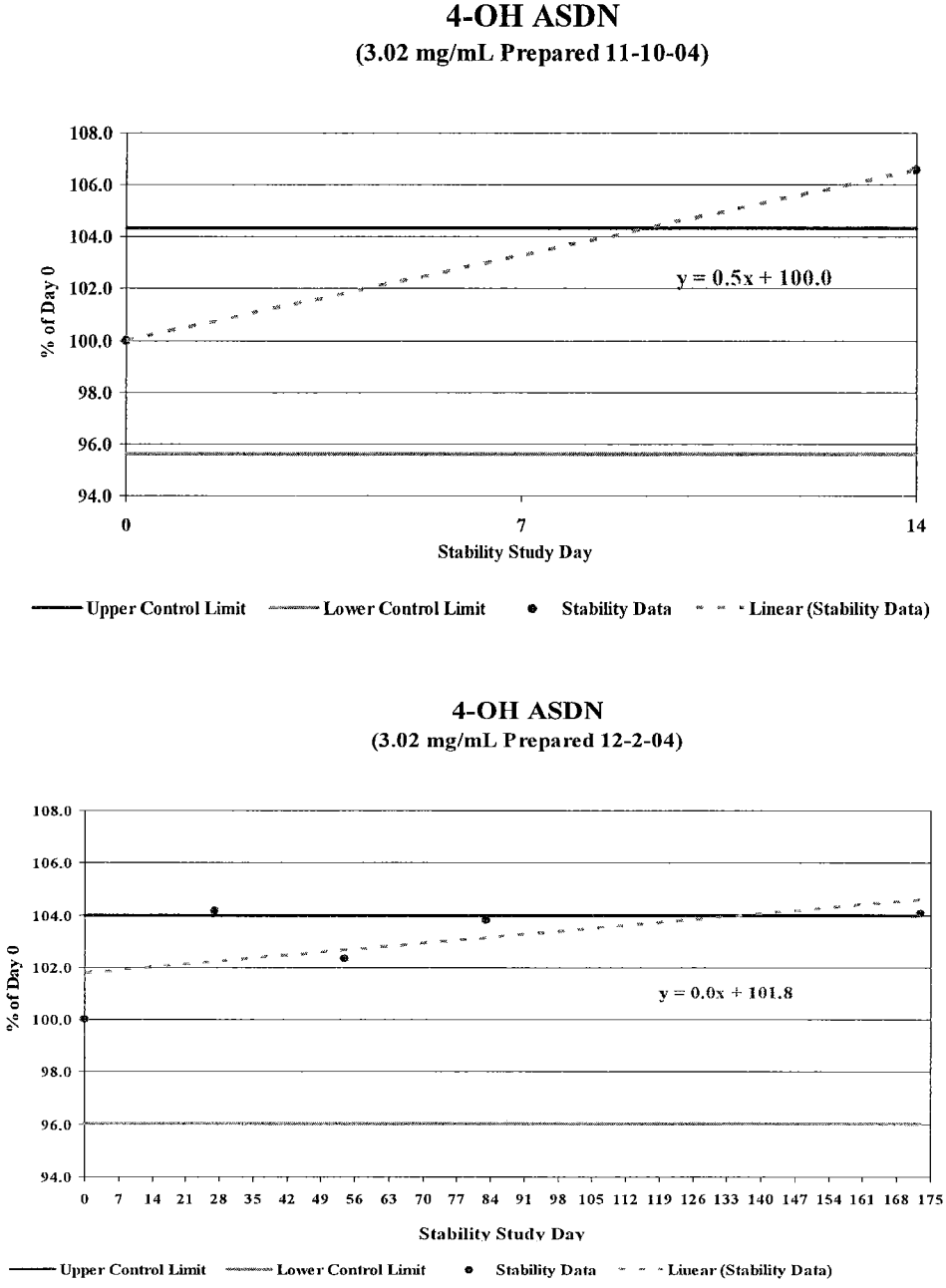


Figure 3 – Control Charts for the Storage Stability Studies

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.

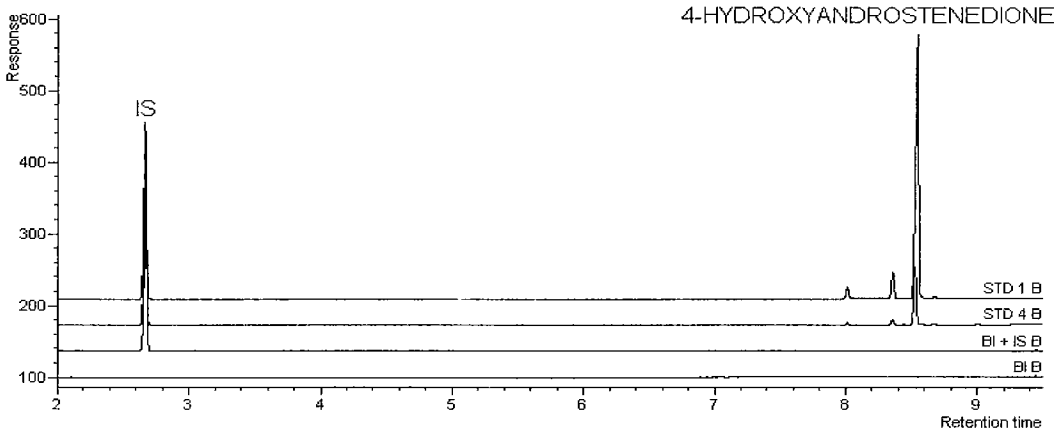


Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from Formulation Analysis

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

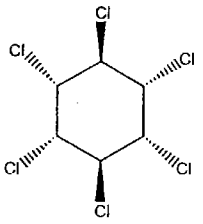
Analytical support for this work was provided by Sandy Runyon, Christina Zielinski, Tudor Fernando, Kevin Carrico, and Darren Brown. The report was written by Denise Contos. Review of the data and report for completeness and accuracy was performed by Maria Evascu.

Battelle
The Business of Innovation

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.:	Mol. Formula:
	290.83 g/mol	$C_6H_6Cl_6$

Prepared By:

Denise A. Contos, M.S.

Approved By:

Steven W. Graves, B.S.
Manager, Chemistry Technical Center

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 C. 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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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.

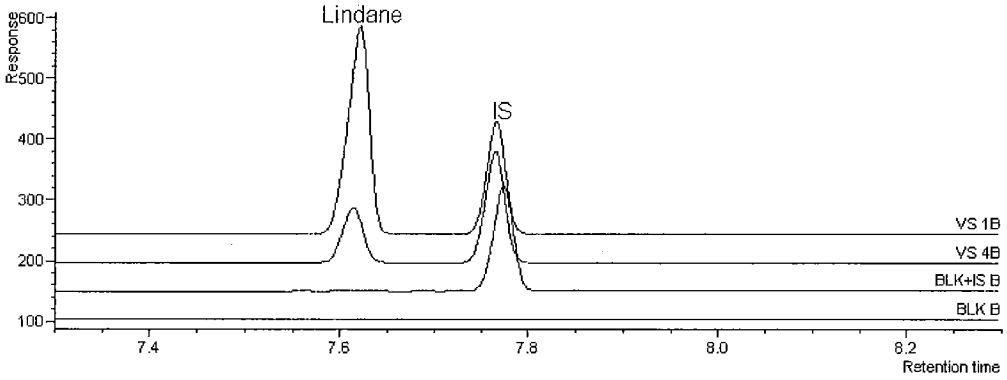

Figure 2 – Representative Overlaid Chromatograms from a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from the Validation (Shown Top to Bottom)

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
	777.3				0.1	
776.3	777.6	776.8	1.1	0.1	0.2	0.1
	775.6				-0.1	
600.2	598.4	NA	NA	NA	-0.3	NA
388.2	387.0	NA	NA	NA	-0.3	NA
	202.8				1.4	
200.1	200.1	200.5	2.1	1.1	0.0	0.2
	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.

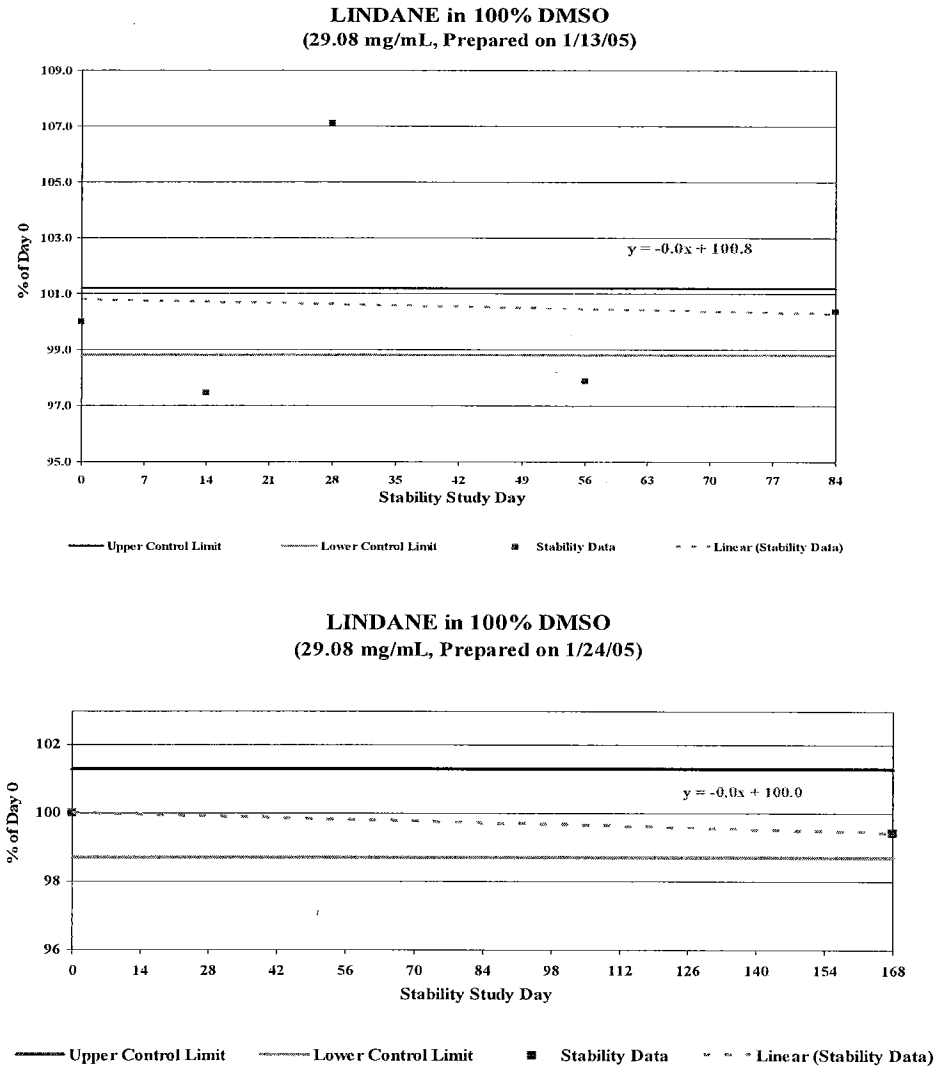

Figure 3 – Control Chart for the Storage Stability Study

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.

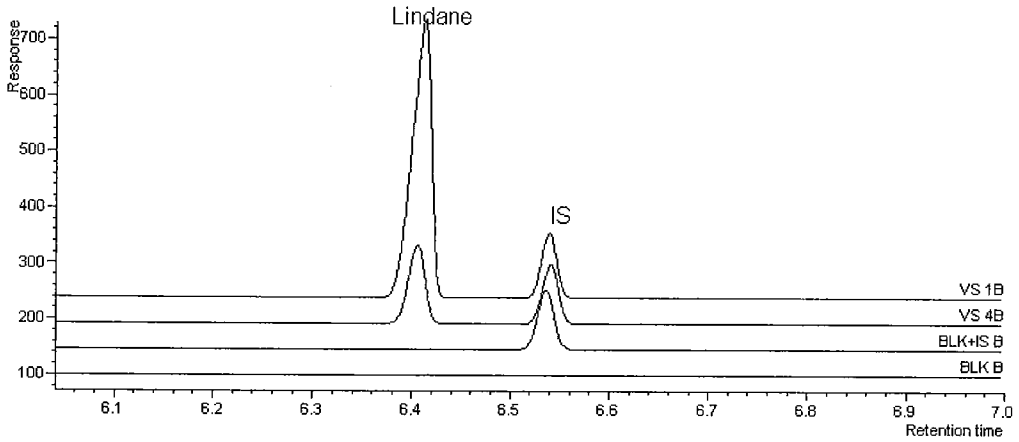

Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from a Formulation Analysis (Shown Top to Bottom)

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 F

Individual Replicate Spreadsheets

Aromatase Assay Spreadsheet

Assay Date	6/1/2005	Test Chemical ID	none	# Concentrations tested			
Technician ID	JG	Replicate #	1-Jan	Microsome type	placental	Microsome ID Battelle	

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0196	29467.35	1503436	
2	0.0199	30933.37	1554441	
3	0.0201	31082.36	1546386	
4	0.0199	31609.92	1588438	
5	0.0200	31344.42	1567221	
			Average DPM/g soln	1551984
			SD	31446
			CV	2.03
			µCi/g soln	0.699

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	8.2274 g
Mass of dilution B used in substrate prep	4.5993 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.570203 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00791 µg/g soln.
µg/g soln.

a. µCi/g soln	0.699
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.

= 0.570203 + 0.00791
= 0.578117 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.209 µCi/µg ASDN

768856 dpm/nmol

Assay Date	6/1/2005	Test Chemical ID	none		# Concentrations tested		0			
Technician ID	JG	Replicate #		1	Microsome type	placental		Microsome ID	Battelle	

								Protein stock (mg	Total volume of	
Standards:	0.25	0.125	0.05	0.025	0.01	0.005	0	BSA)	stock (mL)	Protein stock ID
	0.556	0.342	0.175	0.099	0.055	0.046	0.038	25	100	
	0.563	0.335	0.172	0.098	0.059	0.045	0.034			
	0.566	0.344	0.166	0.101	0.055	0.045	0.036			

Samples:	10	100	Microsomes
	0.047	0.269	0.096
	0.048	0.274	0.094
			0.095

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	
0.25	200	200	0.00025	200	0.0500	0.562	0.526	0.0482	m, b	0.094	-0.001
0.125	100	200	0.00013	200	0.0250	0.340	0.304	0.0275	se_m, se_b	0.004	0.001
0.05	200	1000	0.00005	200	0.0100	0.171	0.135	0.0116	r^2, se_y	0.992	0.002
0.025	100	1000	0.00003	200	0.0050	0.099	0.063	0.0049	F, df	499	4
0.01	40	1000	0.00001	200	0.0020	0.056	0.021	0.0009	ss_{reg}, ss_{resid}	0.002	0.000
0.005	20	1000	0.00001	200	0.0010	0.045	0.009	-0.0001			

Regression results are calculated using the function LINEST

Blank	0.036	r^2=	0.992		
		m=	0.094		
		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
10	0.047	0.011	0.000	200	1	1	0.000	0.000	0.000
10	0.048	0.013	0.000	200	1	1	0.000		
10					1	1			
100	0.269	0.234	0.021	200	1	1	0.000	0.000	0.105
100	0.274	0.238	0.021	200	1	1	0.000		
100					1	1			
Microsomes	0.096	0.060	0.005	200	24	20000	0.019	0.018	18.443
Microsomes	0.094	0.058	0.004	200	24	20000	0.018		
Microsomes	0.095	0.059	0.004	200	24	20000	0.018		

Assay Date	6/1/2005	Test Chemical ID	none	# Concentrations tested	0		
Technician ID	JG	Replicate #	1	Microsome type	placental	Microsome ID	Battelle

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	0.25	0.125	0.05	0.025	0.01	0.005	0	25	100	
	0.556	0.342	0.175	0.099	0.055	0.046	0.038			
	0.563	0.335	0.172	0.098	0.059	0.045	0.034			
	0.566	0.344	0.166	0.101	0.055	0.045	0.036			

Samples:	10	100	Microsomes
	0.047	0.269	0.096
	0.048	0.274	0.094
			0.095

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	
0.25	200	200	0.00025	200	0.0500	0.562	0.526	0.0425	m, b	0.081	0.000
0.125	100	200	0.00013	200	0.0250	0.340	0.304	0.0246	se_m, se_b	0.003	0.000
0.05	200	1000	0.00005	200	0.0100	0.171	0.135	0.0109	r^2, se_y	0.997	0.001
0.025	100	1000	0.00003	200	0.0050	0.099	0.063	0.0051	F, df	963	3
0.01	40	1000	0.00001	200	0.0020	0.056	0.021	0.0016	ss_{reg}, ss_{resid}	0.000	0.000
0.005	20	1000	0.00001	200	0.0010	0.045	0.009	0.0007			

Regression results are calculated using the function LINEST

Blank	0.036	r^2=	0.997	
		m=	0.081	
		b=	0.000	

	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
10	0.047	0.011	0.001	200	1	1	0.000	0.000	0.005
10	0.048	0.013	0.001	200	1	1	0.000		
10					1	1			
100	0.269	0.234	0.019	200	1	1	0.000	0.000	0.095
100	0.274	0.238	0.019	200	1	1	0.000		
100					1	1			
Microsomes	0.096	0.060	0.005	200	24	20000	0.020	0.019	19.479
Microsomes	0.094	0.058	0.005	200	24	20000	0.019		
Microsomes	0.095	0.059	0.005	200	24	20000	0.019		

Assay Date	6/1/2005	Test Chemical ID	none	# Concentrations tested		0			
Technician ID	JG	Replicate #	1	Microsome type		placental	Microsome ID	Battelle	
Standards:	1.5	1	0.75	0.5	0.25	0.13	Blk	Protein stock (mg/10 mL)	Protein stock ID

Samples:

mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results
0.00000	25	0.0000				m, b	
0.00000	25	0.0000				se_m, se_b	
0.00000	25	0.0000				r^2, se_y	
0.00000	25	0.0000				F, df	
0.00000	25	0.0000				ss_{reg}, ss_{resid}	
0.00000	25	0.0000					

Blank

r^2=
m=
b=

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

Assay Date	6/1/2005	Test Chemical ID	none	# Concentrations tested	0	Microsome type	placental	Microsome ID	Battelle	Technician ID	JG	Replicate #	1

Microsome Dilution Details		
Dilution A	0.048	mL microsome Stock used
	3.948	mL total volume
	82.25	dilution factor
Dilution B	2	mL microsome Dilution A used
	20	mL total volume
	10	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	822.5	total dilution factor

Test Chemical Concentrations	
Level	Final Concentration (M)
1	
2	
3	
4	
5	
6	
7	
8	

Protein Concentration (stock microsomes, mg/mL):	19.479
Protein Concentration (dilution added to assay, mg/mL):	0.023683

Assay Date	6/1/2005	Test Chemical ID	none	# Concentrations tested	0	Microsome type	placental	Microsome ID	Battelle	Technician ID	JG	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 (initial)	% 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 (nmol estrogen formed/mg protein/min
Full activity control	1	2	0.5	1	5043.52	10087.04	10141.99	20283.98	0.1	155198	13.07	20149	0.0262	1	0.012	15	0.0738
			0.5	2	5098.47	10196.94			0.1					1		15	
	2	2	0.5	1	5007.63	10015.26	10025.63	20051.26	0.1	155198	12.92	19916	0.0259	1	0.012	15	0.0729
			0.5	2	5018	10036			0.1					1		15	
	3	2	0.5	1	4541.9	9083.8	9536.39	19072.78	0.1	155198	12.29	18938	0.0246	1	0.012	15	0.0693
			0.5	2	4994.49	9988.98			0.1					1		15	
	4	2	0.5	1	5031.76	10063.52	9948.43	19896.86	0.1	155198	12.82	19762	0.0257	1	0.012	15	0.0724
			0.5	2	4916.67	9833.34			0.1					1		15	
Background control	1	2	0.5	1	37.76	75.52	73.12	146.24	0.1	155198	0.09	11	0.0000	1	0.012	15	0.0000
			0.5	2	35.36	70.72			0.1					1		15	
	2	2	0.5	1	33.65	67.3	64.06	128.12	0.1	155198	0.08	-7	0.0000	1	0.012	15	0.0000
			0.5	2	30.41	60.82			0.1					1		15	
	3	2	0.5	1	41.07	82.14	66.12	132.24	0.1	155198	0.09	-3	0.0000	1	0.012	15	0.0000
			0.5	2	25.05	50.1			0.1					1		15	
	4	2	0.5	1	31.64	63.28	66.31	132.62	0.1	155198	0.09	-2	0.0000	1	0.012	15	0.0000
			0.5	2	34.67	69.34			0.1					1		15	
Positive control	1	2	0.5	1	2334.3	4668.6	4733.09	9466.18	0.1	155198	6.10	9331	0.0121	1	0.012	15	0.0342
			0.5	2	2398.79	4797.58			0.1					1		15	
	2	2	0.5	1	2341.73	4683.46	4698.42	9396.84	0.1	155198	6.05	9262	0.0120	1	0.012	15	0.0339
			0.5	2	2356.69	4713.38			0.1					1		15	
	3	2	0.5	1	2253.25	4506.5	4537.31	9074.62	0.1	155198	5.85	8940	0.0116	1	0.012	15	0.0327
			0.5	2	2284.06	4568.12			0.1					1		15	
	4	2	0.5	1	2265.44	4530.88	4565.44	9130.88	0.1	155198	5.88	8996	0.0117	1	0.012	15	0.0329
			0.5	2	2300	4600			0.1					1		15	
Negative Control	1	2	0.5	1	5076.73	10153.46	10321.65	20643.3	0.1	155198	13.30	20508	0.0267	1	0.012	15	0.0751
			0.5	2	5244.93	10489.84			0.1					1		15	
	2	2	0.5	1	4991.62	9983.24	10074.84	20149.68	0.1	155198	12.98	20015	0.0260	1	0.012	15	0.0733
			0.5	2	5083.22	10166.44			0.1					1		15	
	3	2	0.5	1	5013.39	10026.78	9958.33	19916.66	0.1	155198	12.83	19782	0.0257	1	0.012	15	0.0724
			0.5	2	4944.94	9889.86			0.1					1		15	
	4	2	0.5	1	4975.52	9951.04	9846.18	19692.36	0.1	155198	12.69	19558	0.0254	1	0.012	15	0.0716
			0.5	2	4870.66	9741.32			0.1					1		15	
none	1-1	2		1						0		#VALUE!	#VALUE!	1	0.012	15	#VALUE!
				2										1		15	
	1-2	2		1						0		#VALUE!	#VALUE!		0.000	15	#VALUE!
				2												15	
	1-3	2		1						0		#VALUE!	#VALUE!		0.000	15	#VALUE!
				2												15	
	2-1	2		1						0		#VALUE!	#VALUE!		0.000	15	#VALUE!
				2												15	
	2-2	2		1						0		#VALUE!	#VALUE!		0.000	15	#VALUE!
				2												15	
	2-3	2		1						0		#VALUE!	#VALUE!		0.000	15	#VALUE!
				2												15	
	3-1	2		1						0		#VALUE!	#VALUE!		0.000	15	#VALUE!
				2												15	
	3-2	2		1						0		#VALUE!	#VALUE!		0.000	15	#VALUE!
				2												15	
	3-3	2		1						0		#VALUE!	#VALUE!		0.000	15	#VALUE!
				2												15	
	4-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	4-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	4-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	5-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	5-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	5-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	6-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	6-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	6-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	7-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	7-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	7-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	8-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	8-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	8-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													

Assay Date	6/1/2005	Test Chemical ID	none	# Concentrations tested	Microsome 0 type	placental	Microsome ID Battelle	Technician ID JG	Replicate #	1

Control Type	Portion	Average	SD
Full activity	Beginning	0.0733	0.0006
Full activity	End	0.0708	0.0021
Full activity	Overall	0.0721	0.0019
Background	Beginning	0.0000	4.69112E-05
Background	End	0.0000	9.83788E-07
Background	Overall	0.0000	2.8891E-05
Positive	Beginning	0.0340	0.0002
Positive	End	0.0328	0.0001
Positive	Overall	0.0334	0.0007
Negative	Beginning	0.0742	0.0013
Negative	End	0.0720	0.0006
Negative	Overall	0.0731	0.0015

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
NA					

Percent of control values				
	Log[test	Replicate		
Level	substance]	1	2	3
1				
2				
3				
4				
5				
6				
7				
8				

Aromatase Assay Spreadsheet

Assay Date	6/2/2005	Test Chemical ID	none	# Concentrations tested			
Technician ID	JG	Replicate #	2-Jan	Microsome type	placental	Microsome ID Battelle	

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0200	29738.09	1486905	
2	0.0200	31057.67	1552884	
3	0.0197	31490.76	1598516	
4	0.0200	31190.23	1559512	
5	0.0201	32058.83	1594967	
			Average DPM/g soln	1558556
			SD	44971
			CV	2.89
			µCi/g soln	0.702

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	10.1	10		1010.00
Dilution A			100	10.10
Dilution B			10	1.01

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.2115 g
Mass of dilution B used in substrate prep	4.6037 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.566247 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00795 µg/g soln.
µg/g soln.

a. µCi/g soln 0.702
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.

= 0.566247 + 0.00795
= 0.574194 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.223 µCi/µg ASDN

777386 dpm/nmol

Assay Date	6/2/2005	Test Chemical ID	none	# Concentrations tested		0			
Technician ID	JG	Replicate #	2	Microsome type	placental	Microsome ID	Battelle		

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	0.25	0.125	0.05	0.025	0.01	0.005	0	25	100	
	0.545	0.323	0.170	0.096	0.050	0.036	0.028			
	0.551	0.324	0.168	0.093	0.049	0.035	0.026			
	0.556	0.330	0.163	0.096	0.051	0.037	0.027			
Samples:	10	100	m							
	0.049	0.252	0.102							
	0.043	0.252	0.100							
			0.098							

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	
0.25	200	200	0.00025	200	0.0500	0.551	0.524	0.0484	m, b	0.095	-0.001
0.125	100	200	0.00013	200	0.0250	0.326	0.299	0.0270	se_m, se_b	0.004	0.001
0.05	200	1000	0.00005	200	0.0100	0.167	0.140	0.0120	r^2, se_y	0.992	0.002
0.025	100	1000	0.00003	200	0.0050	0.095	0.068	0.0052	F, df	506	4
0.01	40	1000	0.00001	200	0.0020	0.050	0.023	0.0009	ss_{reg}, ss_{resid}	0.002	0.000
0.005	20	1000	0.00001	200	0.0010	0.036	0.009	-0.0004			

Regression results are calculated using the function LINEST

Blank	0.027	r^2=	0.992	
		m=	0.095	
		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
10	0.049	0.022	0.001	200	1	1	0.000	0.000	0.002
10	0.043	0.016	0.000	200	1	1	0.000		
10					1	1			
100	0.252	0.225	0.020	200	1	1	0.000	0.000	0.100
100	0.252	0.225	0.020	200	1	1	0.000		
100					1	1			
m	0.102	0.075	0.006	200	24	20000	0.024	0.023	23.192
m	0.100	0.073	0.006	200	24	20000	0.023		
m	0.098	0.071	0.005	200	24	20000	0.023		

Assay Date	6/2/2005	Test Chemical ID	none	# Concentrations tested		0				
Technician ID	JG	Replicate #	2	Microsome type	placental	Microsome ID	Battelle			

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	0.25	0.125	0.05	0.025	0.01	0.005	0	25	100	
	0.545	0.323	0.170	0.096	0.050	0.036	0.028			
	0.551	0.324	0.168	0.093	0.049	0.035	0.026			
	0.556	0.330	0.163	0.096	0.051	0.037	0.027			
Samples:	10	100	m							
	0.049	0.252	0.102							
	0.043	0.252	0.100							
			0.098							

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	
0.25	200	200	0.00025	200	0.0500	0.551	0.524	0.0429	m, b	0.083	0.000
0.125	100	200	0.00013	200	0.0250	0.326	0.299	0.0244	se_m, se_b	0.004	0.001
0.05	200	1000	0.00005	200	0.0100	0.167	0.140	0.0113	r^2, se_y	0.993	0.001
0.025	100	1000	0.00003	200	0.0050	0.095	0.068	0.0053	F, df	447	3
0.01	40	1000	0.00001	200	0.0020	0.050	0.023	0.0016	ss_{reg}, ss_{resid}	0.000	0.000
0.005	20	1000	0.00001	200	0.0010	0.036	0.009	0.0004			

Regression results are calculated using the function LINEST

Blank	0.027	r^2=	0.993		
		m=	0.083		
		b=	0.000		

	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
10	0.049	0.022	0.001	200	1	1	0.000	0.000	0.006
10	0.043	0.016	0.001	200	1	1	0.000		
10					1	1			
100	0.252	0.225	0.018	200	1	1	0.000	0.000	0.091
100	0.252	0.225	0.018	200	1	1	0.000		
100					1	1			
m	0.102	0.075	0.006	200	24	20000	0.024	0.024	23.582
m	0.100	0.073	0.006	200	24	20000	0.023		
m	0.098	0.071	0.006	200	24	20000	0.023		

Assay Date	6/2/2005	Test Chemical ID	none		# Concentrations tested		0		
Technician ID	JG	Replicate #		2	Microsome type	placental	Microsome ID	Battelle	

Standards:	1.5	1	0.75	0.5	0.25	0.13	Blk	Protein stock (mg/10 mL)	Protein stock ID

Samples:

mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results
0.00000	25	0.0000				m, b	
0.00000	25	0.0000				se_m, se_b	
0.00000	25	0.0000				r^2, se_y	
0.00000	25	0.0000				F, df	
0.00000	25	0.0000				ss_{reg}, ss_{resid}	
0.00000	25	0.0000					

Blank

r^2=
m=
b=

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

Assay Date	6/2/2005	Test Chemical ID	none	# Concentrations tested	0	Microsome type	placental	Microsome ID	Battelle	Technician ID	JG	Replicate #	2

Microsome Dilution Details		
Dilution A	0.048	mL microsome Stock used
	3.948	mL total volume
	82.25	dilution factor
Dilution B	2	mL microsome Dilution A used
	20	mL total volume
	10	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	822.5	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	23.582
Protein Concentration (dilution added to assay, mg/mL):	0.028671

Test Chemical Concentrations	
Level	Final Concentration (M)
1	
2	
3	
4	
5	
6	
7	
8	

Assay Date	6/2/2005	Test Chemical ID	none	# Concentrations tested	0	Microsome type	placental	Microsome ID	Battelle	Technician ID	JG	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)	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 (initial)	% 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 (nmol estrogen formed/mg protein/min
Full activity control	1	2	0.5	1	7549.71	15098.42	15215.5	30431	0.1	155856	19.53	30313	0.0390	1	0.014	15	0.0907
			0.5	2	7665.79	15331.58			0.1					1		15	
	2	2	0.5	1	5294.82	10589.64	10527.63	21055.26	0.1	155856	13.51	20937	0.0269	1	0.014	15	0.0626
			0.5	2	5232.81	10465.62			0.1					1		15	
	3	2	0.5	1	5291.23	10582.46	10533.18	21066.36	0.1	155856	13.52	20948	0.0269	1	0.014	15	0.0627
			0.5	2	5241.95	10483.9			0.1					1		15	
	4	2	0.5	1	5280.24	10560.48	10457.12	20914.24	0.1	155856	13.42	20796	0.0268	1	0.014	15	0.0622
			0.5	2	5176.88	10353.76			0.1					1		15	
Background control	1	2	0.5	1	19.83	39.66	56.82	113.64	0.1	155856	0.07	-4	0.0000	1	0.014	15	0.0000
			0.5	2	36.99	73.98			0.1					1		15	
	2	2	0.5	1	28.06	56.12	57.75	115.5	0.1	155856	0.07	-3	0.0000	1	0.014	15	0.0000
			0.5	2	29.69	59.38			0.1					1		15	
	3	2	0.5	1	29.77	59.54	59.07	118.14	0.1	155856	0.08	0	0.0000	1	0.014	15	0.0000
			0.5	2	29.3	58.6			0.1					1		15	
	4	2	0.5	1	36.03	72.06	62.58	125.16	0.1	155856	0.08	7	0.0000	1	0.014	15	0.0000
			0.5	2	26.55	53.1			0.1					1		15	
Positive control	1	2	0.5	1	2427.54	4855.08	4910.58	9821.16	0.1	155856	6.30	9703	0.0125	1	0.014	15	0.0290
			0.5	2	2483.04	4966.08			0.1					1		15	
	2	2	0.5	1	2394.01	4788.02	4802.66	9605.32	0.1	155856	6.16	9487	0.0122	1	0.014	15	0.0284
			0.5	2	2408.65	4817.3			0.1					1		15	
	3	2	0.5	1	2386.87	4773.74	4751.03	9502.06	0.1	155856	6.10	9384	0.0121	1	0.014	15	0.0281
			0.5	2	2364.16	4728.32			0.1					1		15	
	4	2	0.5	1	2387.2	4774.4	4796.37	9592.74	0.1	155856	6.15	9475	0.0122	1	0.014	15	0.0283
			0.5	2	2409.17	4818.34			0.1					1		15	
Negative Control	1	2	0.5	1	5322.13	10644.26	10656.57	21313.14	0.1	155856	13.67	21195	0.0273	1	0.014	15	0.0634
			0.5	2	5334.44	10668.88			0.1					1		15	
	2	2	0.5	1	5334.58	10669.16	10612.05	21224.1	0.1	155856	13.62	21106	0.0271	1	0.014	15	0.0631
			0.5	2	5277.47	10554.94			0.1					1		15	
	3	2	0.5	1	4975.63	9951.26	9946.4	19892.8	0.1	155856	12.76	19775	0.0254	1	0.014	15	0.0591
			0.5	2	4970.77	9941.54			0.1					1		15	
	4	2	0.5	1	5083.43	10166.86	10237.55	20475.1	0.1	155856	13.14	20357	0.0262	1	0.014	15	0.0609
			0.5	2	5154.12	10308.24			0.1					1		15	
none	1-1	2		1					0.1	155856		#VALUE!	#VALUE!	1	0.014	15	#VALUE!
				2					0.1					1		15	
	1-2	2		1					0.1	155855		#VALUE!	#VALUE!	1	0.014	15	#VALUE!
				2										1		15	
	1-3	2		1						0		#VALUE!	#VALUE!	1	0.014	15	#VALUE!
				2										1		15	
	2-1	2		1						0		#VALUE!	#VALUE!	1	0.014	15	#VALUE!
				2										1		15	
	2-2	2		1						0		#VALUE!	#VALUE!	1	0.014	15	#VALUE!
				2										1		15	
	2-3	2		1						0		#VALUE!	#VALUE!	1	0.014		#VALUE!
				2										1			
	3-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	3-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	3-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	4-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	4-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	4-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	5-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	5-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	5-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	6-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	6-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	6-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	7-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	7-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	7-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	8-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	8-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	8-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													

Assay Date	6/2/2005	Test Chemical ID	none	# Concentrations tested	Microsome 0 type	placental	Microsome ID	Battelle	Technician ID	JG	Replicate #	2

Control Type	Portion	Average	SD
Full activity	Beginning	0.0766	0.0198
Full activity	End	0.0624	0.0003
Full activity	Overall	0.0695	0.0141
Background	Beginning	0.0000	3.93392E-06
Background	End	0.0000	1.48474E-05
Background	Overall	0.0000	1.51038E-05
Positive	Beginning	0.0287	0.0005
Positive	End	0.0282	0.0002
Positive	Overall	0.0285	0.0004
Negative	Beginning	0.0633	0.0002
Negative	End	0.0600	0.0012
Negative	Overall	0.0616	0.0020

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
NA					

Percent of control values				
	Log[test	Replicate		
Level	substance]	1	2	3
1				
2				
3				
4				
5				
6				
7				
8				

Aromatase Assay Spreadsheet

Assay Date	6/30/2005	Test Chemical ID	none	# Concentrations tested		
Technician ID	JG	Replicate #	1-Jan	Microsome type	placental	Microsome ID in vitro

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0201	26022.42	1294648	
2	0.0198	26064.31	1316379	
3	0.0201	27203.34	1353400	
4	0.0200	26615.04	1330752	
5	0.0201	27031.7	1344861	
			Average DPM/g soln	1328008
			SD	23369
			CV	1.76
			µCi/g soln	0.598

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	8.245 g
Mass of dilution B used in substrate prep	4.6309 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.578511 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00677 µg/g soln.
µg/g soln.

a. µCi/g soln	0.598
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.

= 0.578511 + 0.00677
= 0.585283 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.022 µCi/µg ASDN

649842 dpm/nmol

Assay Date	6/30/2005	Test Chemical ID	none	# Concentrations tested	0		
Technician ID	JG	Replicate #	1	Microsome type	placental	Microsome ID	in vitro

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	0.25	0.125	0.05	0.025	0.01	0.005	0	25	100	
	0.549	0.337	0.162	0.116	0.046	0.039	0.034			
	0.565	0.338	0.161	0.135	0.049	0.036	0.025			
	0.560	0.348	0.166	0.106	0.048	0.036	0.027			

Samples:	10	100	m
	0.044	0.249	0.088
	0.041	0.254	0.088
			0.090

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	
0.25	200	200	0.00025	200	0.0500	0.558	0.529	0.0479	m, b	0.094	-0.002
0.125	100	200	0.00013	200	0.0250	0.341	0.312	0.0277	se_m, se_b	0.005	0.001
0.05	200	1000	0.00005	200	0.0100	0.163	0.134	0.0110	r^2, se_y	0.988	0.002
0.025	100	1000	0.00003	200	0.0050	0.119	0.090	0.0069	F, df	326	4
0.01	40	1000	0.00001	200	0.0020	0.048	0.019	0.0002	ss_{reg}, ss_{resid}	0.002	0.000
0.005	20	1000	0.00001	200	0.0010	0.037	0.009	-0.0007			

Regression results are calculated using the function LINEST

Blank	0.029			r^2=	0.988							
				m=	0.094							
				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
10	0.044	0.015	0.000	200	1	1	0.000	0.000	-0.001
10	0.041	0.013	0.000	200	1	1	0.000		
10									
100	0.249	0.220	0.019	200	1	1	0.000	0.000	0.096
100	0.254	0.225	0.019	200	1	1	0.000		
100									
m	0.088	0.059	0.004	200	63	20000	0.006	0.006	6.433
m	0.088	0.059	0.004	200	63	20000	0.006		
m	0.090	0.061	0.004	200	63	20000	0.007		

Assay Date	6/30/2005	Test Chemical ID	none	# Concentrations tested		0				
Technician ID	JG	Replicate #	1	Microsome type	placental	Microsome ID	in vitro			

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	0.25	0.125	0.05	0.025	0.01	0.005	0	25	100	
	0.549	0.337	0.162	0.116	0.046	0.039	0.034			
	0.565	0.338	0.161	0.135	0.049	0.036	0.025			
	0.560	0.348	0.166	0.106	0.048	0.036	0.027			
Samples:	10	100	m							
	0.044	0.249	0.088							
	0.041	0.254	0.088							
			0.090							

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	
0.25	200	200	0.00025	200	0.0500	0.558	0.529	0.0416	m, b	0.079	0.000
0.125	100	200	0.00013	200	0.0250	0.341	0.312	0.0244	se_m, se_b	0.005	0.001
0.05	200	1000	0.00005	200	0.0100	0.163	0.134	0.0103	r^2, se_y	0.987	0.001
0.025	100	1000	0.00003	200	0.0050	0.119	0.090	0.0068	F, df	236	3
0.01	40	1000	0.00001	200	0.0020	0.048	0.019	0.0012	ss_{reg}, ss_{resid}	0.000	0.000
0.005	20	1000	0.00001	200	0.0010	0.037	0.009	0.0003			

Regression results are calculated using the function LINEST

	Blank	0.029		r^2=	0.987							
				m=	0.079							
				b=	0.000							

	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	
10	0.044	0.015	0.001	200	1	1	0.000	0.000		0.004
10	0.041	0.013	0.001	200	1	1	0.000			
10										
100	0.249	0.220	0.017	200	1	1	0.000	0.000		0.087
100	0.254	0.225	0.017	200	1	1	0.000			
100										
m	0.088	0.059	0.004	200	63	20000	0.007	0.007		6.978
m	0.088	0.059	0.004	200	63	20000	0.007			
m	0.090	0.061	0.004	200	63	20000	0.007			

Assay Date	6/30/2005	Test Chemical ID	none	# Concentrations tested		0			
Technician ID	JG	Replicate #	1	Microsome type	placental	Microsome ID	in vitro		

Standards:	1.5	1	0.75	0.5	0.25	0.13	Blk	Protein stock (mg/10 mL)	Protein stock ID

Samples:

mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results
0.00000	25	0.0000				m, b	
0.00000	25	0.0000				se_m, se_b	
0.00000	25	0.0000				r^2, se_y	
0.00000	25	0.0000				F, df	
0.00000	25	0.0000				ss_{reg}, ss_{resid}	
0.00000	25	0.0000					

Blank

r^2=
m=
b=

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

Assay Date	6/30/2005	Test Chemical ID	none	# Concentrations tested	0	Microsome type	placental	Microsome ID	in vitro	Technician ID	JG	Replicate #	1

Microsome Dilution Details		
Dilution A	0.094	mL microsome Stock used
	2.994	mL total volume
	31.85106	dilution factor
Dilution B	2	mL microsome Dilution A used
	20	mL total volume
	10	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	318.5106	total dilution factor

Test Chemical Concentrations	
Level	Final Concentration (M)
1	
2	
3	
4	
5	
6	
7	
8	

Protein Concentration (stock microsomes, mg/mL):	6.978
Protein Concentration (dilution added to assay, mg/mL):	0.021908

Assay Date	6/30/2005	Test Chemical ID	none	# Concentrations tested	0	Microsome type	placental	Microsome ID	In vitro	Technician ID	JG	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 (initial)	% 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 (nmol estrogen formed/mg protein/min
Full activity control	1	2	0.5	1	2633.36	5266.72	5272.05	10544.1	0.1	132801	7.94	10418	0.0160	1	0.011	15	0.0468
			0.5	2	2638.69	5277.38			0.1					1		15	
	2	2	0.5	1	2615.48	5230.96	5293.93	10587.86	0.1	132801	7.97	10462	0.0161	1	0.011	15	0.0490
			0.5	2	2678.45	5356.9			0.1					1		15	
	3	2	0.5	1	2614.87	5229.74	5206.62	10413.24	0.1	132801	7.84	10287	0.0158	1	0.011	15	0.0462
			0.5	2	2591.75	5183.5			0.1					1		15	
	4	2	0.5	1	2500.61	5001.22	5010.92	10021.84	0.1	132801	7.55	9896	0.0152	1	0.011	15	0.0463
			0.5	2	2510.31	5020.62			0.1					1		15	
Background control	1	2	0.5	1	33.81	67.62	71.42	142.84	0.1	132801	0.11	17	0.0000	1	0.011	15	0.0001
			0.5	2	37.61	75.22			0.1					1		15	
	2	2	0.5	1	25.18	50.36	60.76	121.52	0.1	132801	0.09	-5	0.0000	1	0.011	15	0.0000
			0.5	2	35.58	71.16			0.1					1		15	
	3	2	0.5	1	31.93	63.86	55.46	110.92	0.1	132801	0.08	-15	0.0000	1	0.011	15	-0.0001
			0.5	2	23.53	47.06			0.1					1		15	
	4	2	0.5	1	31.21	62.42	64.62	129.24	0.1	132801	0.10	3	0.0000	1	0.011	15	0.0000
			0.5	2	33.41	66.82			0.1					1		15	
Positive control	1	2	0.5	1	1273.08	2546.16	2518.47	5036.94	0.1	132801	3.79	4911	0.0076	1	0.011	15	0.0230
			0.5	2	1245.39	2490.78			0.1					1		15	
	2	2	0.5	1	1233.74	2467.48	2473.69	4947.38	0.1	132801	3.73	4821	0.0074	1	0.011	15	0.0226
			0.5	2	1239.95	2479.9			0.1					1		15	
	3	2	0.5	1	1192.16	2384.32	2424.31	4848.62	0.1	132801	3.65	4722	0.0073	1	0.011	15	0.0221
			0.5	2	1232.15	2464.3			0.1					1		15	
	4	2	0.5	1	1235.77	2471.54	2482.94	4965.88	0.1	132801	3.74	4840	0.0074	1	0.011	15	0.0227
			0.5	2	1247.17	2494.34			0.1					1		15	
Negative Control	1	2	0.5	1	2545.06	5090.12	5115.42	10230.84	0.1	132801	7.70	10105	0.0155	1	0.011	15	0.0473
			0.5	2	2570.36	5140.72			0.1					1		15	
	2	2	0.5	1	2546.98	5093.96	5070.51	10141.02	0.1	132801	7.64	10015	0.0154	1	0.011	15	0.0469
			0.5	2	2523.53	5047.06			0.1					1		15	
	3	2	0.5	1	2579.04	5158.08	5150.98	10301.96	0.1	132801	7.76	10176	0.0157	1	0.011	15	0.0477
			0.5	2	2571.94	5143.88			0.1					1		15	
	4	2	0.5	1	2472.95	4945.9	4994.8	9989.6	0.1	132801	7.52	9863	0.0152	1	0.011	15	0.0462
			0.5	2	2521.85	5043.7			0.1					1		15	
none	1-1	2	0.5	1					0.1	132801		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1								
	1-2	2	0.5	1					0.1	132801		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	1-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	2-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	2-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	2-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	3-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	3-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	3-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	4-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	4-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	4-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	5-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	5-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	5-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	6-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	6-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	6-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	7-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	7-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	7-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	8-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	8-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	8-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													

Assay Date	6/30/2005	Test Chemical ID	none	# Concentrations tested	Microsome 0 type	placental	Microsome ID in vitro	Technician ID	JG	Replicate #	1

Control Type	Portion	Average	SD
Full activity	Beginning	0.0489	0.0001
Full activity	End	0.0473	0.0013
Full activity	Overall	0.0481	0.0012
Background	Beginning	0.0000	7.05938E-05
Background	End	0.0000	6.06603E-05
Background	Overall	0.0000	6.29114E-05
Positive	Beginning	0.0228	0.0003
Positive	End	0.0224	0.0004
Positive	Overall	0.0226	0.0004
Negative	Beginning	0.0471	0.0003
Negative	End	0.0469	0.0010
Negative	Overall	0.0470	0.0006

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
NA					

Percent of control values				
	Log[test	Replicate		
Level	substance]	1	2	3
1				
2				
3				
4				
5				
6				
7				
8				

Aromatase Assay Spreadsheet

Assay Date	7/1/2005	Test Chemical ID	none	# Concentrations tested			
Technician ID	JG	Replicate #	2-Jan	Microsome type	placental	Microsome ID	

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0202	29661.52	1468392	
2	0.0200	29953.85	1497693	
3	0.0200	30309.03	1515452	
4	0.0202	30322.52	1501115	
5	0.0202	31298.18	1549415	
			Average DPM/g soln	1506413
			SD	29498
			CV	1.96
			µCi/g soln	0.679

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	8.2247 g
Mass of dilution B used in substrate prep	4.6069 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.571332 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00768 µg/g soln.
µg/g soln.

a. µCi/g soln 0.679
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.

= 0.571332 + 0.00768
= 0.579014 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.172 µCi/µg ASDN

745123 dpm/nmol

Assay Date	7/1/2005	Test Chemical ID	none	# Concentrations tested	0		
Technician ID	JG	Replicate #	2	Microsome type	placental	Microsome ID	0

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	0.25	0.125	0.05	0.025	0.01	0.005	0	25	100	
	0.547	0.335	0.151	0.087	0.048	0.037	0.033			
	0.541	0.333	0.154	0.091	0.048	0.036	0.032			
	0.552	0.325	0.153	0.094	0.056	0.039	0.026			
Samples:	10	100	m							
	0.040	0.250	0.088							
	0.040	0.249	0.093							
			0.092							

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	
0.25	200	200	0.00025	200	0.0500	0.547	0.517	0.0482	m, b	0.095	-0.001
0.125	100	200	0.00013	200	0.0250	0.331	0.301	0.0278	se_m, se_b	0.004	0.001
0.05	200	1000	0.00005	200	0.0100	0.153	0.122	0.0109	r^2, se_y	0.993	0.002
0.025	100	1000	0.00003	200	0.0050	0.091	0.061	0.0050	F, df	535	4
0.01	40	1000	0.00001	200	0.0020	0.050	0.020	0.0012	ss_{regr}, ss_{resid}	0.002	0.000
0.005	20	1000	0.00001	200	0.0010	0.037	0.007	-0.0001			

Regression results are calculated using the function LINEST

Blank	0.030		r^2=	0.993								
			m=	0.095								
			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
10	0.040	0.009	0.000	200	1	1	0.000	0.000	0.001
10	0.040	0.010	0.000	200	1	1	0.000		
10					1	1			
100	0.250	0.220	0.020	200	1	1	0.000	0.000	0.100
100	0.249	0.219	0.020	200	1	1	0.000		
100						1			
m	0.088	0.058	0.005	200	63	20000	0.008	0.008	7.944
m	0.093	0.062	0.005	200	63	20000	0.008		
m	0.092	0.061	0.005	200	63	20000	0.008		

Assay Date	7/1/2005	Test Chemical ID	none	# Concentrations tested		0				
Technician ID	JG	Replicate #	2	Microsome type	placental	Microsome ID	0			

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	0.25	0.125	0.05	0.025	0.01	0.005	0	25	100	
	0.547	0.335	0.151	0.087	0.048	0.037	0.033			
	0.541	0.333	0.154	0.091	0.048	0.036	0.032			
	0.552	0.325	0.153	0.094	0.056	0.039	0.026			
Samples:	10	100	m							
	0.040	0.250	0.088							
	0.040	0.249	0.093							
			0.092							

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	
0.25	200	200	0.00025	200	0.0500	0.547	0.517	0.0425	m, b	0.082	0.000
0.125	100	200	0.00013	200	0.0250	0.331	0.301	0.0249	se_m, se_b	0.001	0.000
0.05	200	1000	0.00005	200	0.0100	0.153	0.122	0.0103	r^2, se_y	1.000	0.000
0.025	100	1000	0.00003	200	0.0050	0.091	0.061	0.0052	F, df	6542	3
0.01	40	1000	0.00001	200	0.0020	0.050	0.020	0.0019	ss_{reg}, ss_{resid}	0.000	0.000
0.005	20	1000	0.00001	200	0.0010	0.037	0.007	0.0008			

Regression results are calculated using the function LINEST

Blank	0.030	r^2=	1.000		
		m=	0.082		
		b=	0.000		

	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
10	0.040	0.009	0.001	200	1	1	0.000	0.000	0.005
10	0.040	0.010	0.001	200	1	1	0.000		
10					1	1			
100	0.250	0.220	0.018	200	1	1	0.000	0.000	0.091
100	0.249	0.219	0.018	200	1	1	0.000		
100						1			
m	0.088	0.058	0.005	200	63	20000	0.008	0.008	8.229
m	0.093	0.062	0.005	200	63	20000	0.008		
m	0.092	0.061	0.005	200	63	20000	0.008		

Technician ID	JG	Replicate #	2	Microsome type	placental	Microsome ID	0

								Protein stock	
Standards:	1.5	1	0.75	0.5	0.25	0.13	Blk	(mg/10 mL)	Protein stock ID

Samples:

mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results
0.00000	25	0.0000				m, b	
0.00000	25	0.0000				se_m, se_b	
0.00000	25	0.0000				r^2, se_y	
0.00000	25	0.0000				F, df	
0.00000	25	0.0000				ss_{reg}, ss_{resid}	
0.00000	25	0.0000					

Blank — r^2= m= b=

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

Assay Date	7/1/2005	Test Chemical ID	none	# Concentrations tested	0	Microsome type	placental	Microsome ID	0	Technician ID	JG	Replicate #	2

Microsome Dilution Details		
Dilution A	0.094	mL microsome Stock used
	2.994	mL total volume
	31.851064	dilution factor
Dilution B	2	mL microsome Dilution A used
	20	mL total volume
	10	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	318.51064	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	8.229
Protein Concentration (dilution added to assay, mg/mL):	0.025836

Test Chemical Concentrations	
Level	Final Concentration (M)
1	
2	
3	
4	
5	
6	
7	
8	

Assay Date	7/1/2005	Test Chemical ID	none	# Concentrations tested		Microsome type	placental	Microsome ID		Technician ID	JG	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)	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 (initial)	% 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 (nmo estrogen formed/mg protein/min
Full activity control	1	2	0.5	1	3264.95	6529.9	6498.06	12996.12	0.1	150641	8.63	12834	0.0172	1	0.013	15	0.0444
			0.5	2	3233.11	6466.22			0.1					1		15	
	2	2	0.5	1	3006.87	6013.74	6065.77	12131.54	0.1	150641	8.05	11969	0.0161	1	0.013	15	0.0414
			0.5	2	3058.9	6117.8			0.1					1		15	
	3	2	0.5	1	2558.42	5116.84	5156.29	10312.58	0.1	150641	6.85	10150	0.0136	1	0.013	15	0.0351
			0.5	2	2597.87	5195.74			0.1					1		15	
	4	2	0.5	1	2998.59	5997.18	6072.08	12144.16	0.1	150641	8.06	11982	0.0161	1	0.013	15	0.0415
			0.5	2	3073.49	6146.98			0.1					1		15	
Background control	1	2	0.5	1	30.44	60.88	66.12	132.24	0.1	150641	0.09	-30	0.0000	1	0.013	15	-0.0001
			0.5	2	35.68	71.36			0.1					1		15	
	2	2	0.5	1	41.09	82.18	81.57	163.14	0.1	150641	0.11	1	0.0000	1	0.013	15	0.0000
			0.5	2	40.48	80.96			0.1					1		15	
	3	2	0.5	1	39.64	79.28	90.07	180.14	0.1	150641	0.12	18	0.0000	1	0.013	15	0.0001
			0.5	2	50.43	100.86			0.1					1		15	
	4	2	0.5	1	45.94	91.88	87.32	174.64	0.1	150641	0.12	12	0.0000	1	0.013	15	0.0000
			0.5	2	41.38	82.76			0.1					1		15	
Positive control	1	2	0.5	1	1455.43	2910.86	2878.45	5756.9	0.1	150641	3.82	5594	0.0075	1	0.013	15	0.0194
			0.5	2	1423.02	2846.04			0.1					1		15	
	2	2	0.5	1	1381.1	2762.2	2774.61	5549.22	0.1	150641	3.68	5387	0.0072	1	0.013	15	0.0187
			0.5	2	1393.51	2787.02			0.1					1		15	
	3	2	0.5	1	1430.75	2861.5	2832.15	5664.3	0.1	150641	3.76	5502	0.0074	1	0.013	15	0.0191
			0.5	2	1401.4	2802.8			0.1					1		15	
	4	2	0.5	1	1384.89	2769.78	2839.11	5678.22	0.1	150641	3.77	5516	0.0074	1	0.013	15	0.0191
			0.5	2	1454.22	2908.44			0.1					1		15	
Negative Control	1	2	0.5	1	3095.03	6190.06	6144.45	12288.9	0.1	150641	8.16	12125	0.0163	1	0.013	15	0.0420
			0.5	2	3049.42	6098.84			0.1					1		15	
	2	2	0.5	1	2916.26	5832.52	5817.31	11634.62	0.1	150641	7.72	11472	0.0154	1	0.013	15	0.0397
			0.5	2	2901.05	5802.1			0.1					1		15	
	3	2	0.5	1	3033.35	6066.7	6057.89	12115.78	0.1	150641	8.04	11953	0.0160	1	0.013	15	0.0414
			0.5	2	3024.54	6049.08			0.1					1		15	
	4	2	0.5	1	2965.41	5930.82	5957.73	11915.46	0.1	150641	7.91	11753	0.0158	1	0.013	15	0.0407
			0.5	2	2992.32	5984.64			0.1					1		15	
none	1-1	2		1						0		#VALUE!	#VALUE!		0.000	15	#VALUE!
				2													
	1-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	1-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	2-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	2-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	2-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	3-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	3-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	3-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	4-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	4-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	4-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	5-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	5-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	5-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	6-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	6-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	6-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	7-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	7-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	7-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	8-1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	8-2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	8-3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													

Assay Date	7/1/2005	Test Chemical ID	none	# Concentrations tested	0	Microsome type	placental	Microsome ID	0	Technician ID	JG	Replicate #	2

Control Type	Portion	Average	SD
Full activity	Beginning	0.0429	0.0021
Full activity	End	0.0383	0.0045
Full activity	Overall	0.0406	0.0039
Background	Beginning	-0.0001	7.5666E-05
Background	End	0.0001	1.34681E-05
Background	Overall	0.0000	7.4129E-05
Positive	Beginning	0.0190	0.0005
Positive	End	0.0191	0.0000
Positive	Overall	0.0190	0.0003
Negative	Beginning	0.0409	0.0016
Negative	End	0.0410	0.0005
Negative	Overall	0.0410	0.0010

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
NA					

Percent of control values				
	Log[test	Replicate		
Level	substance]	1	2	3
1				
2				
3				
4				
5				
6				
7				
8				

Aromatase Assay Spreadsheet

Assay Date	6/1/2005	Test Chemical ID	none	# Concentrations tested			
Technician ID	JG	Replicate #	ptn only	Microsome type	placental	Microsome ID	Battelle

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.
1			
2			
3			
4			
5			
			Average DPM/g soln
			SD
			CV
			µCi/g soln

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				#DIV/0!
Dilution A				#DIV/0!
Dilution B				#DIV/0!

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution		g
Mass of dilution B used in substrate prep		g
Concentration of nonradiolabeled ASDN in substrate soln.	#DIV/0!	µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = µg/g soln.
µg/g soln.

a. µCi/g soln
b. Specific activity of [^{3}H]ASDN (µCi/mmol)
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.

= #DIV/0! +
= #DIV/0! µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= µCi/µg ASDN

dpm/nmol

Assay Date	6/1/2005	Test Chemical ID	none	# Concentrations tested	0		
Technician ID	JG	Replicate #	ptn only	Microsome type	placental	Microsome ID	Battelle

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	0.25	0.125	0.05	0.025	0.01	0.005	0	25	100	
	0.560	0.332	0.158	0.094	0.045	0.031	0.026			
	0.568	0.344	0.168	0.086	0.049	0.034	0.023			
	0.552	0.336	0.156	0.089	0.047	0.038	0.029			

Samples:	10	100	m
	0.054	0.260	0.327
	0.052	0.254	0.322
			0.323

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	
0.25	200	200	0.00025	200	0.0500	0.560	0.534	0.0481	m, b	0.092	-0.001
0.125	100	200	0.00013	200	0.0250	0.337	0.311	0.0277	se_m, se_b	0.004	0.001
0.05	200	1000	0.00005	200	0.0100	0.161	0.135	0.0115	r^2, se_y	0.992	0.002
0.025	100	1000	0.00003	200	0.0050	0.090	0.064	0.0049	F, df	479	4
0.01	40	1000	0.00001	200	0.0020	0.047	0.021	0.0010	ss_{reg}, ss_{resid}	0.002	0.000
0.005	20	1000	0.00001	200	0.0010	0.035	0.009	-0.0002			

Regression results are calculated using the function LINEST

Blank	0.026	r^2=	0.992
		m=	0.092
		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
10	0.054	0.028	0.002	200	1	1	0.000	0.000	0.008
10	0.052	0.025	0.001	200	1	1	0.000		
10					1	1			
100	0.260	0.234	0.021	200	1	1	0.000	0.000	0.102
100	0.254	0.228	0.020	200	1	1	0.000		
100					1	1			
m	0.327	0.301	0.027	200	12	2000	0.022	0.022	22.027
m	0.322	0.296	0.026	200	12	2000	0.022		
m	0.323	0.297	0.026	200	12	2000	0.022		

Assay Date	6/1/2005	Test Chemical ID	none	# Concentrations tested	0		
Technician ID	JG	Replicate #	ptn only	Microsome type	placental	Microsome ID	Battelle

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	0.25	0.125	0.05	0.025	0.01	0.005	0	25	100	
	0.560	0.332	0.158	0.094	0.045	0.031	0.026			
	0.568	0.344	0.168	0.086	0.049	0.034	0.023			
	0.552	0.336	0.156	0.089	0.047	0.038	0.029			

Samples:	10	100	m
	0.054	0.260	0.327
	0.052	0.254	0.322
			0.323

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
0.25	200	200	0.00025	200	0.0500	0.560	0.534	0.0423
0.125	100	200	0.00013	200	0.0250	0.337	0.311	0.0247
0.05	200	1000	0.00005	200	0.0100	0.161	0.135	0.0107
0.025	100	1000	0.00003	200	0.0050	0.090	0.064	0.0051
0.01	40	1000	0.00001	200	0.0020	0.047	0.021	0.0017
0.005	20	1000	0.00001	200	0.0010	0.035	0.009	0.0007

Blank	0.026	r^2=	0.998	
		m=	0.079	
		b=	0.000	

Variables	Regression results	
m, b	0.079	0.000
se_m, se_b	0.002	0.000
r^2, se_y	0.998	0.001
F, df	1451	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
10	0.054	0.028	0.002	200	1	1	0.000	0.000	0.011
10	0.052	0.025	0.002	200	1	1	0.000		
10					1	1			
100	0.260	0.234	0.019	200	1	1	0.000	0.000	0.092
100	0.254	0.228	0.018	200	1	1	0.000		
100					1	1			
m	0.327	0.301	0.024	200	12	2000	0.020	0.020	19.687
m	0.322	0.296	0.023	200	12	2000	0.020		
m	0.323	0.297	0.024	200	12	2000	0.020		

Assay Date	6/1/2005	Test Chemical ID	none	# Concentrations tested		0			
Technician ID	JG	Replicate #	ptn only	Microsome type		placental	Microsome ID	Battelle	
Standards:	1.5	1	0.75	0.5	0.25	0.13	Blk	Protein stock (mg/10 mL)	Protein stock ID

Samples:

mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results
0.00000	25	0.0000				m, b	
0.00000	25	0.0000				se_m, se_b	
0.00000	25	0.0000				r^2, se_y	
0.00000	25	0.0000				F, df	
0.00000	25	0.0000				ss_{reg}, ss_{resid}	
0.00000	25	0.0000					
Blank		r^2=					Regression results are calculated using the function LINEST
		m=					
		b=					

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

Assay Date	6/1/2005	Test Chemical ID	none	# Concentrations tested	0	Microsome type	placental	Microsome ID	Battelle	Technician ID	JG	Replicate #	ptn only

Microsome Dilution Details		
Dilution A		mL microsome Stock used
		mL total volume
	#DIV/0!	dilution factor
Dilution B		mL microsome Dilution A used
		mL total volume
	#DIV/0!	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	#DIV/0!	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	
Protein Concentration (dilution added to assay, mg/mL):	#DIV/0!

Test Chemical Concentrations	
Level	Final Concentration (M)
1	
2	
3	
4	
5	
6	
7	
8	

Assay Date	6/1/2005	Test Chemical ID	none	# Concentrations tested		Microsome type	placental	Microsome ID	Battelle	Technician ID	JG	Replicate #	ptn only

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 (initial)	% conversion to product	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed	Volume diluted microsomes used in assay tubs (mL)	Final [protein] in assay (mg/mL)	Incubation time (min)	Aromatase activity (nmo estrogen formed/mg protein/min
Full activity control	1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
Background control	1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
Positive control	1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
Negative Control	1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
none	1-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	1-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	1-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	5-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	5-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	5-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	6-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	6-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	6-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	7-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	7-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	7-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	8-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	8-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	8-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													

Assay Date	6/1/2005	Test Chemical ID	none	# Concentrations tested	0	Microsome type	placental	Microsome ID	Battelle	Technician ID	JG	Replicate #	ptn only

Control Type	Portion	Average	SD
Full activity	Beginning	#VALUE!	#VALUE!
Full activity	End	#VALUE!	#VALUE!
Full activity	Overall	#VALUE!	#VALUE!
Background	Beginning	#VALUE!	#VALUE!
Background	End	#VALUE!	#VALUE!
Background	Overall	#VALUE!	#VALUE!
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					

	Percent of control values			
	Log[test	Replicate		
Level	substance]	1	2	3
1				
2				
3				
4				
5				
6				
7				
8				

Aromatase Assay Spreadsheet

Assay Date	6/2/2005	Test Chemical ID		# Concentrations tested			
Technician ID	JG	Replicate #	ptn only	Microsome type	placental	Microsome ID Battelle	

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.
1			
2			
3			
4			
5			
			Average DPM/g soln
			SD
			CV
			µCi/g soln

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				#DIV/0!
Dilution A				#DIV/0!
Dilution B				#DIV/0!

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution		g
Mass of dilution B used in substrate prep		g
Concentration of nonradiolabeled ASDN in substrate soln.	#DIV/0!	µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = µg/g soln.
µg/g soln.

a. µCi/g soln
b. Specific activity of [^{3}H]ASDN (µCi/mmol)
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.

= #DIV/0! +
= #DIV/0! µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= µCi/µg ASDN

dpm/nmol

Assay Date	6/2/2005	Test Chemical ID	0	# Concentrations tested	0		
Technician ID	JG	Replicate #	ptn only	Microsome type	placental	Microsome ID	Battelle

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	0.25	0.125	0.05	0.025	0.01	0.005	0	25	100	
	0.552	0.336	0.163	0.096	0.050	0.034	0.024			
	0.541	0.381	0.163	0.099	0.045	0.035	0.024			
	0.554	0.331	0.169	0.094	0.052	0.035	0.021			

Samples:	10	100	m
	0.048	0.258	0.442
	0.046	0.250	0.427
			0.417

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	
0.25	200	200	0.00025	200	0.0500	0.549	0.526	0.0473	m, b	0.093	-0.002
0.125	100	200	0.00013	200	0.0250	0.350	0.327	0.0288	se_m, se_b	0.006	0.002
0.05	200	1000	0.00005	200	0.0100	0.165	0.142	0.0116	r^2, se_y	0.985	0.003
0.025	100	1000	0.00003	200	0.0050	0.097	0.074	0.0052	F, df	257	4
0.01	40	1000	0.00001	200	0.0020	0.049	0.026	0.0008	ss_{reg}, ss_{resid}	0.002	0.000
0.005	20	1000	0.00001	200	0.0010	0.035	0.012	-0.0006			

Regression results are calculated using the function LINEST

Blank	0.023	r^2=	0.985		
		m=	0.093		
		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
10	0.048	0.025	0.001	200	1	1	0.000	0.000	0.003
10	0.046	0.023	0.000	200	1	1	0.000		
10									
100	0.258	0.235	0.020	200	1	1	0.000	0.000	0.099
100	0.250	0.227	0.019	200	1	1	0.000		
100									
m	0.442	0.419	0.037	200	12	2000	0.031	0.030	30.109
m	0.427	0.404	0.036	200	12	2000	0.030		
m	0.417	0.394	0.035	200	12	2000	0.029		

Assay Date	6/2/2005	Test Chemical ID	0	# Concentrations tested	0		
Technician ID	JG	Replicate #	ptn only	Microsome type	placental	Microsome ID	Battelle

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	0.25	0.125	0.05	0.025	0.01	0.005	0	25	100	
	0.552	0.338	0.163	0.096	0.050	0.034	0.024			
	0.541	0.381	0.163	0.099	0.045	0.035	0.024			
	0.554	0.331	0.169	0.094	0.052	0.035	0.021			

Samples:	10	100
	0.048	0.258
	0.046	0.250

m	
0.442	
0.427	
0.417	

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
0.25	200	200	0.00025	200	0.0500	0.549	0.526	0.0399
0.125	100	200	0.00013	200	0.0250	0.350	0.327	0.0247
0.05	200	1000	0.00005	200	0.0100	0.165	0.142	0.0106
0.025	100	1000	0.00003	200	0.0050	0.097	0.074	0.0054
0.01	40	1000	0.00001	200	0.0020	0.049	0.026	0.0017
0.005	20	1000	0.00001	200	0.0010	0.035	0.012	0.0006
			Blank	0.023				

Variables	Regression results	
m, b	0.076	0.000
se_m, se_b	0.002	0.000
r^2, se_y	0.998	0.001
F, df	1489	3
ss_{reg}, ss_{resid}	0.000	0.000

Regression results are calculated using the function LINEST

r^2=	0.998		
m=	0.076		
b=	0.000		

	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
10	0.048	0.025	0.002	200	1	1	0.000	0.000	0.008
10	0.048	0.023	0.001	200	1	1	0.000		
10									
100	0.258	0.235	0.018	200	1	1	0.000	0.000	0.087
100	0.250	0.227	0.017	200	1	1	0.000		
100									
m	0.442	0.419	0.032	200	12	2000	0.026	0.026	25.629
m	0.427	0.404	0.031	200	12	2000	0.026		
m	0.417	0.394	0.030	200	12	2000	0.025		

Assay Date	6/2/2005	Test Chemical ID		0	# Concentrations tested		0		
Technician ID	JG	Replicate #	ptn only		Microsome type	placental	Microsome ID	Battelle	
Standards:	1.5	1	0.75	0.5	0.25	0.13	Blk	Protein stock (mg/10 mL)	Protein stock ID

Samples:

mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results
0.00000	25	0.0000				m, b	
0.00000	25	0.0000				se_m, se_b	
0.00000	25	0.0000				r^2, se_y	
0.00000	25	0.0000				F, df	
0.00000	25	0.0000				ss_{reg}, ss_{resid}	
0.00000	25	0.0000					

Blank

r^2=
m=
b=

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

Assay Date	6/2/2005	Test Chemical ID		0	# Concentrations tested	0	Microsome type	placental	Microsome ID	Battelle	Technician ID	JG	Replicate # ptn only

Microsome Dilution Details		
Dilution A		mL microsome Stock used
		mL total volume
	#DIV/0!	dilution factor
Dilution B		mL microsome Dilution A used
		mL total volume
	#DIV/0!	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	#DIV/0!	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	
Protein Concentration (dilution added to assay, mg/mL):	#DIV/0!

Test Chemical Concentrations	
Level	Final Concentration (M)
1	
2	
3	
4	
5	
6	
7	
8	

Assay Date	8/2/2005	Test Chemical ID	0	# Concentrations tested	0	Microsome type	placental	Microsome ID	Battelle	Technician ID	JG	Replicate #	pm only

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 (initial)	% 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 (nmo estrogen formed/mg protein/min
Full activity control	1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
Background control	1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
Positive control	1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
Negative Control	1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
0	1-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	1-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	1-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	5-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	5-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	5-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	6-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	6-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	6-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	7-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	7-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	7-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	8-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	8-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	8-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													

Assay Date	6/2/2005	Test Chemical ID		0	# Concentrations tested	Microsome 0 type	placental	Microsome ID Battelle	Technician ID JG	Replicate #	ptn only

Control Type	Portion	Average	SD
Full activity	Beginning	#VALUE!	#VALUE!
Full activity	End	#VALUE!	#VALUE!
Full activity	Overall	#VALUE!	#VALUE!
Background	Beginning	#VALUE!	#VALUE!
Background	End	#VALUE!	#VALUE!
Background	Overall	#VALUE!	#VALUE!
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					

	Percent of control values			
	Log[test	Replicate		
Level	substance]	1	2	3
1				
2				
3				
4				
5				
6				
7				
8				

Aromatase Assay Spreadsheet

Assay Date	6/30/2005	Test Chemical ID		# Concentrations tested		
Technician ID	JG	Replicate #	ptn only	Microsome type	placental	Microsome ID In Vitro

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.
1			
2			
3			
4			
5			
			Average DPM/g soln
			SD
			CV
			µCi/g soln

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				#DIV/0!
Dilution A				#DIV/0!
Dilution B				#DIV/0!

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution		g
Mass of dilution B used in substrate prep		g
Concentration of nonradiolabeled ASDN in substrate soln.	#DIV/0!	µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = µg/g soln.
µg/g soln.

a. µCi/g soln
b. Specific activity of [^{3}H]ASDN (µCi/mmol)
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.

= #DIV/0! +
= #DIV/0! µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= µCi/µg ASDN

dpm/nmol

Assay Date	6/30/2005	Test Chemical ID	0	# Concentrations tested	0				
Technician ID	JG	Replicate #	ptn only	Microsome type	placental		Microsome ID	In Vitro	

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	0.25	0.125	0.05	0.025	0.01	0.005	0	25	100	
	0.551	0.334	0.155	0.090	0.051	0.033	0.025			
	0.560	0.333	0.158	0.088	0.046	0.039	0.026			
	0.560	0.337	0.154	0.089	0.050	0.035	0.027			
Samples:	10	100	m							
	0.043	0.245	0.204							
	0.043	0.251	0.197							
			0.195							

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
0.25	200	200	0.00025	200	0.0500	0.557	0.531	0.0483
0.125	100	200	0.00013	200	0.0250	0.335	0.308	0.0277
0.05	200	1000	0.00005	200	0.0100	0.156	0.129	0.0111
0.025	100	1000	0.00003	200	0.0050	0.089	0.063	0.0049
0.01	40	1000	0.00001	200	0.0020	0.049	0.023	0.0012
0.005	20	1000	0.00001	200	0.0010	0.036	0.009	-0.0001
			Blank	0.026				

Variables	Regression results	
m, b	0.093	-0.001
se_m, se_b	0.004	0.001
r^2, se_y	0.993	0.002
F, df	555	4
ss_{reg}, ss_{resid}	0.002	0.000

Regression results are calculated using the function LINEST

r^2= 0.993
m= 0.093
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
10	0.043	0.016	0.001	200	1	1	0.000	0.000	0.003
10	0.043	0.017	0.001	200	1	1	0.000		
10									
100	0.245	0.218	0.019	200	1	1	0.000	0.000	0.098
100	0.251	0.225	0.020	200	1	1	0.000		
100									
m	0.204	0.177	0.016	200	16	2000	0.010	0.009	9.414
m	0.197	0.171	0.015	200	16	2000	0.009		
m	0.195	0.169	0.015	200	16	2000	0.009		

Assay Date	6/30/2005	Test Chemical ID	0	# Concentrations tested	0				
Technician ID	JG	Replicate #	ptn only	Microsome type	placental	Microsome ID	In Vitro		

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	0.25	0.125	0.05	0.025	0.01	0.005	0	25	100	
	0.551	0.334	0.155	0.090	0.051	0.033	0.025			
	0.560	0.333	0.158	0.088	0.046	0.039	0.026			
	0.560	0.337	0.154	0.089	0.050	0.035	0.027			
Samples:	10	100	m							
	0.043	0.245	0.204							
	0.043	0.251	0.197							
			0.195							

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	
0.25	200	200	0.00025	200	0.0500	0.557	0.531	0.0427	m, b	0.080	0.000
0.125	100	200	0.00013	200	0.0250	0.335	0.308	0.0248	se_m, se_b	0.001	0.000
0.05	200	1000	0.00005	200	0.0100	0.156	0.129	0.0104	r^2, se_y	0.999	0.000
0.025	100	1000	0.00003	200	0.0050	0.089	0.063	0.0051	F, df	3922	3
0.01	40	1000	0.00001	200	0.0020	0.049	0.023	0.0019	ss_{reg}, ss_{resid}	0.000	0.000
0.005	20	1000	0.00001	200	0.0010	0.036	0.009	0.0008			

Regression results are calculated using the function LINEST

Blank	0.026	r^2=	0.999		
		m=	0.080		
		b=	0.000		

	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
10	0.043	0.016	0.001	200	1	1	0.000	0.000	0.007
10	0.043	0.017	0.001	200	1	1	0.000		
10									
100	0.245	0.218	0.018	200	1	1	0.000	0.000	0.089
100	0.251	0.225	0.018	200	1	1	0.000		
100									
m	0.204	0.177	0.014	200	16	2000	0.009	0.009	8.689
m	0.197	0.171	0.014	200	16	2000	0.009		
m	0.195	0.169	0.014	200	16	2000	0.009		

Assay Date	6/30/2005	Test Chemical ID	0	# Concentrations tested	0				
Technician ID	JG	Replicate #	ptn only	Microsome type	placental	Microsome ID	In Vitro		

								Protein stock	
Standards:	1.5	1	0.75	0.5	0.25	0.13	Blk	(mg/10 mL)	Protein stock ID
Samples:									

mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results
0.00000	25	0.0000				m, b	
0.00000	25	0.0000				se_m, se_b	
0.00000	25	0.0000				r^2, se_y	
0.00000	25	0.0000				F, df	
0.00000	25	0.0000				ss_{reg}, ss_{resid}	
0.00000	25	0.0000					

Blank

r^2=
m=
b=

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

Assay Date	6/30/2005	Test Chemical ID		# Concentrations tested	0	Microsome type	placental	Microsome ID	In Vitro	Technician ID	JG	Replicate #	ptn only

Microsome Dilution Details

Dilution A	16	mL microsome Stock used
	2000	mL total volume
	125	dilution factor
Dilution B		mL microsome Dilution A used
		mL total volume
	#DIV/0!	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	#DIV/0!	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	
Protein Concentration (dilution added to assay, mg/mL):	#DIV/0!

Test Chemical Concentrations	
Level	Final Concentration (M)
1	
2	
3	
4	
5	
6	
7	
8	

Assay Date	9/30/2005	Test Chemical ID	0	# Concentrations tested	0	Microsome type	placental	Microsome ID	In Vitro	Technician ID	JG	Replicate #	pth only

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 (initial)	% 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 (nmol estrogen formed/mg protein/min
Full activity control	1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
Background control	1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
Positive control	1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
Negative Control	1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
0	1-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	1-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	1-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	5-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	5-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	5-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	6-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	6-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	6-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	7-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	7-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	7-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	8-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	8-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	8-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													

Assay Date	6/30/2005	Test Chemical ID	0	# Concentrations tested	0	Microsome type	placental	Microsome ID	In Vitro	Technician ID	JG	Replicate #	ptn only

Control Type	Portion	Average	SD
Full activity	Beginning	#VALUE!	#VALUE!
Full activity	End	#VALUE!	#VALUE!
Full activity	Overall	#VALUE!	#VALUE!
Background	Beginning	#VALUE!	#VALUE!
Background	End	#VALUE!	#VALUE!
Background	Overall	#VALUE!	#VALUE!
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					

Percent of control values				
	Log[test	Replicate		
Level	substance]	1	2	3
1				
2				
3				
4				
5				
6				
7				
8				

Aromatase Assay Spreadsheet

Assay Date	7/1/2005	Test Chemical ID	none	# Concentrations tested		
Technician ID	JG	Replicate #	ptn only	Microsome type	placental	Microsome ID In Vitro

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.
1			
2			
3			
4			
5			
			Average DPM/g soln
			SD
			CV
			µCi/g soln

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		0.00
Dilution A			100	0.00
Dilution B			10	0.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution		g
Mass of dilution B used in substrate prep		g
Concentration of nonradiolabeled ASDN in substrate soln.	#DIV/0!	µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = µg/g soln.
µg/g soln.

a. µCi/g soln
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.

= #DIV/0! +
= #DIV/0! µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= µCi/µg ASDN

dpm/nmol

Assay Date	7/1/2005	Test Chemical ID	none	# Concentrations tested	0		
Technician ID	JG	Replicate #	ptn only	Microsome type	placental	Microsome ID	In Vitro

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	0.25	0.125	0.05	0.025	0.01	0.005	0	25	100	
	0.532	0.326	0.150	0.092	0.057	0.036	0.030			
	0.536	0.326	0.156	0.094	0.051	0.035	0.027			
	0.543	0.321	0.154	0.091	0.051	0.036	0.027			
Samples:	10	100	m							
	0.043	0.253	0.193							
	0.047	0.251	0.187							
			0.185							

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	
0.25	200	200	0.00025	200	0.0500	0.537	0.509	0.0482	m, b	0.097	-0.001
0.125	100	200	0.00013	200	0.0250	0.325	0.297	0.0277	se_m, se_b	0.004	0.001
0.05	200	1000	0.00005	200	0.0100	0.153	0.125	0.0110	r^2, se_y	0.993	0.002
0.025	100	1000	0.00003	200	0.0050	0.092	0.064	0.0051	F, df	531	4
0.01	40	1000	0.00001	200	0.0020	0.053	0.025	0.0013	ss_{reg}, ss_{resid}	0.002	0.000
0.005	20	1000	0.00001	200	0.0010	0.036	0.008	-0.0003			
			Blank	0.028	r^2=	0.993					
					m=	0.097					
					b=	-0.001					

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
10	0.043	0.015	0.000	200	1	1	0.000	0.000	0.003
10	0.047	0.019	0.001	200	1	1	0.000		
10					1	1			
100	0.253	0.225	0.021	200	1	1	0.000	0.000	0.103
100	0.251	0.223	0.021	200	1	1	0.000		
100						1			
m	0.193	0.165	0.015	200	16	2000	0.009	0.009	9.028
m	0.187	0.159	0.014	200	16	2000	0.009		
m	0.185	0.157	0.014	200	16	2000	0.009		

Assay Date	7/1/2005	Test Chemical ID	none	# Concentrations tested		0				
Technician ID	JG	Replicate #	ptn only	Microsome type		placental	Microsome ID	In Vitro		

								Protein stock (mg	Total volume of	
Standards:	0.25	0.125	0.05	0.025	0.01	0.005	0	BSA)	stock (mL)	Protein stock ID
	0.532	0.326	0.150	0.092	0.057	0.036	0.030	25	100	
	0.536	0.326	0.156	0.094	0.051	0.035	0.027			
	0.543	0.321	0.154	0.091	0.051	0.036	0.027			

Samples:	10	100	m
	0.043	0.253	0.193
	0.047	0.251	0.187
			0.185

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
0.25	200	200	0.00025	200	0.0500	0.537	0.509	0.0426
0.125	100	200	0.00013	200	0.0250	0.325	0.297	0.0248
0.05	200	1000	0.00005	200	0.0100	0.153	0.125	0.0104
0.025	100	1000	0.00003	200	0.0050	0.092	0.064	0.0053
0.01	40	1000	0.00001	200	0.0020	0.053	0.025	0.0020
0.005	20	1000	0.00001	200	0.0010	0.036	0.008	0.0006

Variables	Regression results	
m, b	0.084	0.000
se_m, se_b	0.002	0.000
r^2, se_y	0.999	0.000
F, df	2410	3
ss_{reg}, ss_{resid}	0.000	0.000

Regression results are calculated using the function LINEST

Blank	0.028	r^2=	0.999	
		m=	0.084	
		b=	0.000	

	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
10	0.043	0.015	0.001	200	1	1	0.000	0.000	0.007
10	0.047	0.019	0.001	200	1	1	0.000		
10					1	1			
100	0.253	0.225	0.019	200	1	1	0.000	0.000	0.094
100	0.251	0.223	0.019	200	1	1	0.000		
100						1			
m	0.193	0.165	0.014	200	16	2000	0.009	0.008	8.345
m	0.187	0.159	0.013	200	16	2000	0.008		
m	0.185	0.157	0.013	200	16	2000	0.008		

Assay Date	7/1/2005	Test Chemical ID	none	# Concentrations tested		0			
Technician ID	JG	Replicate #	ptn only	Microsome type		placental	Microsome ID	In Vitro	
Standards:	1.5	1	0.75	0.5	0.25	0.13	Blk	Protein stock (mg/10 mL)	Protein stock ID

Samples:

mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results
0.00000	25	0.0000				m, b	
0.00000	25	0.0000				se_m, se_b	
0.00000	25	0.0000				r^2, se_y	
0.00000	25	0.0000				F, df	
0.00000	25	0.0000				ss_{reg}, ss_{resid}	
0.00000	25	0.0000					

Blank

r^2=
m=
b=

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

Assay Date	7/1/2005	Test Chemical ID	none	# Concentrations tested	0	Microsome type	placental	Microsome ID	In Vitro	Technician ID	JG	Replicate #	ptn only

Microsome Dilution Details		
Dilution A		mL microsome Stock used
		mL total volume
	#DIV/0!	dilution factor
Dilution B		mL microsome Dilution A used
		mL total volume
	#DIV/0!	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	#DIV/0!	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	
Protein Concentration (dilution added to assay, mg/mL):	#DIV/0!

Test Chemical Concentrations	
Level	Final Concentration (M)
1	
2	
3	
4	
5	
6	
7	
8	

Assay Date	7/1/2005	Test Chemical ID	none	# Concentrations tested	0	Microsome type	placental	Microsome ID	In Vitro	Technician ID	JG	Replicate #	ptn only

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 (initial)	% 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 (nmol estrogen formed/mg protein/min
Full activity control	1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
Background control	1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
Positive control	1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
Negative Control	1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
none	1-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	1-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	1-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	2-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	3-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	4-3	2		1						# /ALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	5-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	5-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	5-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	6-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	6-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	6-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	7-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	7-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	7-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	8-1	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	8-2	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													
	8-3	2		1						#VALUE!		#VALUE!	#VALUE!		#DIV/0!		#VALUE!
				2													

Assay Date	7/1/2005	Test Chemical ID	none	# Concentrations tested	Microsome 0 type	placental	Microsome ID	In Vitro	Technician ID	JG	Replicate #	ptn only

Control Type	Portion	Average	SD
Full activity	Beginning	#VALUE!	#VALUE!
Full activity	End	#VALUE!	#VALUE!
Full activity	Overall	#VALUE!	#VALUE!
Background	Beginning	#VALUE!	#VALUE!
Background	End	#VALUE!	#VALUE!
Background	Overall	#VALUE!	#VALUE!
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					

	Percent of control values			
	Log[test	Replicate		
Level	substance]	1	2	3
1				
2				
3				
4				
5				
6				
7				
8				

Appendix G

Statistical Analysis Report (BioSTAT Consultants, Inc.)

VALIDATION OF THE PLACENTAL AROMATASE ASSAY USING PREPARED MICROSOMES (WA 4-16, TASK 6)

EPA Contract No.: 68-W-01-023

WIL-431008

Statistical Analysis Summary

For protein determinations there were four independent replicate determinations with each microsome source, using the same assay method. The average protein concentration for the Battelle microsomes was compared with the average protein concentration for the In Vitro microsomes by a two-sample t-test. The degrees of freedom were calculated by Satterthwaite's method for separate variances.

	Source		
Statistic	Battelle	In Vitro	Difference (Batelle-In Vitro)
N	4	4	
Mean	22.0943	8.0603	14.0340
SD	3.0190	0.7475	2.1992
T-Test			0.0018

The aromatase activity values (nmol/mg ptn/min) were analyzed for comparisons of aromatase activity in the control values between the controls based on Battelle microsomes and those based on In Vitro microsomes. For each type of control there are two replicates with four repetitions per replicate based on Battelle microsomes and two replicates with four repetitions per replicate based on In Vitro microsomes. Average activity of Battelle and In Vitro microsomes were compared by a two factor analysis of variance (ANOVA) with a fixed term for microsome source and a random term for replicate within microsome source.

		Source	
Activity	Statistic	Battelle	In Vitro
Background	LSM	-0.00000	-0.00000
	LSM s.e.	0.00002	0.00002
	95% CI - lower	-0.00007	-0.00007
	95% CI - upper	0.00007	0.00007
	Source = 1.0000		
	Replicate(Source) = .		
Full Enzyme	LSM	0.07082	0.04435
	LSM s.e.	0.00278	0.00278
	95% CI - lower	0.05885	0.03239
	95% CI - upper	0.08278	0.05632
	Source = 0.0214		
	Replicate(Source) = 0.4570		
NEGATIVE	LSM	0.06737	0.04398
	LSM s.e.	0.00458	0.00458
	95% CI - lower	0.04765	0.02427
	95% CI - upper	0.08709	0.06370
	Source = 0.0690		
	Replicate(Source) = 0.1614		
POSITIVE	LSM	0.03094	0.02082
	LSM s.e.	0.00216	0.00216
	95% CI - lower	0.02164	0.01151
	95% CI - upper	0.04025	0.03012
	Source = 0.0803		
	Replicate(Source) = 0.1601		

APPENDIX E

INTERLABORATORY STATISTICAL ANALYSIS REPORT

DRAFT REPORT

PREPARATION AND CHARACTERIZATION OF HUMAN PLACENTAL MICROSOMES

INTERLABORATORY STATISTICAL ANALYSIS

EPA CONTRACT NUMBER 68-W-01-023
WORK ASSIGNMENT 4-16, TASK 6

February 24, 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
Interlaboratory Statistical Analysis

EPA CONTRACT NUMBER 68-W-01-023
WORK ASSIGNMENT 4-16, TASK 6

Paul I. Feder, Author	Date
Zhenxu J. Ma, Reviewer	Date

Quality Assurance Statement

Study Number: G608316

This study was inspected by the Quality Assurance Unit and reports were submitted to the work Assignment Leader and Management as follows:

Phase Inspected	Inspection Date	Date Reported to Work Assignment Leader/Management
Interlaboratory statistical analysis report	2/23/2006	2/23/2006

Quality Assurance Unit	Date
Terri L Pollock	2-28-06
Terri L Pollock	

Summary and Conclusions

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.

Introduction and Background

In Task 6 of the Placental Aromatase Validation Study, Battelle and In Vitro Technologies each prepared microsomes. They each carried out two independent replicates of a positive control inhibition study with 4-OH ASDN using their own microsomes. There was no inter-laboratory comparison of results for the inhibition study.

Battelle supplied microsomes to In Vitro Technologies, RTI, and WIL Laboratories and In Vitro Technologies supplied microsomes to Battelle, RTI, and WIL Laboratories. Each laboratory determined aromatase activity and protein concentrations of each microsomal preparation and compared the aromatase activity and the protein concentrations between the two microsome sources by analysis of variance and two sample t-tests.

This report discusses the methods and results of the interlaboratory statistical analyses performed on the aromatase activity and protein concentration data obtained by each of the four laboratories to compare the microsomes prepared by Battelle with those prepared by In Vitro Technologies. Intra-laboratory statistical analyses were carried out at each laboratory based on each laboratory's test data, according to a statistical analysis plan prepared by the Data Coordinating Center. The inter-laboratory statistical analysis discussed in this report combines summary values developed in the intra-laboratory

analyses and assesses the relationships among them, the extent of inter-laboratory variation, and overall consensus estimates. This report discusses the methods used and the results obtained from combining the intra-laboratory statistical analysis results.

The inter-laboratory analysis compares the differences in responses between the microsomes prepared at Battelle and In Vitro Technologies (Battelle minus In Vitro) as reported in the intra-laboratory analyses, across the four laboratories. The responses included in the inter-laboratory analysis are: aromatase activity (nmol/mg protein/min) of the full enzyme activity, background activity, negative control, and positive control responses averaged across replicates and the average protein concentration (mg/mL).

Test Organization

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 1 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).

The intra-laboratory analysis summary information upon which the inter-laboratory analysis is based, is displayed in Appendix A, Tables A-1, A-2.

Table 1. Number of Replicates at Each Test Laboratory for Each Response Type

		Test Laboratory			
	Microsome Source	**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

Objectives

The objectives of the inter-laboratory statistical analysis are 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 Analysis Methods

Statistical analyses were carried out for each of the five endpoints displayed in Table 1: 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 intra-laboratory analyses. The analysis of variance resulted in a weighted average across all the laboratories and its

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

associated standard error as well as an estimate of the laboratory-to-laboratory component of variation. The weights included in the weighted averages incorporated 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)[3].

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 1.

---

[3] 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.

In several places entries in the tables in the inter-laboratory analysis report tables may differ from corresponding entries in the intra-laboratory 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 intra-laboratory analyses and the inter-laboratory analysis in rounding in intermediate calculations.

Statistical Analysis Results

Aromatase Activity

Table 2 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 1 to 4. Each figure includes reference lines corresponding to the overall average.

Table 3 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 4 displays the within laboratory standard errors for each laboratory for the background activity, full enzyme activity, negative, and positive controls. Table 4 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 2 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 Technologies 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 3 shows among laboratory CVs (excluding background activity controls) in the range from 32.5% to 46.2%, depending on control type. Table 4 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 (excluding background activity controls) is between 106.6% and 125.2%.

Protein Concentration

Tables 5 to 7 display the same summary information as Tables 2-4, for protein concentration.

Table 5 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 6 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 3. Table 7 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 2. 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 3. 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 4. 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 5. 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]) 6.00[3] df (10.1510, 19.2464)[4]	14.0340(1.55508) 3.37 df (9.3763, 18.6917)	11.3420(0.72900) 6.39 df (9.5840, 13.1000)	16.7300(2.53540) 3.14 df (8.8574, 24.6026)	13.5370[5](1.18610[6]) 4.22[7] df (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 6. 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 7. 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.

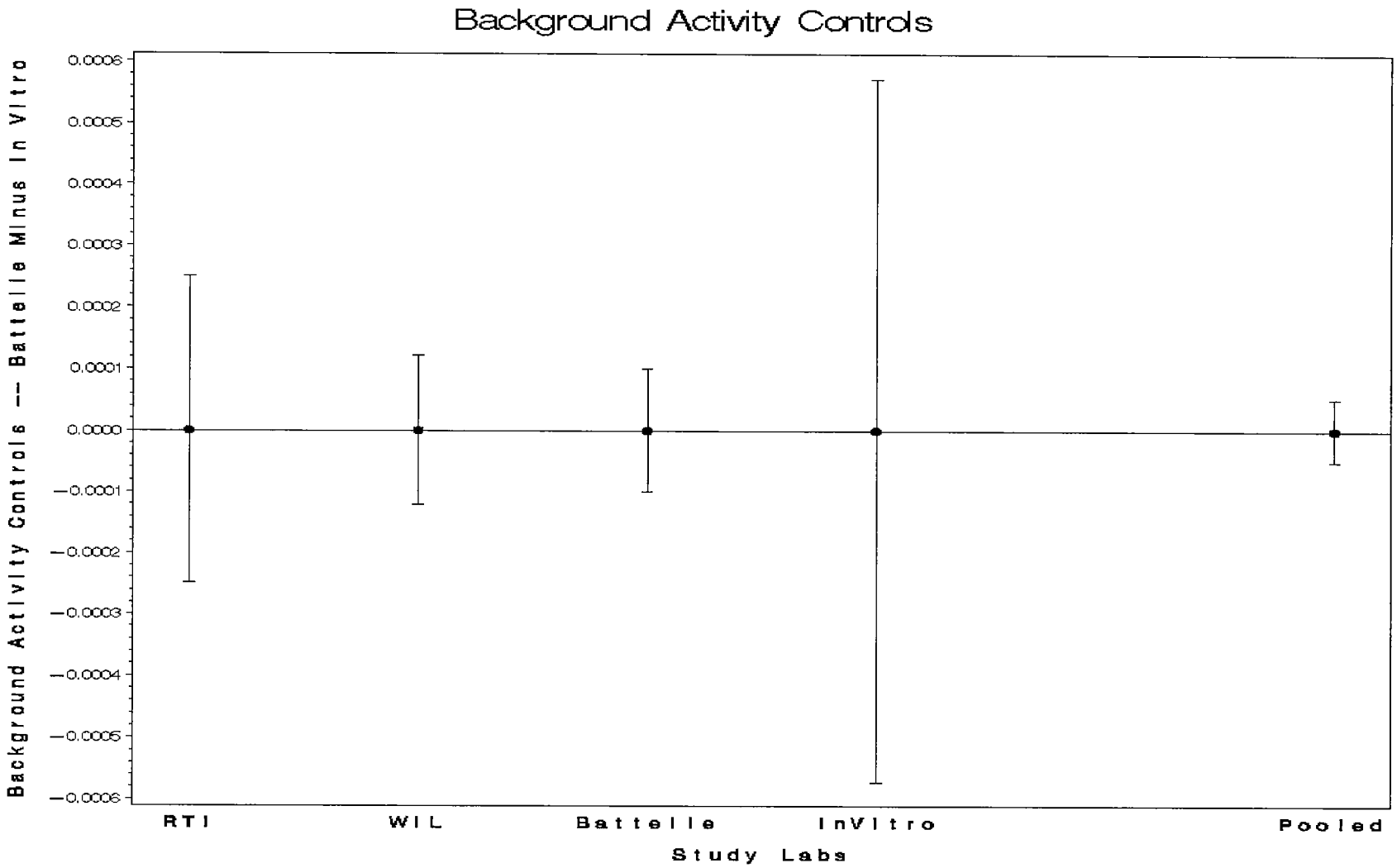

Figure 1. **Background Activity Controls (nmol/mg protein/min). Parameter Estimates and Their Associated 95% Confidence Intervals for Microsome Source Difference (Battelle Minus In Vitro) in the Placental Aromatase Assay. By Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to theAverage Across Laboratories.**

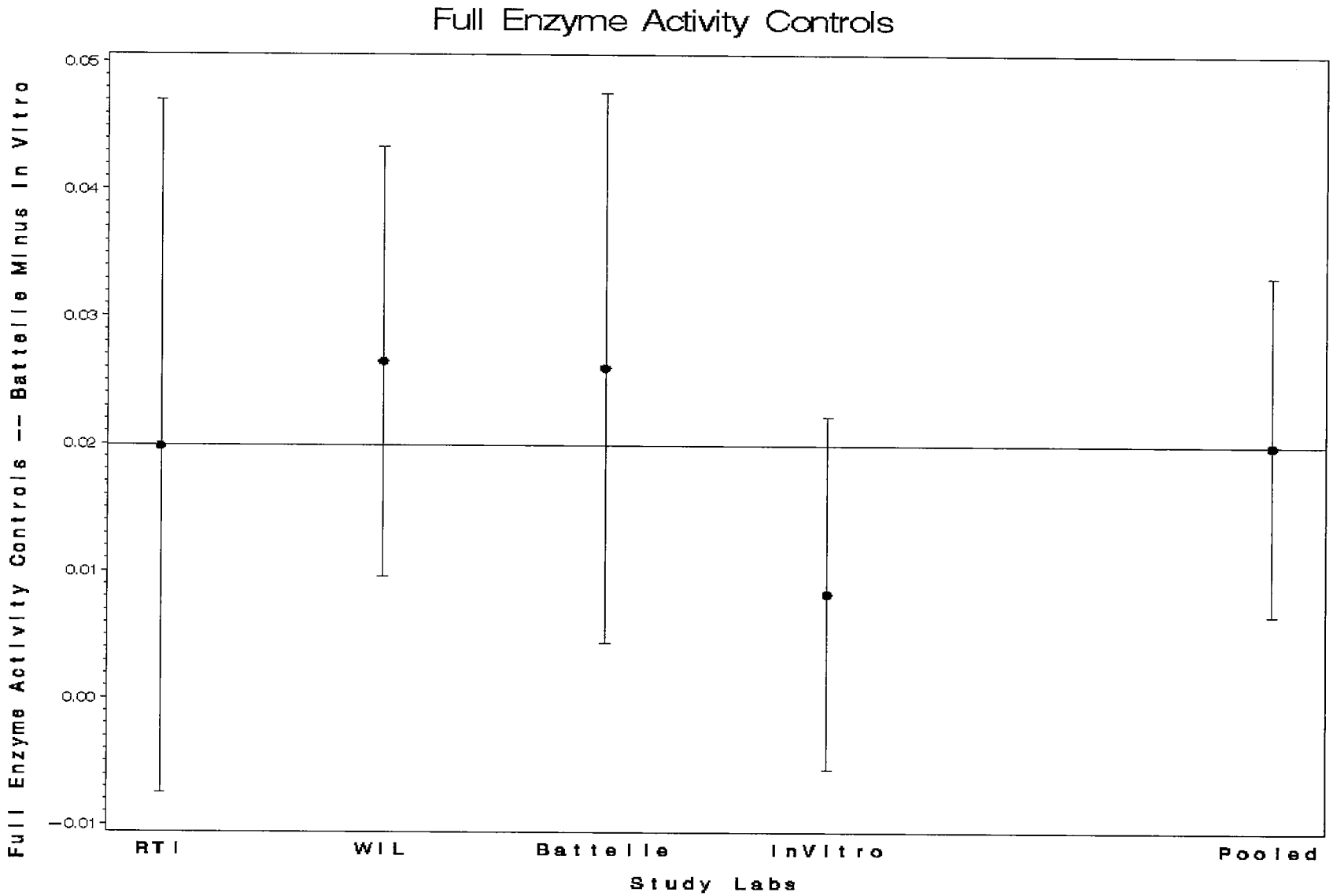

Figure 2. **Full Enzyme Activity Controls (nmol/mg protein/min). Parameter Estimates and Their Associated 95% Confidence Intervals for Microsome Source Difference (Battelle Minus In Vitro) in the Placental Aromatase Assay. By Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds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

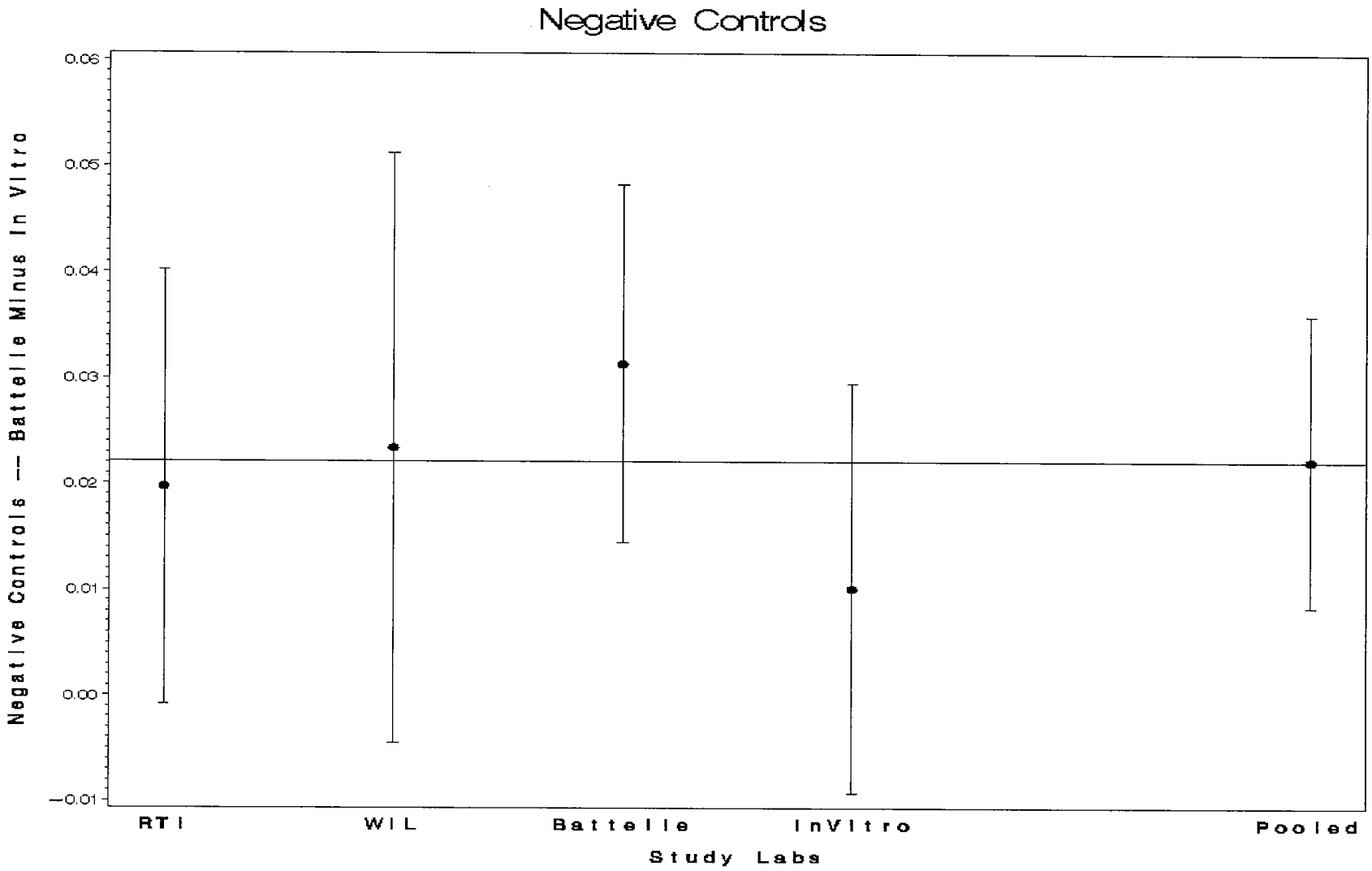

Figure 3. **Negative Controls (nmol/mg protein/min). Parameter Estimates and Their Associated 95% Confidence Intervals for Microsome Source Difference (Battelle Minus In Vitro) in the Placental Aromatase Assay. By Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average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

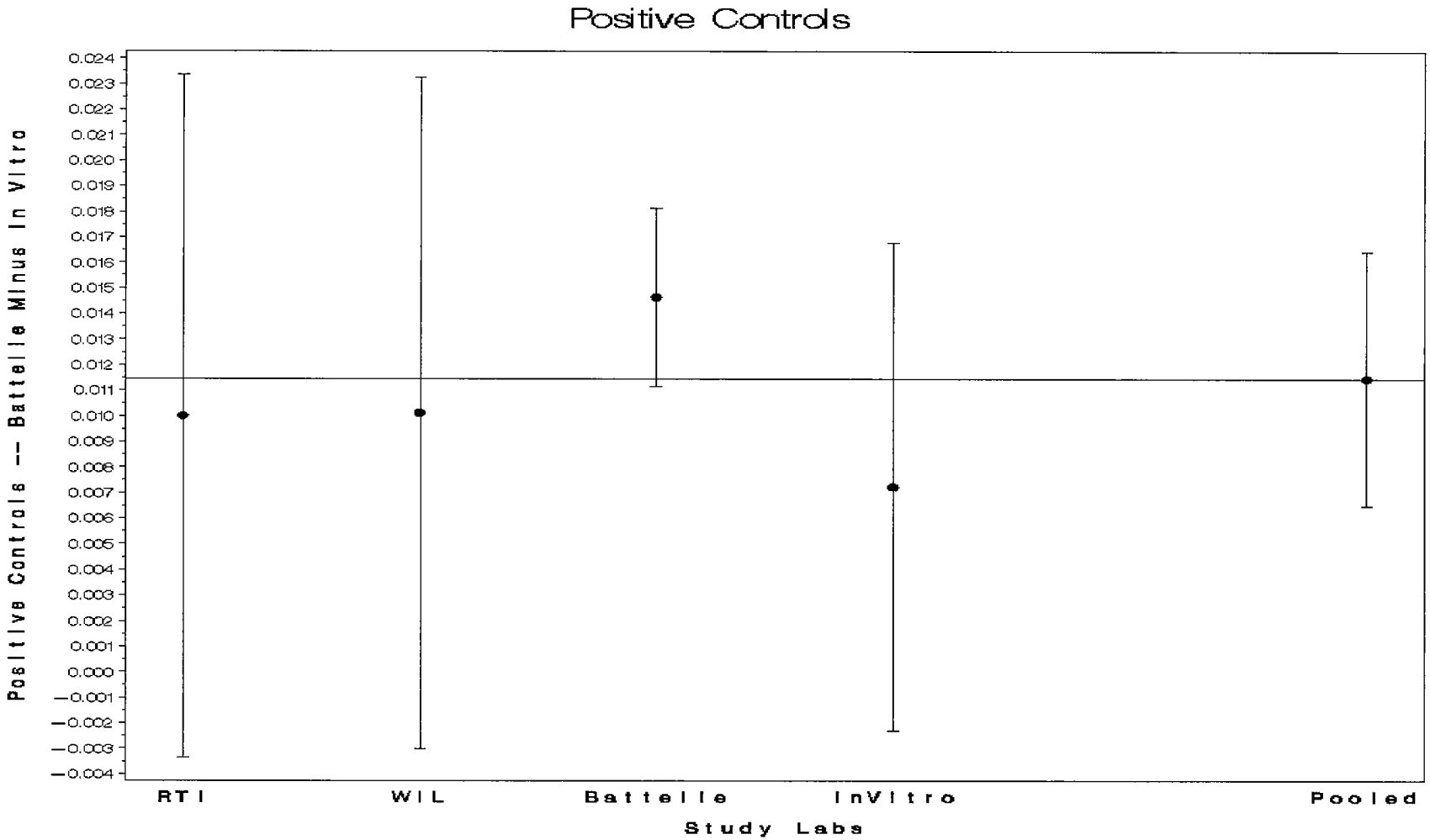

Figure 4. Positive Controls (nmol/mg protein/min). Parameter Estimates and Their Associated 95% Confidence Intervals for Microsome Source Difference (Battelle Minus In Vitro) in the Placental Aromatase Assay. By Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average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

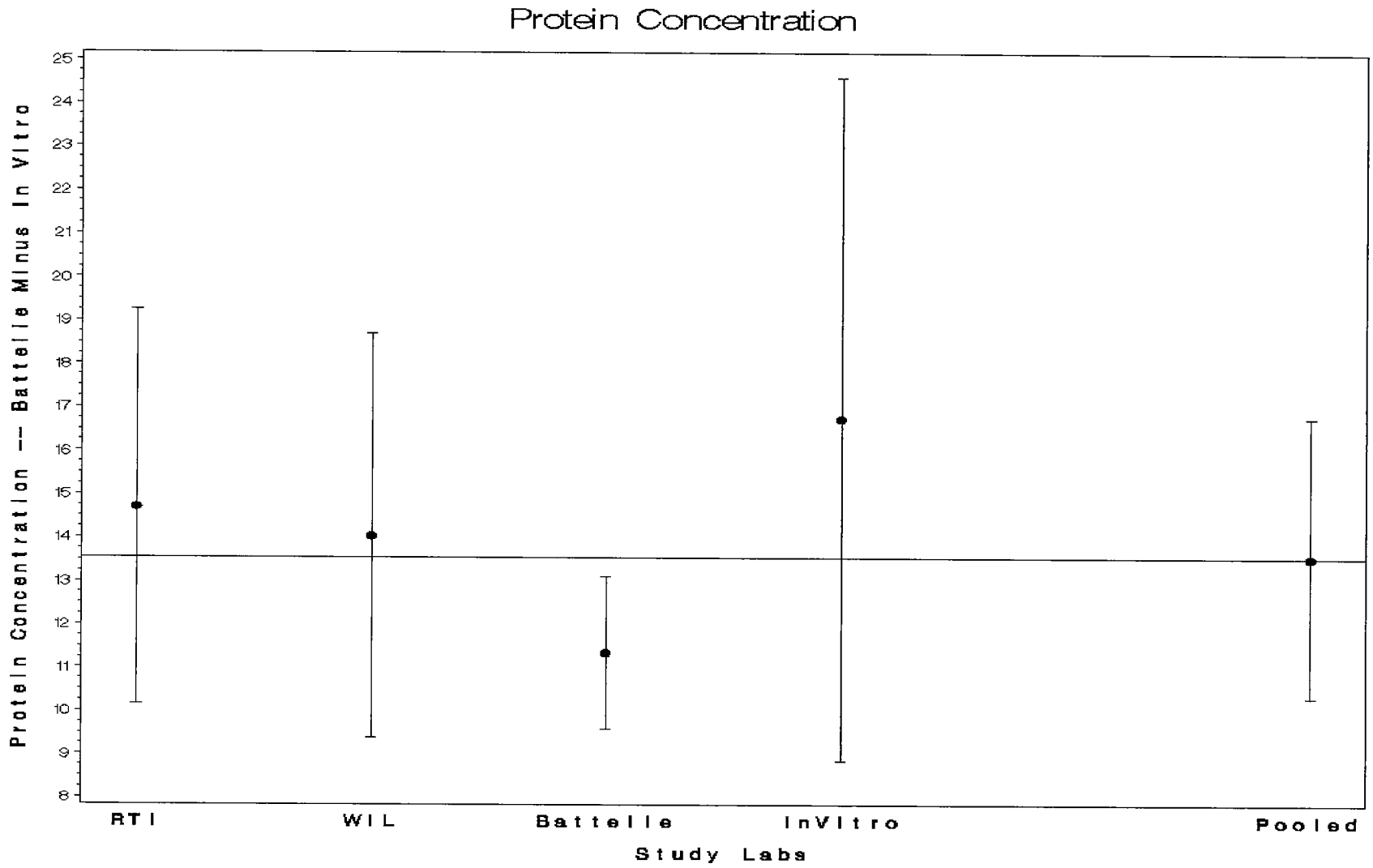

Figure 5. **Protein Concentration (mg/mL). Parameter Estimates and Their Associated 95% Confidence Intervals for Microsome Source Difference (Battelle Minus In Vitro) in the Placental Aromatase Assay. By Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average Across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

Appendix A

Table A-1. Aromatase Activity Control Data (nmol/mg protein/min). By Test Laboratory, Control Type, and Microsome Source.

Test_Lab	Ctl_Type	Source	LSMean	StdErr	df
Battelle	Full_Act	BAT	0.05355	.004489	4
	Full_Act	IVT	0.02759	.006348	4
	Full_Act	DIFF	0.02596	.007775	4
	Bkgd_Act	BAT	-0.00000	.000021	4
	Bkgd_Act	IVT	0.00000	.000029	4
	Bkgd_Act	DIFF	-0.00000	.000036	4
	Pos	BAT	0.02992	.000572	2
	Pos	IVT	0.01528	.000572	2
	Pos	DIFF	0.01464	.000810	2
	Neg	BAT	0.06008	.002785	2
	Neg	IVT	0.02872	.002785	2
	Neg	DIFF	0.03135	.003939	2
InVitro	Full_Act	BAT	0.04642	.004084	4
	Full_Act	IVT	0.03819	.002887	4
	Full_Act	DIFF	0.00823	.005001	4
	Bkgd_Act	BAT	-0.00000	.000168	4
	Bkgd_Act	IVT	-0.00000	.000119	4
	Bkgd_Act	DIFF	0.00000	.000206	4
	Pos	BAT	0.02594	.002809	4
	Pos	IVT	0.01871	.001986	4
	Pos	DIFF	0.00723	.003440	4
	Neg	BAT	0.04956	.005710	4
	Neg	IVT	0.03943	.004038	4
	Neg	DIFF	0.01012	.006994	4
RTI	Full_Act	BAT	0.05626	.004482	2
	Full_Act	IVT	0.03649	.004482	2
	Full_Act	DIFF	.	.	.
	Bkgd_Act	BAT	-0.00000	.000041	2
	Bkgd_Act	IVT	-0.00000	.000041	2
	Bkgd_Act	DIFF	.	.	.
	Pos	BAT	0.02675	.002195	2
	Pos	IVT	0.01674	.002195	2
	Pos	DIFF	.	.	.
	Neg	BAT	0.05498	.003375	2
	Neg	IVT	0.03529	.003375	2
	Neg	DIFF	.	.	.
WIL	Full_Act	BAT	0.07082	.002780	2
	Full_Act	IVT	0.04435	.002780	2
	Full_Act	DIFF	.	.	.
	Bkgd_Act	BAT	0.00000	.000020	2

Table A-1 (Continued)

Test_Lab	Ctl_Type	Source	LSMean	StdErr	df
WIL	Bkgd_Act	IVT	0.00000	.000020	2
	Bkgd_Act	DIFF	.	.	.
	Pos	BAT	0.03094	.002160	2
	Pos	IVT	0.02082	.002160	2
	Pos	DIFF	.	.	.
	Neg	BAT	0.06737	.004580	2
	Neg	IVT	0.04398	.004580	2
	Neg	DIFF	.	.	

Table A-2. Protein Concentration Data (mg/ml). By Test Laboratory and Microsome Source.

Test_Lab	Source	LSMean	N	StdDev	StdErr	df	Typ
Battelle	BAT	19.9110	6	1.6640	.	5	Sepr
	IVT	8.5690	4	0.5290	.	3	Sepr
InVitro	BAT	24.5450	4	5.0140	.	3	Sepr
	IVT	7.8150	6	0.9270	.	5	Sepr
RTI	BAT	24.0557	4	.	1.3142	6	Comm
	IVT	9.3570	4	.	1.3142	6	Comm
WIL	BAT	22.0943	4	3.0190	.	3	Sepr
	IVT	8.0603	4	0.7475	.	3	Sepr