DRAFT TASK REPORT

VALIDATION OF THE PLACENTAL AROMATASE ASSAY: POSITIVE CONTROL STUDY (WA 4-16, TASK 4)

WA 4-16 Task 4: Conduct Positive Control Studies in the Participating Laboratories

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

WIL-431006

Sponsor:

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Title:	VALIDATION OF THE PLACENTAL AROMATASE ASSAY: POSITIVE CONTROL STUDY (WA 4-16, TASK 4) Task 4: Conduct Positive Control Studies in the Participating Laboratories
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Quality Assurance Unit Statement

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 as approved by the Sponsor, with the following exceptions.

Intra-laboratory 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 H. 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(s) of Inspection	Phase Inspected	Date(s) Findings reported to Study Director	Date(s) Findings reported to management	Auditor
11/29/04	Protocol review	11/29/04	12/20/04	L. Goodrich
12/13/04	Aromatase Assay Sample Preparation	12/13/04	1/27/05	A. Deppe, J. House
12/13/04	Micosomal Praparation for Aromatase Assay	12/13/04	1/27/05	A. Deppe, J. House
12/13/04	Test Article Dilution for Aromatase Assay	12/13/04	1/27/05	A. Deppe, J. House
12/29/04	Study Records (A-1)	1/3/05	2/16/05	L. Goodrich
12/29/04	Study Records (A-2)	1/3/05	2/16/05	L. Goodrich
12/29/04	Study Records (B-1)	1/3/05	2/16/05	L. Goodrich
1/3/05	Aromatase Assay Data Spreadsheets	1/3/05	2/16/05	L. Goodrich
1/17/05	Protocol Amendment I Review	1/17/05	2/16/05	P. Brant
2/22/05	Draft Report (BioSTAT Appendix)	2/22/05	3/25/05	L. Goodrich
2/18,22,23/05	Draft Report (without BioSTAT Appendix)	2/23/05	3/25/05	L. Goodrich

Approval

This study was inspected according to the criteria described above.

Report Audited By:

Lori J. Goodrich Date
Group Supervisor, Quality Assurance

Report Released By:

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

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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 aromatase assay is conducted over a range of concentrations such that a dose response curve can be developed and an IC_{50} calculated to determine the concentration of test chemical required to inhibit aromatase activity by 50%. The general purpose of this assay is to screen potential endocrine disruptors for aromatase inhibition. 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 the known aromatase inhibitor 4-hydroxyandrostenedione (4-OH ASDN) as a positive control (WA 4-16, Task 4).

The results of this positive control study using centrally-prepared placental microsomes and the known aromatase inhibitor 4-OH ASDN were as expected, based on the prevalidation work (WA 2-24 and 4-10) and the training data (WA 4-16, Task 3). Generally, 4-OH ASDN at concentrations ranging from $1x10^{-9}$ M to $1x10^{-6}$ M resulted in a sigmoidal dose response curve ranging from no inhibition (100 percent of control) to almost full inhibition (4.81 percent of control), respectively. The overall IC_{50} for 4-OH ASDN in this study was 47.2 nM, comparable to the Environmental Protection Agency (EPA)-established value of 42 nM in human placental microsomes. 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.

The responsiveness of the placental microsome aromatase assay using the known aromatase inhibitor 4-OH ASDN as a positive control was successfully validated in this laboratory. Additional testing of this placental microsome assay using four potential aromatase inhibitors will be evaluated under WA 4-16, Task 5, WIL-431007 (Thomas-Wohlever, Draft).

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 EPA is implementing an Endocrine Disruptor Screening Program. 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 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 (Brueggemeier and Sloan, Draft).

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 found in breast tissue, and the importance of intratumoral aromatase and local estrogen production is being investigated. 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 1970s and have expanded greatly in the past three decades.

An *in vitro* aromatase assay could easily be used 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 pharmaceutical 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 used 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 an *in vitro* 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 was to use the now optimized assay to

obtain intra- and inter-laboratory assay variability estimates to complete the validation of the human placental microsome aromatase assay.

2.2 Task Description and Objectives

WIL Research Laboratories, LLC was selected as one of the participating laboratories in the inter-laboratory validation of the human placental aromatase assay as part of the Endocrine Disruptor Screening Program. The objective of this task in the validation process overseen by the EPA was to demonstrate the responsiveness of the placental microsome aromatase assay at WIL Research Laboratories, LLC using the known aromatase inhibitor 4-OH ASDN as a positive control.

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-Aldrich (St. Louis, MO) by the Sponsor's Chemical Repository and was then distributed to the participating laboratories. The reported purity was 100%. The radiolabeled androstenedione ([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 also assessed by high performance liquid chromatography by the lead laboratory (RTI International).

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 tube contained 0.1 µCi. This was accomplished by preparing a 100-fold dilution of the radiolabeled stock in buffer. In addition, a 1 mg/mL stock 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 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 is appended to this document (Appendix E).

When the test chemical and assay reagents 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. All documents accompanying the shipment were filed with the log-in sheet.

Test Chemical Information									
Chemical name	Chemical code	Mfr. Purity	CAS No.	Molecular formula	Molecular weight (g/mol)	Stock Solution ID	Target Stock Formulation Concentration (mg/mL)	Vehicle	Storage Conditions
4-Androsten-4-ol-3,17-dione	4-OH ASDN	*	566-48-3	$C_{19}H_{26}O_3$	302.4	MET-0252A	3.02	95% Ethanol	~5°C
* Test chemical characterization data in Appendix E									

The test chemical, 4-OH ASDN, was received as a 0.01 M stock solution in ethanol. This solution was used to create a 0.0001 M secondary stock solution by diluting 20 µL to 2 mL in ethanol. To prepare solutions of appropriate concentration, the dilution scheme outlined in the following table was followed. These dilutions were prepared fresh each day of the assay.

Test Chemical Dilutions					
	4-OH ASDN Stock solution		Diluent (µL Ethanol)	Solution Concentration (M)	Target Concentration in Assay (M)
	µL	M			
4-OH ASDN Concentration 1	1000	1×10^{-4}	0	1×10^{-4}	1×10^{-6}
4-OH ASDN Concentration 2	100	1×10^{-4}	900	1×10^{-5}	1×10^{-7}
4-OH ASDN Concentration 3	50	1×10^{-4}	950	5×10^{-6}	5×10^{-8}
4-OH ASDN Concentration 4	25	1×10^{-4}	975	2.5×10^{-6}	2.5×10^{-8}
4-OH ASDN Concentration 5	100	1×10^{-5}	900	1×10^{-6}	1×10^{-8}
4-OH ASDN Concentration 6	100	1×10^{-6}	900	1×10^{-7}	1×10^{-9}

3.3 Microsomes

Human placental microsomes were received as multiple frozen aliquots from RTI International. The sample ID number was 11343-7, and the microsomes were stored between -70°C to -80°C. Fresh aliquots of microsomes were used for each replicate. Microsomes were thawed rapidly in a water bath maintained at 37°C ± 1°C and rehomogenized using a chilled Potter Elvejhem homogenizer. The microsomes were kept on ice throughout the dilution process and until the pre-incubation phase. The reported protein concentration was 14 mg/mL. A 50-fold dilution was made to obtain a concentration of approximately 0.28 mg/mL. The actual protein concentration of this dilution was determined using the protein assay described in Section 3.5. Another 10-fold dilution was made to achieve the final working stock of microsomes.

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	4011-01
Test/control vehicle A - Ethanol, 95%	Sponsor	SW0045

3.4.1 NADPH

Nicotinamide adenine dinucleotide phosphate (NADPH, reduced form tetrasodium salt) is a required cofactor for aromatase activity. As such, it is included in excess in the aromatase assay. To prepare a 5 mg/mL stock solution, 0.025 g NADPH was weighed, transferred to a 5-mL volumetric flask and diluted to volume with 0.1 M phosphate buffer (see Section 3.4.2). Adding 100 µL of this 5 mg/mL NADPH solution to each reaction tube (2 mL total assay volume) resulted in a final assay concentration of 0.3 mM. NADPH was prepared fresh every assay day and was stored on ice until added 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 0.1 M phosphate buffer, pH 7.4. The assay buffer was stored refrigerated for up to one month.

3.5 Protein Determination

The protein concentration of the microsome preparation was determined for each replicate of the aromatase assay on the day of the assay. A six-point standard curve was prepared, ranging from approximately 0.12 to 1.4 mg protein/mL. The protein standards were made from bovine serum albumin (BSA). The protein concentration was determined using a DC Protein Assay kit purchased from Bio-Rad (Hercules, CA). To a 25-µL aliquot of unknown or standard, 125 µL of Bio-Rad DC Protein Kit Reagent A was added and mixed. Next, 1 mL of Bio-Rad 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 color developing reaction was stable for about 1 hour. Each sample (unknown and standards) was transferred to appropriate cuvettes and the absorbance at a wavelength of 750 nm was measured using a spectrophotometer. The protein concentration of the microsomal sample was determined from the absorbance value using linear regression to the absorbance of the protein standards.

3.6 Cytochrome P450 Aromatase (CYP19) Activity

The Cytochrome P450 Aromatase (CYP19) Activity procedure is summarized in Appendix B. Aromatase activity was determined via an *in vitro* screening assay using human placental microsomes supplied by RTI International. The assays were performed in 13x100 mm glass test tubes. Each test tube was uniquely labeled with the replicate and repetition, and the group information summarized in the following table as necessary to differentiate the tubes. In addition to tubes containing test chemical, full enzyme activity controls (tubes including vehicle but no test chemical) and background activity controls (tubes containing no NADPH) were used to determine 100% and 0% activity, respectively.

Assay Design-Single Replicate			
Sample Type/Group	Repetitions (test tubes)	Description	Test Chemical Concentration (M final)
Full Enzyme Activity Control - Beginning	2	Complete assay[a] with test chemical vehicle control	N/A
Background Activity Control - Beginning	2	Complete assay with test chemical vehicle control omitting NADPH	N/A
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}
Full Enzyme Activity Control - End	2	Complete assay with test chemical vehicle control	N/A
Background Activity Control - End	2	Complete assay with test chemical vehicle control omitting NADPH	N/A

a = The Complete assay contains buffer, propylene glycol, microsomal protein, [^{3}H]ASDN+ASDN 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 to make 1 mL total volume. Microsomes were diluted to the appropriate concentration as detailed in Section 3.3 (Microsomes) and used within 2 hours of thawing (see following table).

Microsome Thaw Time				
Replicate	Removed From Freezer	Start Time Of 5 Minutes Incubation Prior To Assay Start	Minutes Elapsed Between Removal From Freezer To Start Of 5 Minute Incubation	Time Last Assay Tube Quenched
1	10:21 AM	11:06 AM	45	11:26 AM
2	10:55 AM	11:41 AM	46	12:00 PM
3	11:40 AM	12:16 PM	36	12:43 PM

The reaction mixture and the microsomes were incubated independently at 37°C ± 1°C for 5 minutes. The reactions were initiated by the addition of diluted microsomes (1 mL) to each test tube. Each assay was incubated at 37°C ± 1°C for 15 minutes. At the conclusion of the reaction time, each reaction was quenched by the addition of 2 mL methylene chloride. The tubes were mixed by vortex action for approximately 5 seconds and placed on ice until all tubes were quenched. The tubes were then mixed by vortex action an additional 20 to 25 seconds to extract unreacted ASDN. The tubes were centrifuged for 10 minutes at approximately 162 x *g* to facilitate separation of the organic and aqueous layers. The methylene chloride layers were removed and discarded; the aqueous layers were extracted two more times, each time with 2 mL of methylene chloride. The aqueous layers were transferred to vials and duplicate aliquots (0.5 mL) were transferred to 20-mL liquid scintillation counting vials. Liquid scintillation cocktail (Ultima Gold, Packard, approximately 10 mL) was added to each counting vial and the vials shaken to mix.

Analysis of the samples was performed using a liquid scintillation counter (LSC). Radiolabel found in the aqueous fractions represents ^3H_2O formed from the hydrolysis of [^{3}H]ASDN. One H_2O molecule is released per molecule of ASDN converted to estrogen in a

stereospecific reaction. Thus, the amount of estrogen product formed can be determined by dividing the total amount of ^3H_2O formed by the specific activity of the [^{3}H]ASDN substrate (expressed in DPM/nmol). Results are presented as the activity (velocity) of the enzyme reaction. The activity of the enzyme reaction was expressed in nmol (mg protein)$^{-1}$min^{-1} and described in Section 3.7.

Each assay replicate was performed on the day shown in the following table. The same technician performed each replicate. Two unsuccessful replicates were completed on December 9 and 14, 2004; consequently, the results of those two replicates were not statistically analyzed and are not presented with the rest of the results. The results of the unsuccessful replicates were documented in the study records and are summarized in Appendix G. Additional replicates were authorized by the EPA and completed following identification and removal of the source of the problem.

Assay Dates by Technician		
Replicate	Date	Technician
1	12/13/04	JG
2	12/17/04	JG
3	12/27/04	JG

3.7 Data Analysis

Relevant data were entered into the latest version of the Microsoft® Excel spreadsheet Aromatase_Master_Version1.2.xls (where 1 and 2 denote version number designation) for calculation of aromatase activity and percent of control. Data recorded included assay date and replicate number, technician, chemical and log chemical concentration, total DPM-background DPM and percent activity. The spreadsheet calculated DPM/mL for each aliquot of extracted aqueous incubation mixture and average DPM/mL and total DPM for each aqueous portion after extraction. Total DPM present in the assay tube at initiation was calculated by multiplying the volume of substrate solution added to the incubation by the substrate solution radiochemical content (DPM/mL). Background DPM was calculated as the average DPM present in the aqueous portion for the background tubes, and was subtracted from the total DPM for all samples to provide the 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)$^{-1}$min^{-1} and was calculated by dividing the amount of estrogen product formed (nmol) by the milligrams of microsomal protein used and 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. Because background DPM were subtracted from all samples and controls, total average background DPM equaled 0 for each replicate. Thus, the average percent activity across the four background activity repeat tubes must necessarily equal 0 within each replicate. The average percent activity across the four full enzyme activity repeat tubes must necessarily equal 100 within each replicate. Although percent of control values ideally vary between 0% near high inhibitor concentrations and 100% near low inhibitor concentrations, individual experimental percent of control activity values will sometimes extend below 0% or above 100%. For curve fitting, observed individual percent activity values above 100% were set to 99.5%, and values below 0% were set to 0.5%

Percent of control activity data was exported to Prism v. 4.02 (GraphPad, San Diego, CA) for curve fitting of the percent of control activity (y) versus log test chemical concentration (x) data using the following equation:

$$Y = 100/(1+10^{((LogIC50-X)*Hill\ Slope)})$$

The software incorporated a weighting factor for the percent of activity values of 1/Y. As shown, the curve fitting equation uses the fixed value of 100 as the numerator. An alternative is to use a four-parameter equation that estimates the top and bottom plateau from the percent of control activity values. Fixing the top and bottom boundary allowed for estimation of the IC_{50} value on inhibition curves that may not span the entire inhibition range from 100% to 0%. Each response curve was classified in the following manner: complete curves show inhibition from 0% to 100% for test chemical and incomplete curves show at least 50% inhibition but do not span the entire range and chemicals that result in less than 80% inhibition across the tested concentration range are considered not to be inhibitors. Concentration response fits were carried out for each replicate and the concentration ranges of 4-OH ASDN tested resulted in a complete inhibition curve. The resultant μ (log IC_{50}) and β (slope) summarized the extent of aromatase inhibition. The average and standard error for log IC_{50} and slope were analyzed using a one-way random effects analysis of variance model. For each replicate the estimated log IC_{50} (μ), the IC_{50}, β (slope) and the within replicate standard deviation of μ and β are displayed in a table (Section 4.6, IC_{50} and Slope Determination).

Slope (β) and log IC_{50} (μ) were also compared across replicates based on random effects analysis of variance, treating the replicates as random effects with mean β_{AVG} and μ_{AVG} using the following equation.

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

The average response curve across replicates was plotted along with the average data for each replicate. The associated 95% confidence interval from the analysis of variance within and across replicates is also displayed on separate plots for β and μ .

Microsoft® Excel was used to calculate mathematical averages, standard deviations and standard errors of the mean in order to assess the variation among repetitions (within a single replicate) and between replicates.

Within each replicate, quadruplicate repetitions were made of the background activity and the full enzyme activity control. Half the repetitions were performed at the beginning of the replicate assay and half at the end of the replicate assay. The controls at the beginning were equivalent to those at the end. The average of the four background activity samples 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 two beginning controls and the two end controls were plotted by replicate with the plotting symbol distinguishing between beginning and end, and with reference line 0% (background activity) or 100% (full enzyme activity control) respectively. These plots display the extent of consistency across replicates with respect to average value and variability and provide comparisons of beginning versus end of each replicate.

Two-way analysis of variance was performed separately for the full enzyme activity control tubes and the background activity tubes. The factors in the analysis of variance were replicate, portion (beginning or end) and replicate by portion interaction. The error corresponds to repetition within replicate and portion. The response was percent of control aromatase activity. Because the daily replicates are controlled, the portion main effect and portion by replicate interaction are non-significant. Note that the replicate effects are necessarily zero because of the constrained totals within each replicate. For the purposes of evaluation, replicate was treated as a fixed effect. The portion by replicate interaction was not significant. The portion effect within each replicate and the portion effect averaged across replicates, and associated 95% confidence intervals, are also presented graphically.

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

The radiochemical purity (see Appendix D - Radiochemical Purity Report) of the [^{3}H]ASDN was determined by RTI International to be 97%. The final concentration of the substrate (ASDN + ^{3}H-ASDN) used in replicates 1, 2 and 3 was 0.588, 0.579 and 0.590 µg ASDN/g of solution, respectively. The specific activity of the substrate for each replicate is shown in the following table. The concentration and specific activity of the substrate was used to calculate the aromatase activity in the assay.

Substrate Results				
Radiochemical Code	Radiochemical Identification	Radiochemical Stock Concentration (µCi/g)	Assay Substrate (ASDN + 3H-ASDN) Final Concentration (µg/g)	Substrate Solution Specific Activity (DPM/nmol)
[^{3}H]-ASDN	MET-0251A	0.745	0.588	805162
[^{3}H]-ASDN	MET-0251A	0.755	0.579	829299
[^{3}H]-ASDN	MET-0251A	0.793	0.589	855106

4.2 Stock Formulation Analysis (Test Chemical)

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 provided to the laboratories for this study was within the acceptance criteria for both average concentration and percent relative standard deviation between analyses. In addition, the formulation was found to be stable for 173 days.

Test Chemical Stock Solution Results			
Test Chemical Code	Test Chemical ID	Stock Solution Concentration	Stock Solution Expiration Date
4-OH ASDN	MET-0252A	3.02 mg/mL	5/24/05

4.3 Protein Analysis (Microsomes)

The protein concentration of each microsomal preparation was determined on the day that the microsomes were used in the assay. The concentrations of microsomes used were 12.1, 12.3 and 15.7 mg/mL for replicates 1, 2 and 3, respectively.

Protein Analysis Results		
Replicate	Assay Date	Protein stock concentration (mg/mL, measured)
1	12/13/04	12.1
2	12/17/04	12.3
3	12/27/04	15.7

4.4 Control Aromatase Activity

Full Enzyme Activity Controls were conducted in duplicate at the beginning and end of each assay. The following table presents the mean beginning and end activities within and among the replicates. Figure 1 (Summary of Full Enzyme Activity Results) presents the individual full enzyme aromatase activity values and the percent of control values for each replicate and a graphical representation of the data. For Replicate 1, 2 and 3, the mean percent of control (beginning/end) was (92.7, 107.3), (99.3, 100.7) and (104.9, 95.1), respectively, as shown in Figure 1 indicating that the conditions were constant throughout each replicate. The overall percent of control among the replicates for the beginning and end of the assay was 98.4 and 101.6, respectively.

Full Enzyme Activity Control Aromatase Activity										
Replicate	mean FULL[a] Beginning (nmol/mg protein/min)	mean FULL[a] End nmol/mg protein/min)	Within Replicate FULL				Overall FULL			
			Mean	SD	sem	%CV	Mean	SD	sem	%CV
1	0.074	0.086	0.080	0.012	0.0006	15	0.072	0.012	0.003	17
2	0.077	0.078	0.077	0.005	0.0002	6.1				
3	0.062	0.056	0.059	0.004	0.002	7.3				

[a] FULL=Full Enzyme Activity Control

Background Activity Controls were conducted in duplicate at the beginning and end of each assay. Figure 2 (Summary of Background Activity Results) presents the individual background aromatase activity values and the percent of control values for each replicate and a graphical representation of the data. The aromatase activity in these control samples was negligible, indicating that there was no background activity (caused by nonspecific turnover of reactant to product, or unintentional NADPH contamination) that might interfere with the interpretation of the results. There were negligible differences between the beginning and end background aromatase activity values per replicate, indicating that the conditions were constant throughout each replicate test (see Appendix F, Individual Replicate Spreadsheets).

4.5 Test Chemical Aromatase Activity

Increasing the concentration of the test chemical (4-OH ASDN) inhibited control aromatase activity in a dose responsive manner. Low intra-assay variability was characterized by a coefficient of variance of less than 5% across triplicate samples (tubes) at all concentrations and replicates.

Test Chemical Aromatase Activity-Percent of Control									
Test chemical	Replicate	Log [test chemical]	Percent of Control			Overall Percent of Control			
			Tube 1	Tube 2	Tube 3	Mean	sd	sem	%CV
4-OH ASDN	1	-6.00	4.74	4.91	5.15	4.93	0.21	0.12	4.18
4-OH ASDN	1	-7.00	31.69	32.33	33.05	32.36	0.68	0.39	2.10
4-OH ASDN	1	-7.30	50.46	51.33	47.32	49.70	2.11	1.22	4.25
4-OH ASDN	1	-7.60	68.28	69.89	68.92	69.03	0.81	0.47	1.18
4-OH ASDN	1	-8.00	88.27	89.68	86.83	88.26	1.42	0.82	1.61
4-OH ASDN	1	-9.00	102.96	105.89	105.95	104.93	1.71	0.99	1.63
4-OH ASDN	2	-6.00	4.73	4.49	4.67	4.63	0.13	0.07	2.76
4-OH ASDN	2	-7.00	31.94	30.86	30.41	31.07	0.79	0.45	2.53
4-OH ASDN	2	-7.30	47.39	46.80	46.82	47.01	0.33	0.19	0.71
4-OH ASDN	2	-7.60	66.43	66.22	66.70	66.45	0.24	0.14	0.36
4-OH ASDN	2	-8.00	88.16	87.59	86.34	87.36	0.93	0.54	1.07
4-OH ASDN	2	-9.00	102.42	102.36	101.53	102.10	0.50	0.29	0.49
4-OH ASDN	3	-6.00	4.78	5.05	4.73	4.86	0.17	0.10	3.54
4-OH ASDN	3	-7.00	31.61	32.00	31.93	31.84	0.21	0.12	0.66
4-OH ASDN	3	-7.30	43.41	46.03	47.72	45.72	2.18	1.26	4.76
4-OH ASDN	3	-7.60	62.98	59.33	62.85	61.72	2.07	1.20	3.36
4-OH ASDN	3	-8.00	83.62	81.21	77.70	80.84	2.98	1.72	3.68
4-OH ASDN	3	-9.00	94.30	93.99	93.99	94.10	0.18	0.10	0.19

In addition, the inter-assay coefficient of variation in percent of control across replicates was less than 6%. The highest concentration of 4-OH ASDN (1×10^{-6} M) inhibited aromatase activity to a mean of 4.81 overall percent of control. Decreasing concentrations of 4-OH ASDN resulted in decreased enzyme inhibition characterized by increased percent of control activity. This inhibition by 4-OH ASDN was a sigmoidal dose response (see Figure 3, Figure 4 and Figure 5). The lowest concentration of 4-OH ASDN (1×10^{-9} M) did not inhibit aromatase activity (100% of control; see Appendix F, Individual Replicate Spreadsheets).

Mean Test Chemical Aromatase Activity-Percent of Control								
Test Chemical	Log [test Chemical]	Mean Percent of Control			Overall Percent of Control			
		Replicate 1	Replicate 2	Replicate 3	Mean	SD	sem	%CV
4-OH ASDN	-6.00	4.93	4.63	4.86	4.81	0.16	0.09	3.27
4-OH ASDN	-7.00	32.36	31.07	31.84	31.76	0.65	0.37	2.04
4-OH ASDN	-7.30	49.70	47.01	45.72	47.48	2.03	1.17	4.28
4-OH ASDN	-7.60	69.03	66.45	61.72	65.73	3.71	2.14	5.64
4-OH ASDN	-8.00	88.26	87.36	80.84	85.49	4.05	2.34	4.74
4-OH ASDN	-9.00	104.93	102.10	94.10	100.38	5.62	3.24	5.60

4.6 IC_{50} and Slope Determination

Based on the curve-fit of the percent of control aromatase activity across six concentrations of 4-OH ASDN, the calculated IC_{50} values were 51.8, 47.5 and 42.7 nM for Replicates 1, 2 and 3, respectively (see Figure 6, Plot of Average 4-OH ASDN Inhibition Per Replicate). The overall curve-fit for the data from the three replicates resulted in an IC_{50} of 47.3 nM based on an arithmetic calculation, and using the statistical method described in Section 3.7., the overall IC_{50} based on μ_{AVG} and β_{AVG} was 47.2 nM (see Figure 7, Overall 4-OH ASDN Inhibition Response Curve). The Log IC_{50} (μ) and 95% confidence interval is plotted per replicate and across replicates in Figure 8. The inter-assay variability (%CV) of the arithmetic IC_{50} determination was less than 10% (9.63). The β (slope) and 95% confidence interval is plotted per replicate and across replicates in Figure 9. The inter-assay variability (%CV) of the slope determination was less than 7%.

IC_{50} and Slope Results								
Test chemical	Replicate	Log IC_{50} (μ) (se)	IC_{50} (nM)	Slope (β) (se)	Overall[a]			
					IC_{50} (nM)	SD	sem	%CV
4-OH ASDN	1	-7.29 (0.01)	51.8	-1.0478 (0.0242)	47.3	4.6	2.6	9.63
	2	-7.32 (0.01)	47.5	-1.0389 (0.0226)				
	3	-7.37 (0.01)	42.7	-0.9343 (0.0190)				
[a] Arithmetic calculations								

4.7 Statistical Analysis

The Full Enzyme Activity and Background Activity Control values among replicates were analyzed by two-way analysis of variance with the percent of control aromatase activity as the response variable. Replicate by portion (beginning and end) interactions were not significant. P-values for replicate, portion and replicate by portion are presented in Appendix H (Statistical Analysis Report). The mean percent of control aromatase activity values are presented graphically by portion (beginning and end) in Figures 1B and 2B. In addition, estimates for the LSMeans and 95% confidence intervals are presented for percent of control aromatase activity across replicates.

The Log IC_{50} (μ) and slope (β) were subjected to random effects analysis of variance to determine the within replicate standard error (see table in Section 4.6, IC_{50} and Slope Determination). Log IC_{50} and slope were also compared across replicates based on random effects analysis of variance using μ_{AVG} and β_{AVG}. The overall Log IC_{50} was -7.326 (95% confidence interval of -7.431 to -7.222) resulting in the final IC_{50} value of 47.2 nM. The overall slope was -1.007 (95% confidence interval of -1.164 to -0.850).

5.0 Discussion

The results of this positive control study using centrally-prepared placental microsomes and the known aromatase inhibitor 4-OH ASDN were as expected based on the prevalidation work (WA 2-24 and 4-10) and the training data (WA 4-16, Task 3). Generally, 4-OH ASDN at concentrations ranging from $1x10^{-9}$ M to $1x10^{-6}$ M resulted in a sigmoidal dose response curve starting at no inhibition (100% of control) to almost full inhibition (4.81% of control), respectively. Therefore, response curves from Replicates 1, 2 and 3 were considered complete curves. The overall IC_{50} in this study for 4-OH ASDN was 47.2 nM (arithmetic value), comparable to the EPA-established value of 42 nM in human placental microsomes. 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.

The inclusion of the Full Enzyme and Background Activity Controls did contribute to successful troubleshooting when the results of the assay were not as expected. There were two unsuccessful replicates where the Full Enzyme Activity Controls were much lower than expected, particularly in the end portion of the assay. This suggested that the microsomes were somehow compromised. Closer inspection of the method revealed that the two unsuccessful replicates used a repeat pipettor to dispense the diluted microsomes to initiate the assay incubation. The first successful replicate (conducted between the unsuccessful replicates) did not use a repeat pipettor. Subsequent EPA-authorized replicates (2 and 3) using a normal air-displacement pipet gave the expected results. The hypothesis is that the repeat pipettor compromised the microsomes, either through shear forces due to larger volume (several mL to fill the reservoir vs. 100 μL per individual assay) through the pipet tip or unpredictable settling of the microsome suspension between the beginning and end of the assay.

6.0 Conclusion

The responsiveness of the placental microsome aromatase assay using the known aromatase inhibitor 4-hydroxyandrostenedione as a positive control was successfully validated in this laboratory. Additional testing of this placental microsome assay using four potential aromatase inhibitors will be evaluated under WA 4-16, Task 5, WIL-431007 (Thomas-Wohlever, Draft).

7.0 References

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

Thomas-Wohlever, J.A. Validation of the Placental Aromatase Assay for Endocrine Disruptor Screening (WA 4-16, TASK 5) (Study No. WIL-431007). WIL Research Laboratories, LLC, Ashland, OH, **Draft.**

8.0 Key Personnel and Report Submission

Report Submitted By:

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Report Reviewed By:

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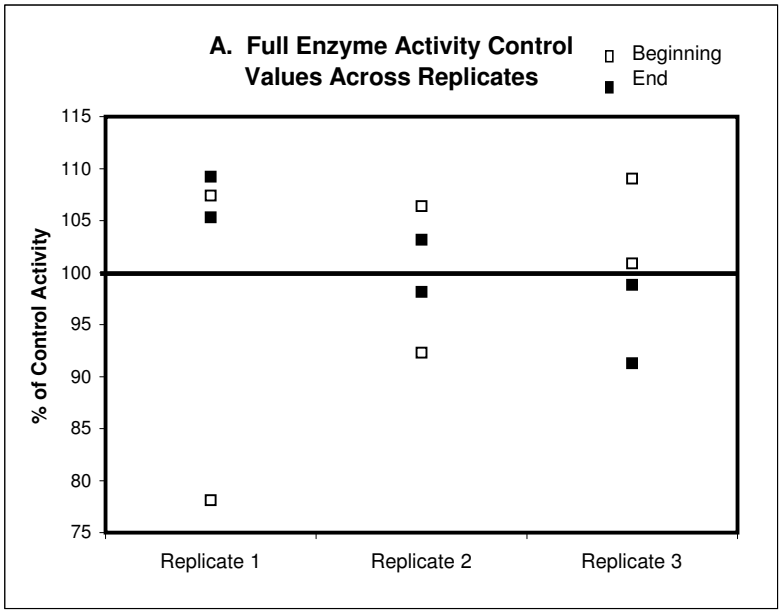
Daniel W. Sved, Ph.D. Director, Metabolism and Analytical Chemistry	Date
Robert A. Wally, B.S., RAC Technical Manager, Regulatory Affairs and Services	Date

Figures 1 - 9

Figure 1. Summary of Full Enzyme Activity Results

	Replicate 1				Replicate 2				Replicate 3				OVERALL	
	Aromatase Activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase Activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase Activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Portion Mean Across Replicates	Portion Percent of Control Across Replicates
Begin	0.062256996	78.09			0.071163	92.31			0.059323115	100.87				
	0.085608192	107.38	0.074	92.7	0.082018918	106.39	0.077	99.3	0.064123057	109.03	0.062	104.9	0.071	98.4
End	0.087069758	109.22			0.075672172	98.15			0.058121066	98.82				
	0.083949043	105.30	0.086	107.3	0.079527863	103.16	0.078	100.7	0.053681413	91.28	0.056	95.1	0.073	101.6
Average % Activity:		100				100				100				



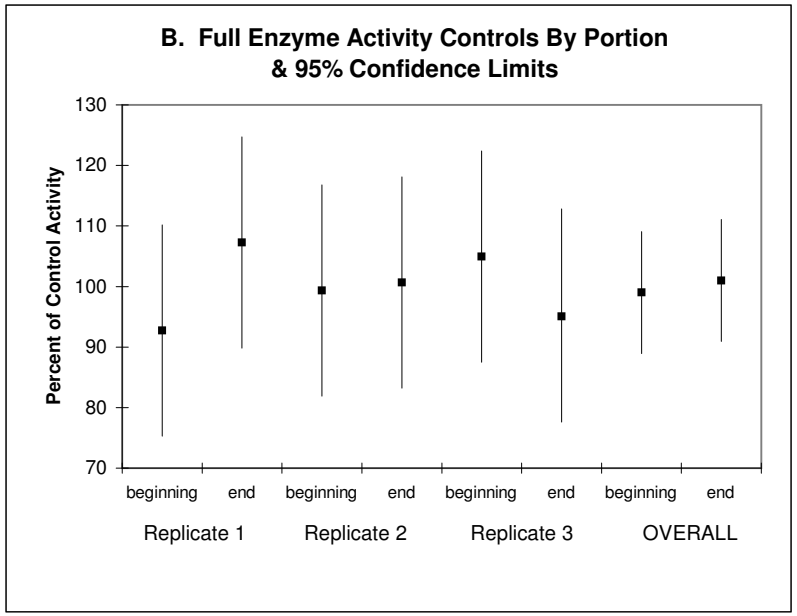
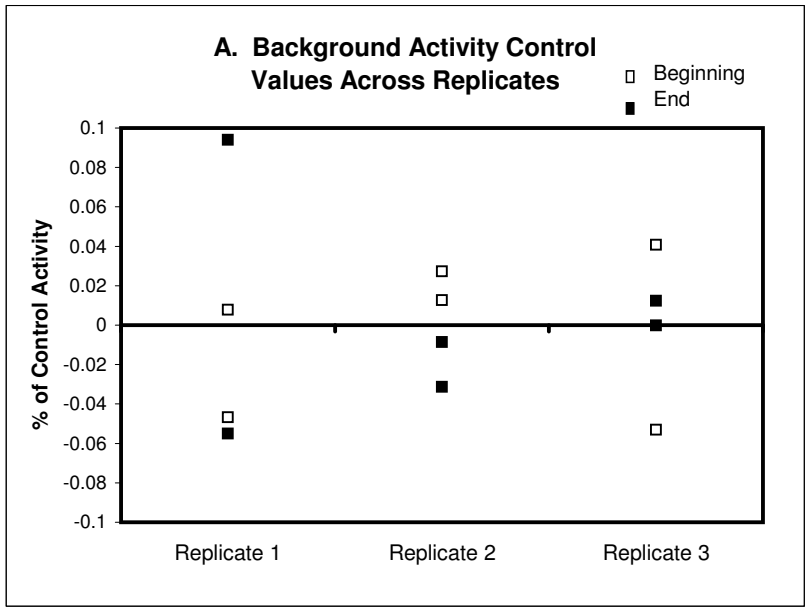


Figure 2. Summary of Background Enzyme Activity Results

	Replicate 1				Replicate 2				Replicate 3				OVERALL	
	Aromatase activity (nmol estrogen formed/mg protein/min	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min	% of Control	Portion Mean	Portion % of Control	Portion Mean Across Replicates	Portion Percent of Control Across Replicates
Begin	6.15E-06	0.008			2.10E-05	0.027			-3.12E-05	-0.053				
	-3.73E-05	-0.047	-1.56E-05	-0.020	9.82E-06	0.013	1.54E-05	0.020	2.40E-05	0.041	-3.60E-06	-0.006	-1.252E-06	-0.0017
End	7.50E-05	0.094			-2.42E-05	-0.031			-8.65E-08	0.000				
	-4.39E-05	-0.055	1.56E-05	0.020	-6.58E-06	-0.009	-1.54E-05	-0.020	7.28E-06	0.012	3.60E-06	0.006	1.252E-06	0.0017
Sum of Background Activity:		0				0				1.2E-16				



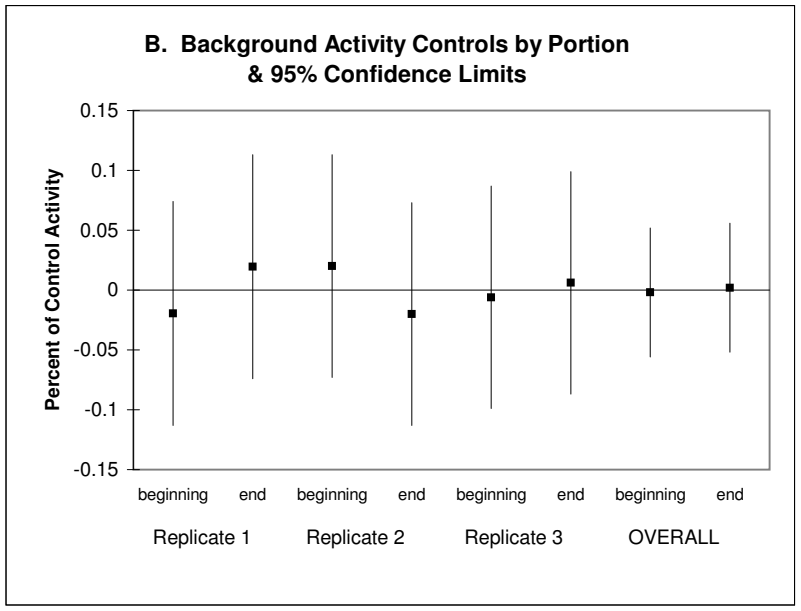
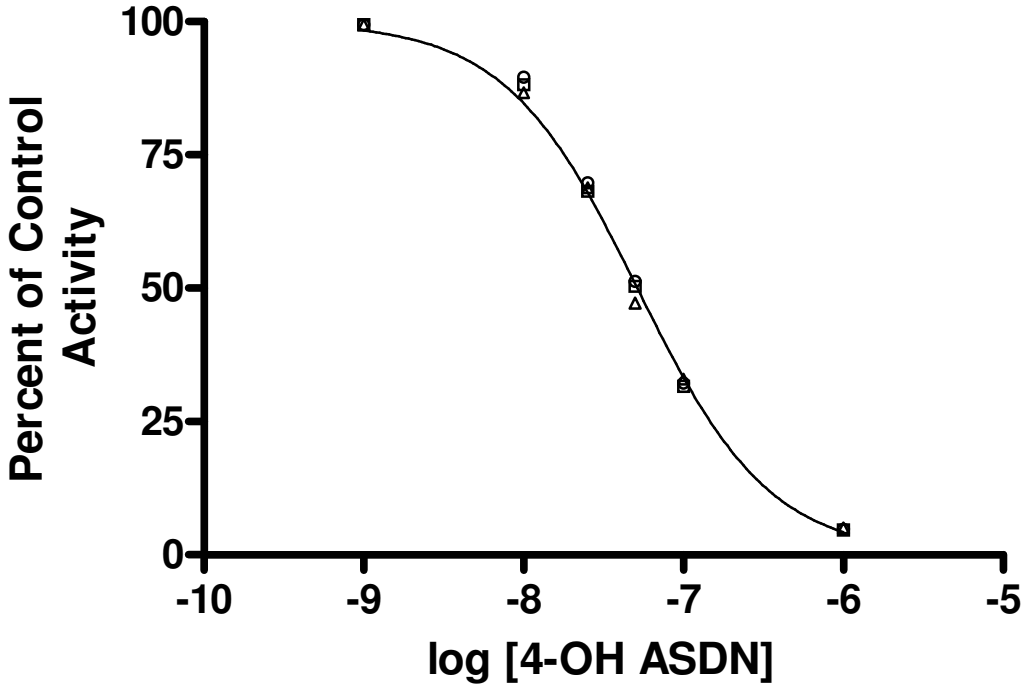
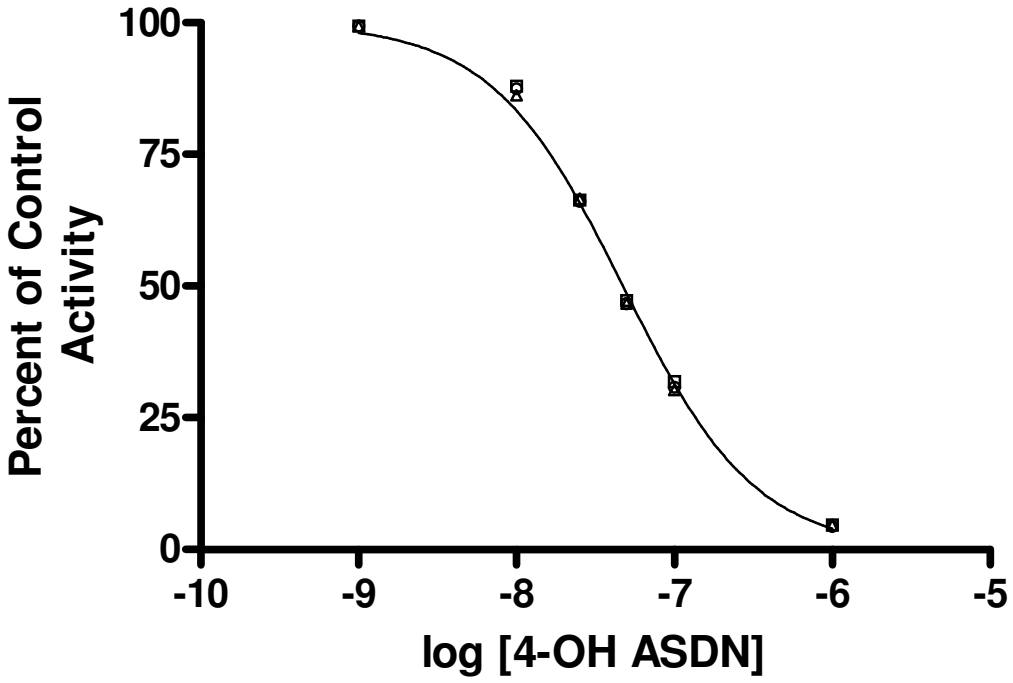


Figure 3. Plot of 4-OH ASDN Inhibition Replicate 1



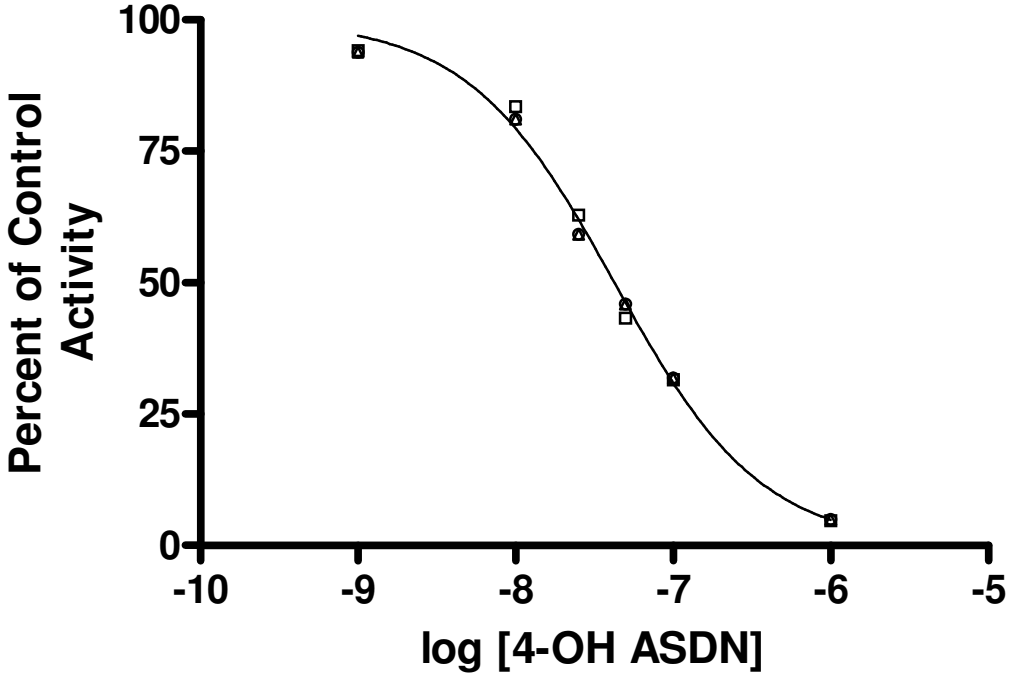
log [4-OH ASDN]	Replicate1: % Control Activity		
	Y1	Y2	Y3
-6.00	4.7	4.9	5.2
-7.00	31.7	32.3	33.0
-7.30	50.5	51.3	47.3
-7.60	68.3	69.9	68.9
-8.00	88.3	89.7	86.8
-9.00	99.5	99.5	99.5

Figure 4. Plot of 4-OH ASDN Inhibition Replicate 2



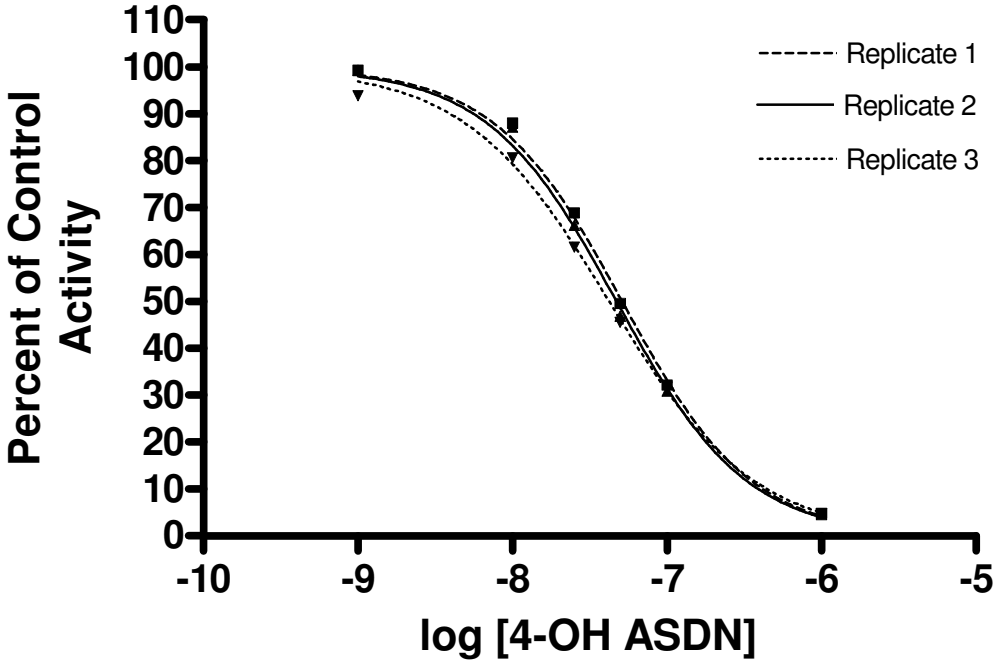
log [4-OH ASDN]	Replicate2: % Control Activity		
	Y1	Y2	Y3
-6.00	4.7	4.5	4.7
-7.00	31.9	30.9	30.4
-7.30	47.4	46.8	46.8
-7.60	66.4	66.2	66.7
-8.00	88.2	87.6	86.3
-9.00	99.5	99.5	99.5

Figure 5. Plot of 4-OH ASDN Inhibition Replicate 3



log [4-OH ASDN]	Replicate3: % Control Activity		
	Y1	Y2	Y3
-6.00	4.78	5.05	4.73
-7.00	31.61	32.00	31.93
-7.30	43.41	46.03	47.72
-7.60	62.98	59.33	62.85
-8.00	83.62	81.21	77.70
-9.00	94.30	93.99	93.99

Figure 6. Plot of Average 4-OH Inhibition Per Replicate



	Replicate 1	Replicate 2	Replicate 3
aromatase eqn			
Best-fit values			
LOGEC50	-7.2860	-7.3226	-7.3695
HILLSLOPE	-1.0478	-1.0389	-0.93430
Std. Error			
LOGEC50	0.010722	0.010142	0.010841
HILLSLOPE	0.024192	0.022643	0.019025
95% Confidence Intervals			
LOGEC50	-7.3087 to -7.2632	-7.3441 to -7.3011	-7.3925 to -7.3465
HILLSLOPE	-1.0991 to -0.99649	-1.0869 to -0.99092	-0.97463 to -0.89396
Goodness of Fit			
Degrees of Freedom	16	16	16
R^2 (unweighted)	0.99658	0.99663	0.99557
Weighted Sum of Squares (1/Y)	1.1901	1.0612	1.0307
Absolute Sum of Squares	64.770	63.901	71.311
Sy.x	2.0120	1.9985	2.1111
Data			
Number of X values	6	6	6
Number of Y replicates	3	3	3
Total number of values	18	18	18
Number of missing values	0	0	0

Figure 7. Overall 4-OH ASDN Inhibition Response Curve

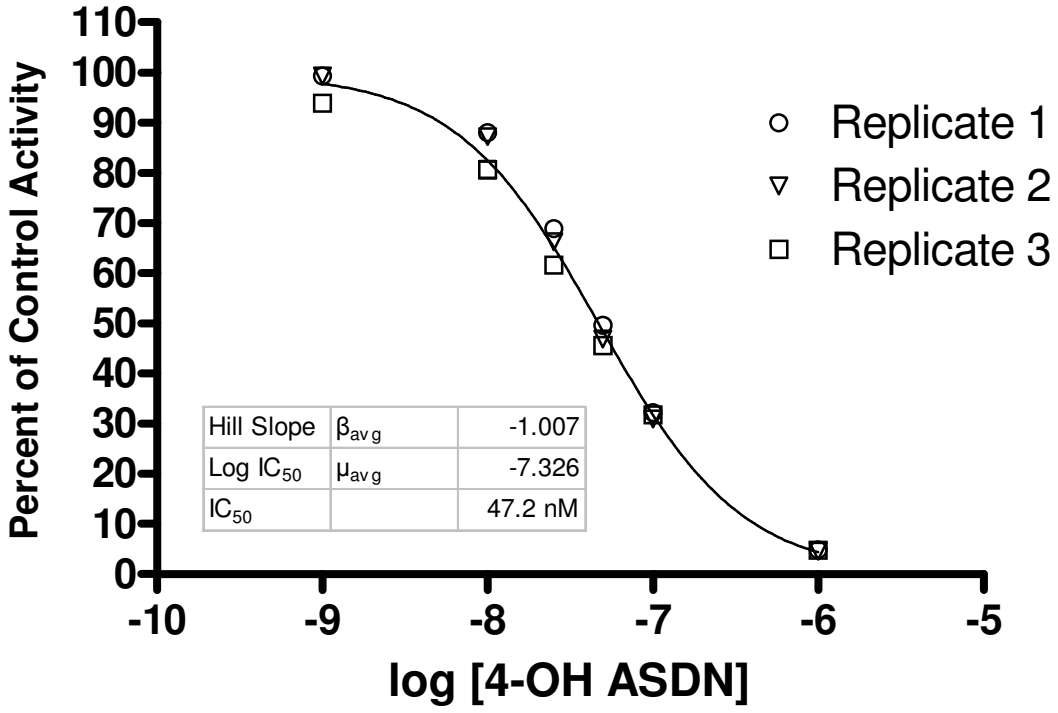


Figure 8. Log IC_{50} (µ) Results

		Replicate	Values	Std. Error	95% Confidence Level low	high
µ	LOGIC50	1	-7.29	0.01	-7.31	-7.26
µ	LOGIC50	2	-7.32	0.01	-7.34	-7.30
µ	LOGIC50	3	-7.37	0.01	-7.39	-7.35
µ (avg)	AVG LogIC_{50}		-7.33	0.02	-7.43	-7.22

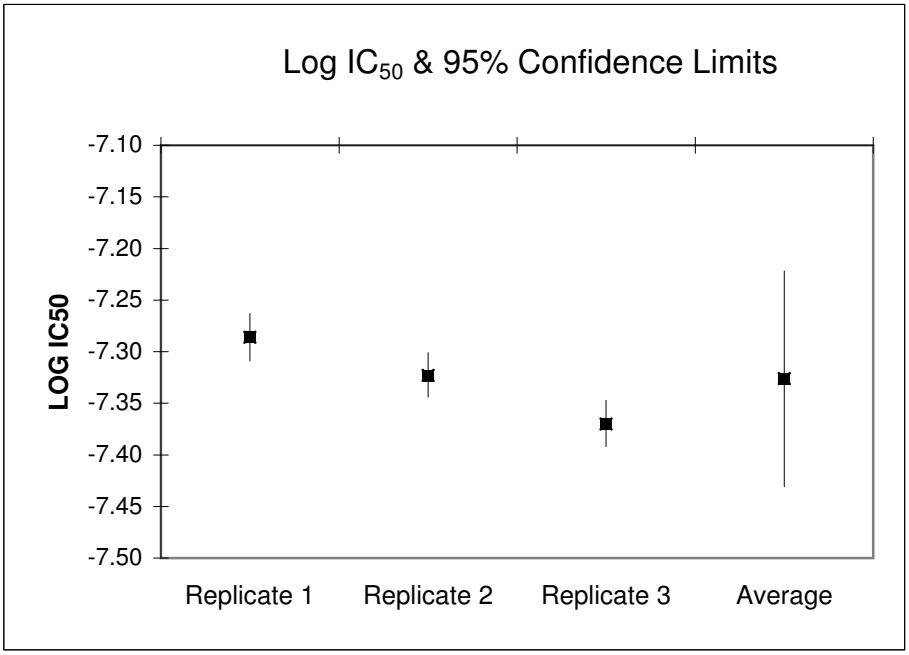
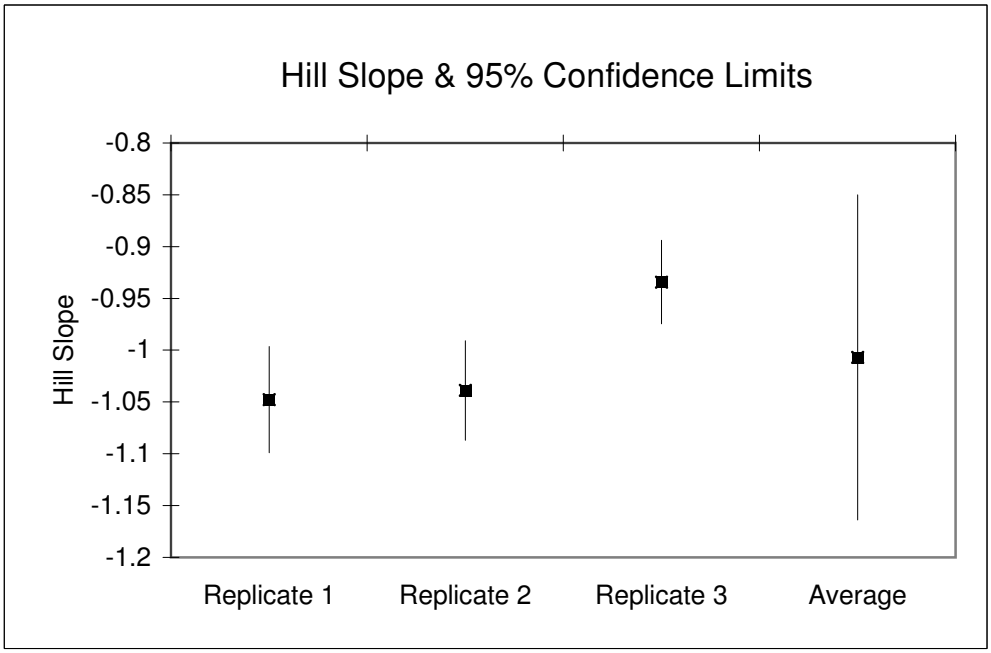


Figure 9. Hill Slope (β) Results

		Replicate	Values	Std. Error	95% Confidence Level	
					low	high
β	HILLSLOPE	1	-1.0478	0.0242	-1.0991	-0.9965
β	HILLSLOPE	2	-1.0389	0.0226	-1.0869	-0.9909
β	HILLSLOPE	3	-0.9343	0.0190	-0.9746	-0.8940
β (avg)	AVG HILLSLOPE		-1.007	0.036	-1.164	-0.850



Appendix A

Study Protocol (with Amendments and Deviations)

Study Number: WIL-431006

PROTOCOL AMENDMENT I

Sponsor: Battelle Memorial Institute

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

A. Title of Study:

Validation of the Placental Aromatase Assay: Positive Control Study
(WA 4-16, Task 4)

B. Protocol Additions/Modifications:

1) **4.2.3 Lot Number:**

This section is changed to the following:

SW0045

2) **8 IC50 DETERMINATION OF AROMATASE ASSAY RESULTS:**

The following is added to this section:

Two additional independent experimental replicates (fourth and fifth) were conducted under the same conditions as the first three replicates with the following specification. These additional replicates used a normal positive displacement pipet to dispense the diluted microsomes into the assay tubes to initiate the assay (no repeat pipettor will be used for this step).

3) **9 DATA ANALYSIS:**

This entire section is changed to the following:

The data to be reported will include the following information: assay date and run number, technician, chemical and log chemical concentration, total DPM-background DPM, and % activity. The average of the DPMs for the background tubes should be subtracted from the tubes with Total DPMs to provide DPMs for specific aromatase activity. A spreadsheet will be developed by the lead laboratory that will be used to process the data into a final form for analysis and evaluation. A working document detailing the conversion of the data from DPMs to nmol, as well as the actual methods for

calculations of the final aromatase activity will be distributed to the laboratories. This process is briefly summarized below.

The spreadsheet calculates DPM/mL for each aliquot of extracted aqueous incubation mixture and average DPM/mL and total DPM for each aqueous portion (after extraction). Multiplication of the volume (mL) of substrate solution added to the incubation by the substrate solution radiochemical content (DPM/mL) yields the total DPM present in the assay tube at initiation. The total DPM remaining in the aqueous portion after extraction divided by the total DPM present in the assay tube at initiation times 100 yields the percent of the substrate that was converted to product. The total DPM remaining in the aqueous portion after extraction is corrected for background by subtracting the average DPM present in the aqueous portion of the background activity tubes (for that day/assay). This corrected DPM is then converted to nmol product formed by dividing by the substrate specific activity (DPM/nmol). 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 (nmol) by the product of mg microsomal protein used times the incubation time. Average activity in the full enzyme activity control samples for a given Study is calculated. Percent of control activity remaining in the presence of various inhibitor concentrations is calculated by dividing the aromatase activity at a given concentration by the average full enzyme activity control and multiplying by 100.

IC_{50} will be calculated using Prism (Version 3 or higher) software to fit the percent of control activity and log concentration data to a curve using the following equation:

$$Y=100/(1+10^{((LogIC50-X)*HillSlope)})$$

Where: X is the logarithm of concentration
Y is the percent activity

The data will be formatted as follows (data from replicates 1 and 3 will be presented in an appendix to the report):

- One spreadsheet or table will display the DPMs for all assay tubes, calculations of activity (nmol (mg protein)$^{-1}$min^{-1}) etc.
- Another table will present the results of the analysis of variability of the assay and will include :

(1) the variation between repetitions within a single replicate of the assay,
(2) the day to day (replicate-to-replicate) variation, and
(3) technician variation

- Graphs of activity versus log chemical concentration.
- Table of IC_{50}s by date, run, technician, assay method.

4) **10 STATISTICAL ANALYSES:**

This entire section is changed to the following:

10.1 <u>Concentration Response Fits for the Test Substance:</u>

For the test substance multiple independent replicates of the concentration response curve fit were carried out. The number of replicates conducted were five. Only the data from the second, fourth and five replicates will be analyzed as described below (the first and third replicates conducted will not be statistically analyzed). For reporting purposes, replicates 2, 4 and 5 may be referred to as replicates 1, 2 and 3 in the final report. Full enzyme activity and background activity control percent activity values will be compared across daily replicate tests for each test substance.

For each replicate two repeat tubes of the full enzyme activity controls and the background activity controls will be prepared prior to the preparation of the repetitions of the inhibitor compound and two repeat tubes of the full enzyme activity controls and the background activity controls will be prepared after the repetitions of the inhibitor compound are prepared. Three repetitions will be prepared for each level of the inhibitor compound (4-OH ASDN).

For each repetition at each level the Excel database spreadsheet will include total DPMs per tube (corrected for background DPMs) and total aromatase activity per tube. The aromatase activity is calculated as the (background corrected) DPM, normalized by the specific activity of the [^{3}H]ASDN, the mg of protein of the aromatase, and the incubation time. The aromatase activity is corrected for the background DPMs, as measured by the average of the background activity tubes. Percent activity is the (background corrected) aromatase activity divided by the average of the aromatase activity in the full enzyme activity control tubes, multiplied 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. The total DPM values are not corrected for background.

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 individual experimental percent of control activity 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 replicate at each inhibitor concentration. Concentration is expressed on the log scale. In agreement with past convention, logarithms will be common logarithms (i.e. base 10). Let X denote the logarithm of the concentration of inhibitor compound (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 inhibitor concentration
β ≡ slope of the concentration response curve (β will be negative)
$\mu \equiv \log_{10}IC_{50}$ (IC_{50} is the concentration corresponding to percent of control activity equal to 50%)

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

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

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

The response curve will be fitted by weighted least squares nonlinear regression analysis with weights equal to 1/Y. 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%.[1]

The concentration response fits will be carried out 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 the inhibitor 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 log10IC50 or β can be calculated based on a one-way random effects analysis of variance model fit.

For each test substance and 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 is indicated as:

"C" Complete. i.e. ranging from essentially 0 percent to 100 percent of control.
"II" Incomplete. But can interpolate to $\log_{10}IC_{50}$.
"IX" Incomplete. But must extrapolate to $\log_{10}IC_{50}$.

Replicates for which a concentration response curve cannot be fitted (and so an IC_{50} cannot be estimated) will be referred to as "noninhibitors".

10.2 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 inhibitor compound 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 inhibitor concentration on the same plot. Plotting symbols will distinguish among

[1] This adjustment tacitly assumes an upper bound of 100% and a lower bound of 0%. Fixing these bounds rather than permitting PRISM to fit variable Top and Bottom parameters permits estimation of the IC_{50} concentration on inhibition curves that do not span the entire inhibition range from 100% to 0%.

replicates. The fitted concentration response curve for each replicate will be superimposed on the plot. On a separate plot the average percent of control values for each replicate will be plotted versus logarithm of inhibitor compound concentration. The average concentration response curve across replicates will be superimposed on the same plot.

For each replicate treat (β, μ) as a random variable with mean (β_{avg}, μ_{avg}). Let X and Y ($0 < Y < 100$) denote logarithm of concentration and percent of control, as defined above.

The average response curve is

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

Slope (β) and $\log_{10}IC_{50}$ (μ) will also be compared across replicates based on random effects analysis of variance, treating the replicates as random effects. β and μ are estimated, separately within each replicate, and plotted along with the average across replicates and associated 95% confidence interval across replicates (including replicate-to-replicate variation).

10.3 Full Enzyme Activity and Background Activity Control Values Across Replicates

Within each replicate, quadruplicate repetitions will be made of the background activity tubes and the full enzyme activity control tubes. Half the repetitions will be carried out at the beginning of the replicate and half at the end. If the conditions are constant 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 combined across replicates and expressed as percent of full enzyme activity control activity. The average of the four background activity samples 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 two beginning controls and the two end controls will be plotted by replicate with plotting symbol distinguishing between beginning and end, and with reference line 0% (background activity) or 100% (full enzyme activity control) respectively. These plots will display the extent of consistency across replicates with respect to average value and variability and will provide comparisons of beginning versus end of each replicate.

Two-way analysis of variance will be carried out, separately for the full enzyme activity control tubes and the background activity tubes. The factors in the analysis of variance will be replicate, portion (beginning or end), replicate by portion interaction. The error corresponds to repetition within replicate and portion. The response will be percent of control aromatase activity. If the daily replicates are in control the portion main effect and portion by replicate interaction should be non-significant. Note that the replicate effects will necessarily be zero because of the constrained totals within each replicate. For the purposes of evaluation, replicate will be treated as a fixed effect. If 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, adjusting for simultaneity by Bonferroni's method. The portion effect within each replicate and the portion effect averaged across replicates, and associated 95% confidence intervals, will be presented graphically.

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

10.5 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.

5) **12** **RECORDS TO BE MAINTAINED:**

This first sentence in this section is changed to the following (change in bold):

All specimens and original raw data records, as defined by WIL SOPs and the applicable GLPs, will be stored as described in Section **13** in the Archives at WIL Research Laboratories, LLC

C. Reasons for Protocol Additions/Modifications:

1) The Lot Number has been provided.

2) Based on the results of the first three replicates it was hypothesized that the use of a repeat pipettor negatively affected the outcome of replicates 1 and 3 since replicate 2 worked as expected. Therefore the Sponsor authorized a fourth replicate (see form A-186 dated December 16, 2004) to test this hypothesis. Not using a repeat pipettor resulted in an expected experimental outcome in the fourth replicate, so a fifth replicate was authorized by the Sponsor (see form A-186 dated December 20, 2004).

3) This entire section changed significantly at the request of the Sponsor.

4) This entire section changed significantly at the request of the Sponsor. These changes included only doing statistical analyses on the three experimental replicates that did not use the repeat pipettor.

5) The section referenced in this section was inadvertently listed incorrectly in the original protocol, it has been corrected.

Approved By:

Battelle Memorial Institute

Jerry D. Johnson, Ph.D., D.A.B.T.
Sponsor Representative

1-19-05
Date

Prepared By:

WIL Research Laboratories, LLC

Christopher J. Bowman, Ph.D.
Study Director

1/18/05
Date

Mark D. Nemec, B.S., D.A.B.T.
Director, Developmental and
Reproductive Toxicology

1/18/2005
Date

PROTOCOL

VALIDATION OF THE PLACENTAL AROMATASE ASSAY: POSITIVE CONTROL STUDY (WA 4-16, TASK 4)

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 4: Conduct of the Positive Control Studies in the Participating Laboratories. WIL was selected as one of the participating laboratories in the interlaboratory validation of this human placental microsomal aromatase assay. This protocol describes the set of experiments to be conducted at WIL Research Laboratories, LLC.

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 since the test system is a microsome. The method used for treating the microsomes will be to mix the microsomes, reagents, and test article in a common reaction vessel so that microsomal uptake of the test article can be used to evaluate possible effects on enzymatic activity.

The objective is to demonstrate the responsiveness of the placental microsome aromatase assay using the known aromatase inhibitor, 4-hydroxyandrostenedione as a positive control.

2 PERSONNEL INVOLVED IN THE STUDY:

2.1 Sponsor Representatives:

Jerry D. Johnson, Ph.D., D.A.B.T.
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David P. Houchens, Ph.D.
Program Manager
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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:

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2.4 WIL Deputy Director:

Jennifer Thomas-Wohlever, Ph.D.
Research Scientist, 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

Donald G. Stump, Ph.D., D.A.B.T.
Associate Director, Developmental
and Reproductive Toxicology

Justin Godsey, B.S.
Biologist, Metabolism

Lewis E. Kaufman, M.S., RAC, RQAP-GLP
Director, Regulatory Affairs and Services

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

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.

3 STUDY SCHEDULE:

Proposed First Replicate Assay Date:	December 9, 2004
Proposed Last Replicate Assay Date:	December 20, 2004
Proposed Unaudited Data Submission Date:	December 22, 2004
Proposed Audited Report Date:	January 31, 2005

4 TEST CHEMICAL DATA:

Reserve samples of the test chemical(s) 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 4-Hydroxyandrostenedione:

4.1.1 CAS Number:

566-48-3

4.1.2 Synonym:

4-OH ASDN, 4-Androsten-4-ol-3,17-dione

4.1.3 Molecular Formula/M.W.

$C_{19}H_{26}O_3$; 302.4 g/mol

4.1.4 Supplier:

Sigma-Aldrich

4.1.5 Lot Number:

063K4069

4.1.6 Purity:

99%

4.1.7 Physical Description Test Solution:

To be documented by the Sponsor.

4.1.8 Storage Conditions, Test Solution:

Refrigerated (2-8°C).

4.2 Ethanol (Vehicle):

4.2.1 CAS Number:

64-17-5

4.2.2 Synonyms:

EtOH

4.2.3 Lot Number:

To be provided by the Sponsor.

4.2.4 Purity:

To be provided by the Sponsor.

4.2.5 Storage Conditions:

Room temperature.

Personnel safety data are to be provided by the Sponsor. It is the responsibility of the Sponsor to notify the testing facility of any special handling requirements of the test chemical stock solution. A material safety data sheet (MSDS) will accompany the test chemical stock solution upon arrival at the laboratory.

Test chemical stock solutions formulated in ethanol will be prepared and analyzed by the Sponsor and distributed to the laboratories. Stability of the test chemical stock solutions in ethanol will also be conducted by the Sponsor. Therefore, documentation that the specified test chemicals and lot numbers were used and stored according to the Sponsor's Standard Operating Procedures will be maintained by the Sponsor and stored at

the Sponsor's facility. The chemistry report will be supplied by the Sponsor and included in the final report as an appendix.

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.

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 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

5.1.1 Substrate Name/Supplier

The substrate for the aromatase assay is androstenedione (ASDN). Non-radiolabeled and radiolabeled ASDN will be used. The radiolabeled androstenedione ([1β-3H]-androstenedione, [^{3}H]ASDN) will be shipped directly from the supplier (Perkin Elmer). The non-radiolabeled ASDN will be provided to the laboratories by Battelle's Chemical Respository (CR). The CR will forward all applicable information regarding supplier, lot numbers and reported/measured purity for the substrate to the laboratories and this information will be included in study reports. The radiochemical purity of the [^{3}H]ASDN (of each lot that is used) will be confirmed by the lead laboratory (RTI International). The radiochemical purity of the [^{3}H]ASDN will be greater than approximately 95 percent. If the radiochemical purity is less than 95 percent, then the Sponsor and Study Director will be notified.

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 100-fold 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 (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.

5.2 Microsomes

Placental microsomes will be supplied to each laboratory by the lead laboratory (RTI International). The microsomes must be stored between approximately -70 and -80°C. Microsomes may be thawed and re-frozen up to 4 times if necessary. The approximate protein content of the microsomes will be provided.

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. Use microsomes within 2 hours of setting in the ice bath. The microsomes will be rehomogenized (approximately 5 to 10 passes) using a Potter-Elvejhem homogenizer 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 (no longer than 2 hours) until they are placed in the water bath just prior to their addition to the aromatase assay.

Caution: Microsomes can be denatured by detergents. Therefore, it is important to ensure that all reagent flasks, test tubes, pipettes, etc. that are used in the conduct of the assay are free of detergent residue.

5.3 Other Assay Components

5.3.1 Buffer

The assay buffer is 0.1 M sodium phosphate buffer, pH 7.4. Sodium phosphate monobasic and sodium phosphate dibasic 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 refrigerated (2-8°C) for up to one month.

5.3.2 Propylene Glycol

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

5.3.3 NADPH

NADPH (β-nicotinamide adenine dinucleotide phosphate, reduced form, tetrasodium salt) 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 the microsome preparation will be determined 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. The protein standards will be made from bovine serum albumin (BSA). Protein will be determined by using a DC Protein Assay kit purchased from Bio-Rad (Hercules, CA). 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 minutes 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)

The assays will be performed in 13x100 mm test tubes maintained at 37 ± 1°C in a shaking water bath. 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 the table below.

Human Placental Microsomal Aromatase Assay-Optimized Conditions	
Microsomal Protein	0.0125 mg/mL[a]
NADPH	0.3 mM[a]
[^{3}H]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 five minutes prior to initiation of the assay by the addition of 1 mL of the diluted microsomal suspension. The total assay volume will be 2.0 mL, and the tubes will be incubated for 15 minutes. The incubations will be stopped by the addition of methylene chloride (2.0 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 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.

Results will be presented as the activity (velocity) of the enzyme reaction. 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 dpm/nmol). 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 IC50 DETERMINATION OF AROMATASE ASSAY RESULTS:

Each experiment will test the response of aromatase activity in the presence of six concentrations of 4-OH ASDN. The experiment will be conducted in three

independent replicates. Each concentration of 4-OH ASDN will be run in triplicate tubes in each experimental replicate. See the experimental design table below. Control samples will be included for each study. Full Enzyme Activity Controls will contain substrate, NADPH, propylene glycol, buffer, vehicle (used for preparation of 4-OH ASDN solutions) and microsomes. Background Activity Controls contain all Full Enzyme Activity Control assay components except NADPH and serve as assay blanks. Four Full Enzyme Activity Controls and four Background Activity Controls are included with each experimental replicate and are treated the same as the other samples. The control sets will be split so that two tubes (of each Full Enzyme Activity Controls and Background Activity Control) are run at the beginning and two at the end of each experimental replicate.

Positive control assays 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. The detailed procedure will be documented in the study records.

Experimental Design

Sample type	Repetitions (test tubes)	Description of assay[a]	4-OH ASDN dilution concentration (M stock)	4-OH ASDN concentration (M final)
Full Enzyme Activity Control	4	no 4-OH ASDN, inhibitor vehicle only	N/A	N/A
Background Activity Control	4	no 4-OH ASDN or NADPH, inhibitor vehicle only	N/A	N/A
4-OH ASDN Concentration 1	3	4-OH ASDN added	1×10^{-4}	1×10^{-6}
4-OH ASDN Concentration 2	3	4-OH ASDN added	1×10^{-5}	1×10^{-7}
4-OH ASDN Concentration 3	3	4-OH ASDN added	5×10^{-6}	5×10^{-8}
4-OH ASDN Concentration 4	3	4-OH ASDN added	2.5×10^{-6}	2.5×10^{-8}
4-OH ASDN Concentration 5	3	4-OH ASDN added	1×10^{-6}	1×10^{-8}
4-OH ASDN Concentration 6	3	4-OH ASDN added	1×10^{-7}	1×10^{-9}

[a] All assay tubes contain the following unless otherwise stated: buffer, propylene glycol, microsomal protein, [^{3}H]ASDN and NADPH.

9 DATA ANALYSIS:

The data to be reported will include the following information: assay date and run number, technician, chemical and log chemical concentration, total DPM-background DPM, and % activity. The DPMs for the background tubes should be subtracted from the tubes with Total DPMs to provide DPMs for specific aromatase activity. A spreadsheet will be developed by the lead laboratory that will be used to process the data into a final form for analysis and evaluation. A working document detailing the conversion of the data from DPMs to nmol, as well as the actual methods for calculations of the final aromatase activity will be distributed to the laboratories. This process is briefly summarized below.

The spreadsheet calculates DPM/mL for each aliquot of extracted aqueous incubation mixture and average DPM/mL and total DPM for each aqueous portion (after extraction). Multiplication of the volume (mL) of substrate solution added to the incubation by the substrate solution radiochemical content (DPM/mL) yields the total DPM present in the assay tube at initiation. The total DPM remaining in the aqueous portion after extraction divided by the total DPM present in the assay tube at initiation times 100 yields the percent of the substrate that was converted to product. The total DPM remaining in the aqueous portion after extraction is corrected for background by subtracting the average DPM present in the aqueous portion of the background activity tubes (for that day/assay). This corrected DPM is then converted to nmol product formed by dividing by the substrate specific activity (DPM/nmol). The activity of the enzyme reaction is expressed in nmol (mg protein)-1min-1 and is calculated by dividing the amount of estrogen formed (nmol) by the product of mg microsomal protein used times the incubation time. Average activity in the full enzyme activity control samples for a given Study is calculated. Percent of control activity remaining in the presence of various inhibitor concentrations is calculated by dividing the aromatase activity at a given concentration by the average full enzyme activity control and multiplying by 100.

IC_{50} will be calculated using Prism (Version 3 or higher) software to fit the percent of control activity and log concentration data to a curve using the following equation:

$$Y=Bottom + (Top-Bottom)/(1+10^{((LogIC_{50}-X)*HillSlope)})$$

Where:
X is the logarithm of concentration
Y is the percent activity
Bottom is the lower plateau
Top is the upper plateau.

The data will be formatted as follows:

- One spreadsheet or table will display the DPMs for all assay tubes, calculations of activity (nmol (mg protein)$^{-1}$min^{-1}) etc.
- Another table will present the results of the analysis of variability of the assay and will include :

  (1) the variation between replicates within a single assay,
  (2) the day to day (study-to-study) variation, and
  (3) technician variation.

- Graphs of activity versus log chemical concentration.
- Table of IC_{50}s by date, run, technician, assay method.

10 STATISTICAL ANALYSES

10.1 Concentration Response Fits for the Test Substance

For the test substance multiple independent replicates of the concentration response curve fit will be carried out. The number of replicates will be three. Full enzyme activity and background activity control aromatase values will be compared across daily replicate tests for each test substance.

For each replicate two repeat tubes of the full enzyme activity controls and the background activity samples will be prepared prior to the preparation of the repetitions of the inhibitor compound and two repeat tubes of the full enzyme activity controls and the background activity controls will be prepared after the repetitions of the inhibitor compound are prepared. Three repetitions will be prepared for each level of the inhibitor compound (4-OH ASDN).

For each repetition at each level the Excel database spreadsheet will include total DPMs per tube and total aromatase activity per tube. The aromatase activity is calculated as the DPM, normalized by the specific activity of the [^{3}H]ASDN, the mg of protein of the aromatase, and the incubation time. The aromatase activity is corrected for the background DPMs, as measured by the average of the background activity tubes. Thus the average aromatase activity across the four background activity repeat tubes must necessarily equal 0 within each replicate. The total DPM values are not corrected for background.

For each repetition within each inhibitor concentration, percent of control activity is determined by dividing the aromatase activity for that tube by the average full enzyme activity control 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 individual experimental percent of control activity values will sometimes extend below 0% or above 100%. Thus upper and lower response curve plateaus need to be included in the response curve models.

Concentration response trend curves will be fitted to the percent of control activity values within each of the repeat tubes at each inhibitor concentration. Concentration is expressed on the log scale. In agreement with past convention, logarithms will be common logarithms (i.e. base 10). Let X denote the logarithm of the concentration of inhibitor compound (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
T = upper plateau of the concentration response curve
B = lower plateau of the concentration response curve
DAVG = average DPMs across the repeat tubes with the same inhibitor concentration
B = 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:

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

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 response curve will be fitted by weighted least squares nonlinear regression analysis with weights equal to 1000/DAVG. Model fits will be carried out using Prism software (Version 3 or higher).

The concentration response fits will be carried out 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 the inhibitor will be the 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 can be calculated based on a one-way random effects analysis of variance model fit.

10.2 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 inhibitor compound 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 inhibitor concentration on the same plot. Plotting symbols will distinguish among replicates. The fitted concentration response curve for each replicate will be superimposed on the plot. On a separate plot the average percent of control values for each replicate will be plotted versus logarithm of inhibitor compound concentration. The average concentration response curve across replicates will be superimposed on the same plot with 95 percent confidence intervals on average control values at each observed concentration. Replicate-to-replicate variation will be treated as a random effect for purposes of calculating confidence intervals.

For each replicate treat (β, μ) as a random variable with mean (β_{avg}, μ_{avg}) and covariance Σ ($_{\beta, \mu}$) across replicates. Let B_{avg}, T_{avg} denote the average bottom and top across the replicates. Let

$$Z = (Y - B_{avg})/(T_{avg} - B_{avg})$$

$$L = \log_{10}(Z/(1 - Z)).$$

The average response curve is expressed as

$$L = \beta_{avg}(\mu_{avg} - X)$$

with approximate standard errors of prediction of L at a given X based on Σ ($_{\beta, \mu}$) and propagation of errors. These are used to calculate approximate confidence intervals for predictions at each X. The linearized response curve and associated confidence intervals are back transformed to yield the response curve in terms of percent of control, Y

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

Slope (β) and $\log_{10}IC_{50}$ (μ) will also be compared across replicates based on random effects analysis of variance, treating the replicates as random effects. β and μ are estimated, separately within each replicate, and plotted along with the average and associated 95% confidence interval across replicates.

10.3 Full Enzyme Activity and Background Activity Control Values Across Replicates

Within each replicate, quadruplicate repetitions will be made of the background activity tubes and the full enzyme activity control tubes. Half the repetitions will be carried out at the beginning of the replicate and half at the end. If the conditions are constant 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 combined across replicates and expressed as percent of full enzyme activity control activity. The average of the four background activity samples 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 two beginning controls and the two end controls will be plotted by replicate with plotting symbol distinguishing between beginning and end, and with reference line 0% (background activity) or 100% (full enzyme activity control) respectively. These plots will display the extent of consistency across replicates with respect to average value and variability and will provide comparisons of beginning versus end of each replicate. Two-way analysis of variance will be carried out, separately for the full enzyme activity control tubes and the background activity tubes. The factors in the analysis of variance will be replicate, portion (beginning or end), replicate by portion interaction. The error corresponds to repetition within replicate and portion. The response will be percent of control aromatase activity. If the daily replicates are in control the portion main effect and portion by replicate interaction should be non-significant. Note that the replicate effects will not be estimable because of the constrained totals within each replicate. For the purposes of evaluation, replicate will be treated as a fixed effect. If 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, adjusting for simultaneity by Scheffe's method. The portion effect within each replicate and the portion effect averaged across replicates, and associated 95% confidence intervals, will be presented graphically.

10.4 Variability Assessments

For the inhibitor test compound variability among replicates and variability among repetitions within replicates will be estimated and assessed for statistical significance. The response will be aromatase activity. These analyses will treat inhibitor concentration as a classification variable and will include both the full enzyme activity and background activity groups. The factors in the mixed effects analysis of variance will be concentration group (including full enzyme activity and background activity groups), replicate, replicate by concentration interaction, and residual variation. Residual variation corresponds to repetition within replicate and concentration. Inhibitor concentration will be treated as a

fixed effect. Replicate and replicate by concentration interaction will be treated as random effects. The analysis of variance fit will incorporate weights. The weight for responses in each concentration group will be based on the average of the DPMs across all the replicates and repetitions within replicates associated with that concentration group. The weight for each concentration group will be 1000/[Average DPM].

Normal probability plots will be prepared to identify outlying replicates or repetitions. Deviations of average within replicate from average across replicate results within that concentration group will be ordered and plotted on a normal probability scale. The differences will be normalized by [Average DPM]^{½} for their concentration group to adjust for differing variability across concentration groups. Deviations of repetitions from average across repetitions within replicate and concentration group will be ordered and plotted on a normal probability scale. The differences will be normalized by [Average DPM]^{½} for their concentration group to adjust for differing variability across concentration groups.

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

10.6 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 (e.g. outlying laboratories), the extent of laboratory-to-laboratory variation, and overall consensus estimates among the laboratories.

11 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 will be 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 test 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.

12 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.

13 WORK PRODUCT:

The Sponsor will have title to all documentation records, raw data, slides, 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 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.

14 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."

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. Two electronic copies (PDF format) will be provided; requests for paper copies of the final report may result in additional charges.

15 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.

16 PROTOCOL APPROVAL:

Sponsor approval received via **e-mail** on **December 7, 2004**.

12/8/04
Christopher J. Bowman, Ph.D. Date
Study Director
WIL Research Laboratories, LLC

12/8/2004
Mark D. Nemec, B.S., D.A.B.T. Date
Director, Developmental and
Reproductive Toxicology
WIL Research Laboratories, LLC

Jerry D. Johnson 12-9-04
Jerry D. Johnson, Ph.D., D.A.B.T. Date
Work Assignment Leader/Study Monitor
Endocrine Disruptor Screening Program
Battelle Memorial Institute

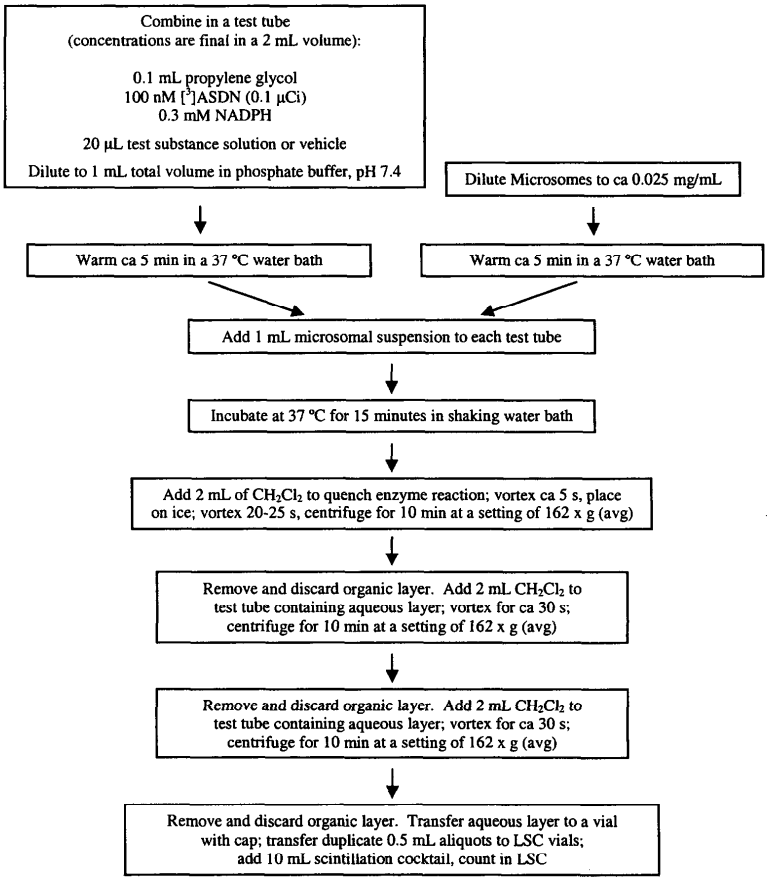
David P. Houchens 12/9/04
David P. Houchens, Ph.D. Date
Program Manager
Endocrine Disruptor Screening Program
Battelle Memorial Institute

17 PROTOCOL REVIEW:

Heather L Osborn 12/8/04
Heather L. Osborn, B.S., RQAP-GLP Date
Manager, Quality Assurance
WIL Research Laboratories, LLC

Terri L Pollock 12-9-04
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 CH2Cl2 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 CH2Cl2 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 CH2Cl2 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

Deviations from the Protocol

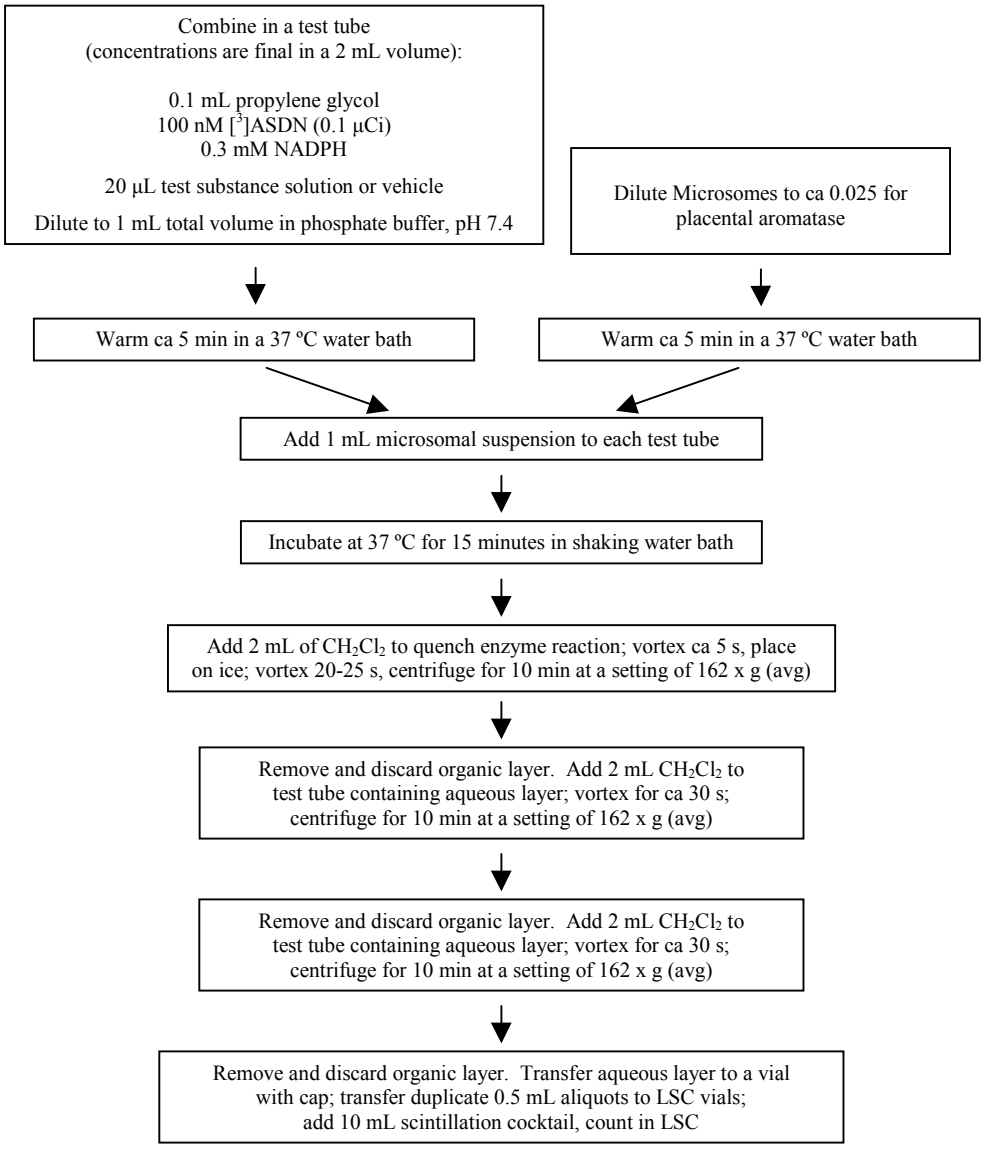
This study was conducted in accordance with the protocol and protocol amendments, except for the following.

- **Protocol Section 6.** states that for the protein assay, a six-point standard curve would be prepared, ranging from 0.13 to 1.5 mg protein/mL. Due to the bovine serum albumin (BSA) standard (Reference 431006A1-2-1) having a concentration of 2.36 mg/mL and not 2.5 mg/mL, the standard curve had a range of 0.12 to 1.4. On December 8, 2004, the BSA standard was prepared at 2.5 mg/mL from a lyophilized BSA assay standard (CP# 04-170) which had a stated concentration of 1.40 mg/mL. The 2.5 mg/mL concentration was calculated incorrectly, using an assay standard concentration of 1.48 mg/mL and not the correct value of 1.40 mg/mL. Therefore, the BSA standard (Reference 431006A1-2-1) had a protein concentration of 2.36 mg/mL and the subsequent standard curve ranged from 0.12 to 1.4 mg protein/mL rather than 0.13 to 1.5 mg protein/mL.

This deviation did not negatively impact the quality or integrity of the data nor the outcome of the study.

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]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 for placental aromatase
Warm ca 5 min in a 37 ºC water bath
Warm ca 5 min in a 37 ºC water bath
Add 1 mL microsomal suspension to each test tube
Incubate at 37 ºC for 15 minutes in shaking water bath
Add 2 mL of CH2Cl2 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 CH2Cl2 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 CH2Cl2 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 (with Amendments and Deviations)

1.0 TITLE AND APPROVAL

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

Task 4 - Conduct Positive Control Studies in the Participating Laboratories

for

EPA CONTRACT NUMBER 68-W-01-023

December 7, 2004

SIGNATURE PAGE

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

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Battelle
Columbus, OH

Signature Date 12-7-04

David P. Houchens, Ph.D.
EDSP Program Manager
Battelle
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Signature Date 12/7/04

Jerry D. Johnson, Ph.D., DABT
EDSP Work Assignment Leader
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Linda J. Phillips, Ph.D.
EPA Project Officer
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2.0 TABLE OF CONTENTS

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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 positive control 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 are to 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 Tasks 4 through 7 of the work assignment.

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

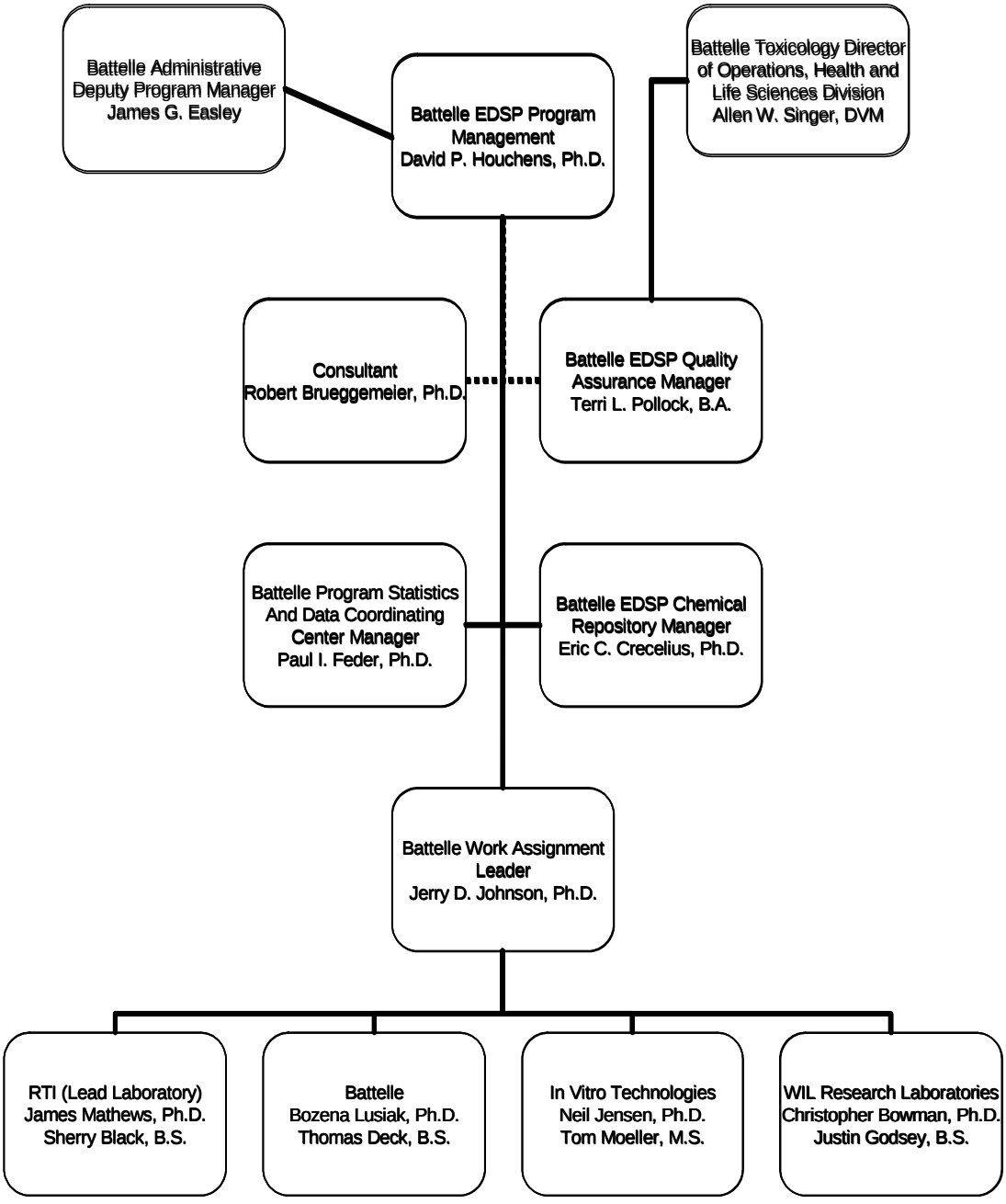

Figure 1. WA 4-16 Project Organization Overview

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 is 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 are 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 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 WA L/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 reports, 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 assures 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 1 summarizes the prevalidation tasks and the laboratory(ies) involved for each experimental task.

Table 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	Prepare/Analyze Microsomes and Conduct Positive Control Study at Two Participating Laboratories; Analyze Microsomes at Lead and One Participating Laboratory	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 = Endodrine 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 $P450_{arom}$ 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 found in breast tissue, and the importance of intratumoral aromatase and local estrogen production is being unraveled. Effective aromatase inhibitors have been developed as therapeutic agents for estrogen-dependent breast cancer to reduce the growth stimulatory effects of estrogens in breast cancer. Investigations on the development of aromatase inhibitors began in the 1970's and have expanded greatly in the past three decades.

An *in vitro* aromatase assay could easily be utilized as an alternative screening method in the Tier 1 Screening Battery to assess the potential effects of various environmental toxicants on aromatase activity. Both *in vitro* subcellular (microsomal) assays and cell-based assays are available for measuring aromatase activity. The *in vitro* subcellular assay using human placental microsomes, is commonly used to evaluate the ability of pharmaceuticals and environmental

chemicals to inhibit aromatase activity. In addition, human JEG-3 and JAR choriocarcinoma cell culture lines, originally isolated from cytotrophoblasts of malignant placental tissues, have been used as *in vitro* systems for measuring the effects of compounds on aromatase activity. These cell lines are also utilized for investigations on the effects of agents in placental toxicology.

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

The human placental microsomal aromatase assay was recommended as the *in vitro* aromatase screening assay to be included in the Tier 1 Screening Battery. This assay will detect environmental toxicants that possess the ability to inhibit aromatase activity. Prevalidation studies on recombinant aromatase (WA 2-24) were conducted to optimize the microsomal aromatase assay protocol for human placenta, demonstrate the utility of the microsomal assay to detect known aromatase inhibitors, and compare the performance of a recombinant assay system and the placental microsomal assays. 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 4 is under the control by 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 protocol included as an attachment. The Task 4 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 will be completed by staff at Battelle, WIL and In Vitro. RTI staff will not conduct any experiments on this task but will be involved in the review of the data produced by the other laboratories. RTI will provide human placental microsomes to the other laboratories for use in this task. Battelle/RTI will provide a boilerplate protocol for this Task to the participating laboratories which they will use to prepare their laboratory-specific protocols. These protocols will contain all necessary technical detail for the conduct of this Task. Briefly, the Task requires 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) will be tested in the aromatase assay at 6 concentrations to construct a dose/response curve from which an IC_{50} may be calculated. Control runs also will be 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) will supply 4-OH ASDN to each laboratory as a stock solution and will conduct all necessary pre-assay chemistry activities for 4-OH ASDN.

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

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

Task 5: Conduct Studies with Centrally Prepared Microsomes

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

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

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

Task 6: Prepare Microsomes in Two Participating Laboratories

There are two activities in this Task. The first, to be conducted by Battelle and In Vitro, requires 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 microsomes preparations to the other three participating laboratories. Each laboratory will measure the protein content and (uninhibited) aromatase activity of the microsomal preparations 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 are also included in each assay set to measure full aromatase activity (without any inhibitor added) and background activity (without NADPH co-factor). Battelle's CR will supply the test chemicals to each laboratory as individual stock solutions and will conduct all necessary pre-assay chemistry activities for the test chemicals.

7.0 QUALITY OBJECTIVES AND CRITERIA

The endpoints for WA 4-16 include the aromatase activity measured in the control and inhibitor samples, the inter- and intralaboratory variance, and the IC_{50} and slope values for each inhibitor tested.

7.1 Data Quality Indicators

7.1.1 Precision

The mean positive control activity for each assay/laboratory should be within the overall mean ± 15% 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 positive control activity between and within laboratories should be statistically equivalent at the $p > 0.1$ level. Any modifications to this criterion would be discussed with the sponsor and added to the QAPP by amendment.

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

7.1.2 Bias

The positive control and background activity samples that are run with each assay are used to control for bias. If the control samples for any assay do not meet the precision criteria described above, the assay may be rerun.

7.1.3 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 may 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 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 will be 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 will conduct the aromatase assay including positive control and background activity samples and a series of samples containing varying amounts of a known aromatase inhibitor (4-OH ASDN). The resultant data will be 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

is 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 must be stored at-70 to -80°C and the freezer temperature records must 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, they will be 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 are 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 for freezing. 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, otherwise the samples should be frozen and stored at about -20°C.

Each test 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 Chemical Solutions

The test 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 (positive and negative) are run with each assay. Acceptance criteria and corrective actions where acceptance criteria are not met are described in Section 7. Replicates are 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.

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 may also be recorded manually on data sheets. All data sheets 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 must be annotated to identify samples with the sequential vial number. Procedures for converting CPM data to DPM data must 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 manually into Prism data files for calculation of IC_{50} and undergo a 100% QC check. 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 are 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 occurs as specified in relevant SOPs. The water bath, pipettes, spectrophotometer, and HPLC equipment are 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 must be inspected for conformance to quality requirements prior to use. All use of the product must be prior to the expiration dates, if applicable. Chemicals are 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 include TSAs and ADQs. Performance Evaluations do 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 are 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 may 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 should 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 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 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 are 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 should 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 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 may format an audit report.

The audit report consists of a cover page for study information and additional page(s) with the audit findings. All pages have header information containing the study protocol number, audit report date, and audit type. The audit report date is 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 contains 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 may 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) 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 is 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 forwards the audit report to management for review. Management adds comments as necessary, signs and dates the report and returns it to the EDSP QA team member. The EDSP QA team member assesses the responses and verifies 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 follows the Stop Work Procedure specified in the EDSP QMP (Section 3.3).

20.7 Independent Assessments

The EDSP Battelle QA Manager (QAM), or designee, may conduct an independent TSA and ADQ during the conduct of this work assignment. Typically one independent audit may be conducted during the work assignment. If major deficiencies are uncovered, additional independent audits may 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, has 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 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 is recorded in the sample chain-of-custody records. Compromised samples are not analyzed. The criteria for validating data are those found in Section 7 (Data Quality Objectives).

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 are 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 constitutes part of the ADQ process performed by EDSP QA team members and described earlier. Verification ensures 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 are 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, are 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

DRAFT PROTOCOL FOR TASK 4

PROTOCOL		Page 1 of 15

EPA Contract No.:
EPA Work Assignment No.:

TITLE: Template Protocol for WA 4-16 Task 4: Conduct of the Positive Control Studies in the Participating Laboratories

SPONSOR:

TESTING FACILITY:

PROPOSED EXPERIMENTAL START DATE:

PROPOSED EXPERIMENTAL END DATE:

AMENDMENTS:

Number	Date	Section(s)	Page(s)
1			
2			
3			
4			
5			

Approved By:

Study Director Date

Jerry Johnson, Ph.D, DABT Date
Battelle Work Assignment Leader

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

Reviewed By:

Quality Assurance Specialist Date

Terri Pollock, B.A. Date
EDSP Quality Assurance Manager

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1.0 OBJECTIVES

Task 4: Conduct of the Positive Control Studies in the Participating Laboratories

The objective of this protocol is to describe procedures for conduct of the aromatase assay using placental microsomes. Positive Control Study refers to the use of 4-hydroxyandrostenedione (4-OH ASDN, a known aromatase inhibitor) in the aromatase assay to demonstrate the responsiveness of the assay to aromatase inhibitors.

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.

Route of administration and reason for its choice: The route of administration is not applicable since the test system is a microsome. The method used for treating the microsomes will be to mix the microsomes, reagents, and test article in a common reaction vessel so that microsomal uptake of the test article can be used to evaluate the effect on enzymatic activity.

2.0 MATERIALS RECEIPT AND/OR PREPARATION

A sufficient supply of chemical reagents, radiolabeled and non-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.

Procedure for identification of the test system: 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.

2.1 Substrate

2.1.1 Substrate Name/Supplier

The substrate for the aromatase assay is androstenedione (ASDN). Non-radiolabeled and radiolabeled ASDN will be used. The non-radiolabeled ASDN and the radiolabeled androstenedione ([1β-^{3}H]-androstenedione, [^{3}H]ASDN) will be provided to the laboratories by Battelle's Chemical Respository (CR). The CR will forward all applicable information regarding supplier, lot numbers and reported/measured purity for the substrate to the laboratories and this information will be included in study reports. The radiochemical purity of the [^{3}H]ASDN (of each lot that is used) will be assessed by the lead laboratory as described in Section 2.1.2

2.1.2 Radiochemical Purity (Lead Laboratory only)

The radiochemical purity of the [^{3}H]ASDN will be 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 will be collected using Waters Millennium^{32} Client/Server Chromatography Data System Software, Version 4.0.

The HPLC method uses a Zorbax SB-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 will be monitored by UV absorbance at 240 nm and by a flow-through radiochemical detector. Eluant fractions will be collected manually into vials containing ca. 10 mL Ultima Gold and assayed for radiochemical content by liquid scintillation spectrometry (LSS). A reference standard of nonradiolabeled ASDN will be analyzed by the same method and coelution of the nonradiolabeled and radiolabeled ASDN will be confirmed.

The radiochemical purity of the [^{3}H]ASDN will be greater than approximately 95 percent. If the radiochemical purity is less than 95 percent, then the Sponsor will be notified.

2.1.3 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 (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-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, solution storage conditions to be determined)

2.2.2 Test Substance Formulation and Analysis

Test substance stock solutions will be prepared and analyzed by the CR and distributed to the laboratories. 4-OH ASDN will be formulated in 95 percent 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 95 percent 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 Microsomes

Placental microsomes will be supplied to each laboratory by the lead laboratory. The microsomes must be stored at -70 to -80°C. The approximate protein content of the microsomes will be provided.

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.

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 (about 5-10 passes) prior to use. The microsomes are diluted in buffer (serial dilutions may be necessary) to an approximate protein concentration of 0.008 mg/mL. The addition of 1 mL of that microsome dilution will result in a final approximate protein concentration of 0.004 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. The microsomes should not be left on ice for longer than approximately 2 hours before proceeding with the assay or the microsomal enzyme activity may be decreased. Under no conditions should microsomes be thawed and 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 (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 the microsome preparation will be determined 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. The protein standards will be made from bovine serum albumin (BSA). Protein will be determined by using a DC Protein Assay kit purchased from Bio-Rad (Hercules, CA). 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

The assays will be performed in 13x100 mm test tubes maintained at 37 ± 1°C in a shaking water bath. 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 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 2.0 mL, and the tubes will be incubated for 15 min. The incubations will be stopped by the addition of methylene chloride (2.0 mL); the tubes will be vortex-mixed for ca. 5 s and placed on ice. The tubes 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 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 1. Optimized Aromatase Assay Conditions

Assay factor (units)	Assay Type	
	Human Placental	Human Recombinant
Microsomal Protein (mg/mL)[a]	0.0125	0.004
NADPH (mM)[a]	0.3	0.3
[^{3}H]ASDN (nM)[a]	100	100
Incubation Time (min)	15	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.

Results will be presented as the activity (velocity) of the enzyme reaction. 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 dpm/nmol). 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.

5.0 USE OF THE AROMATASE ASSAY FOR MEASUREMENT OF IC_{50}

5.1 Positive Control Study

Each study will test the response of aromatase activity to the presence of six concentrations of 4-OH ASDN. This study will be conducted in three independent replicates by each participating laboratory. Each concentration of 4-OH ASDN will be run in triplicate tubes in each Study. See Table 2 for the study design. Full enzyme activity control and background

activity samples will be included for each study. Full enzyme activity controls will contain substrate, NADPH, propylene glycol, buffer, vehicle (used for preparation of 4-OH ASDN solutions) and microsomes. Background activity samples contain all full enzyme activity control assay components except NADPH and serve as assay blanks. Four full enzyme activity control and four background activity samples are included with each Study and are treated the same as the other samples. The controls sets will be split so that two tubes (of each full enzyme activity control and background activity samples) are run at the beginning and two at the end of each study 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 2. Positive Control Study Design

Sample type	Repetitions (test tubes)	Description	4-OH ASDN concentration (M final)
Full enzyme activity control	4	Complete assay[a] with inhibitor vehicle control	N/A
Background Activity	4	Complete assay with inhibitor vehicle control omitting NADPH	N/A
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

5.2 Data Analysis and Presentation

The data to be reported will include the following information: assay date and run number, technician, chemical and log chemical concentration, total DPM-background DPM, and % activity. The DPMs for the background tubes should be subtracted from the tubes with Total DPMs to provide DPMs for specific aromatase activity. A spreadsheet will be developed by the lead laboratory that will be used to process the data into a final form for analysis and evaluation. A working document detailing the conversion of the data from DPMs to nmol, as well as the actual methods for calculations of the final aromatase activity will be distributed to the laboratories. This process is briefly summarized below.

The spreadsheet calculates DPM/mL for each aliquot of extracted aqueous incubation mixture and average DPM/mL and total DPM for each aqueous portion (after extraction). Multiplication of the volume (mL) of substrate solution added to the incubation by the substrate solution radiochemical content (DPM/mL) yields the total DPM present in the assay tube at initiation. The total DPM remaining in the aqueous portion after extraction divided by the total DPM present in the assay tube at initiation times 100 yields the percent of the substrate that was converted to product. The total DPM remaining in the aqueous portion after extraction is corrected for background by subtracting the average DPM present in the aqueous portion of the background activity tubes (for that day/assay). This corrected DPM is then converted to nmol product formed by dividing by the substrate specific activity (DPM/nmol). 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 (nmol) by the product of mg microsomal protein used times the incubation time. Average activity in the positive control samples for a given Study is calculated. Percent of control activity remaining in the presence of various inhibitor concentrations is calculated by dividing the aromatase activity at a given concentration by the average positive control activity and multiplying by 100.

IC_{50} will be calculated using Prism (Version 3.02) software to fit the percent of control activity and log concentration data to a curve using the following equation:

$$Y=Bottom + (Top-Bottom)/(1+10^{((LogIC50-X)*HillSlope))}$$

Where: X is the logarithm of concentration
Y is the percent activity
Bottom is the lower plateau
Top is the upper plateau.

The data will be formatted as follows:

- One spreadsheet or table will display the DPMs for all assay tubes, calculations of activity (nmol (mg protein)$^{-1}$min^{-1}) etc.
- Another table will present the results of the analysis of variability of the assay and will include :

(1) the variation between replicates within a single assay,
(2) the day to day (study-to-study) variation, and
(3) technician variation.

- Graphs of activity versus log chemical concentration.

- Table of IC_{50}s by date, run, technician, assay method.

6.0 STATISTICAL ANALYSES

Concentration-response curves will be fitted to describe trends in the aromatase activity percent of control responses. Full enzyme activity control and background activity values will be compared across daily replicate tests for each test substance.

6.1 Concentration Response Fits for the Test Substance

For the test substance multiple independent replicates of the concentration response curve fit will be carried out. The number of replicates will be three.

For each replicate two repeat tubes of the positive controls and the background activity samples will be prepared prior to the preparation of the repetitions of the inhibitor compound and two repeat tubes of the positive controls and the background activity samples will be prepared after the repetitions of the inhibitor compound are prepared. Three repetitions will be prepared for each level of the inhibitor compound (4-OH ASDN).

For each repetition at each level the Excel database spreadsheet will include total DPMs per tube and total aromatase activity per tube. The aromatase activity is calculated as the DPM, normalized by the specific activity of the [^{3}H]ASDN, the mg of protein of the aromatase, and the incubation time. The aromatase activity is corrected for the background DPMs, as measured by the average of the background activity tubes. Thus the average aromatase activity across the four background activity repeat tubes must necessarily equal 0 within each replicate. The total DPM values are not corrected for background.

For each repetition within each inhibitor concentration, percent of control activity is determined by dividing the aromatase activity for that tube by the average positive control activity 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 individual experimental percent of control activity values will sometimes extend below 0% or above 100%. Thus upper and lower response curve plateaus need to be included in the response curve models,

Concentration response trend curves will be fitted to the percent of control activity values within each of the repeat tubes at each inhibitor concentration. Concentration is expressed on the log scale. In agreement with past convention, logarithms will be common logarithms (i.e

base 10). Let X denote the logarithm of the concentration of inhibitor compound (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
T ≡ upper plateau of the concentration response curve
B ≡ lower plateau of the concentration response curve
DAVG ≡ average DPMs across the repeat tubes with the same inhibitor concentration
β ≡ slope of the concentration response curve (β will be negative)
$\mu \equiv \log_{10}IC_{50}$ (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

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

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 response curve will be fitted by weighted least squares nonlinear regression analysis with weights equal to 1000/DAVG. Model fits will be carried out using Prism software (Version 3 or higher).

The concentration response fits will be carried out for each replicate test within each test compound. 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 an inhibitor compound will be the 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 can be calculated based on a one-way random effects analysis of variance model fit.

6.2 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 inhibitor compound 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 inhibitor concentration on the same plot. Plotting symbols will distinguish among replicates. The fitted concentration response curve for each replicate will be superimposed on the plot. On a separate plot the average percent of control values for each replicate will be plotted versus logarithm of inhibitor compound concentration. The average concentration response curve across replicates will be superimposed on the same plot with 95 percent confidence intervals on average control values at each observed concentration. Replicate-to-

replicate variation will be treated as a random effect for purposes of calculating confidence intervals.

For each replicate treat (β, μ) as a random variable with mean (β_{avg}, μ_{avg}) and covariance $\Sigma_{(\beta, \mu)}$ across replicates. Let B_{avg}, T_{avg} denote the average bottom and top across the replicates. Let

$$Z \equiv (Y - B_{avg})/(T_{avg} - B_{avg})$$

$$L \equiv \log_{10}(Z/(1 - Z)).$$

The average response curve is expressed as

$$L \equiv \beta_{avg}(\mu_{avg} - X)$$

with approximate standard errors of prediction of L at a given X based on $\Sigma_{(\beta, \mu)}$ and propagation of errors. These are used to calculate approximate confidence intervals for predictions at each X. The linearized response curve and associated confidence intervals are back transformed to yield the response curve in terms of percent of control, Y

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

Slope (β) and $\log_{10}IC_{50}$ (μ) will also be compared across replicates based on random effects analysis of variance, treating the replicates as random effects. β and μ are estimated, separately within each replicate, and plotted along with the average and associated 95% confidence interval across replicates.

6.3 Negative and Positive Control Values Across Replicates

Within each replicate, quadruplicate repetitions will be made of the background activity tubes and the 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 constant 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 combined across replicates and expressed as percent of (positive) control activity. The average of the four background activity samples within a replicate must necessarily be 0 and the average of the four positive controls within a replicate must necessarily be 100. The two beginning controls and the two end controls will be plotted by replicate with plotting symbol distinguishing between beginning and end, and with reference line 0% (background activity) or 100% (positive control) respectively. These plots will display the extent of consistency across replicates with respect to average value and variability and will provide comparisons of beginning versus end of each replicate. Two-way analysis of variance will be carried out, separately for the positive control tubes and the background activity tubes. The factors in the analysis of variance will be replicate, portion (beginning or end), replicate by portion interaction. The error corresponds to repetition within replicate and portion. The response will be percent of control aromatase activity. If the daily replicates are in control the

portion main effect and portion by replicate interaction should be nonsignificant. Note that the replicate effects will not be estimable because of the constrained totals within each replicate. For purposes of evaluation replicate will be treated as a fixed effect. If 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, adjusting for simultaneity by Scheffe's method. The portion effect within each replicate and the portion effect averaged across replicates, and associated 95% confidence intervals, will be presented graphically.

6.4 Variability Assessment

For the inhibitor test compound variability among replicates and variability among repetitions within replicates will be estimated and assessed for statistical significance. The response will be aromatase activity. These analyses will treat inhibitor concentration as a classification variable and will include both the positive and background activity groups. The factors in the mixed effects analysis of variance will be concentration group (including positive and background activity groups), replicate, replicate by concentration interaction, and residual variation. Residual variation corresponds to repetition within replicate and concentration. Inhibitor concentration will be treated as a fixed effect. Replicate and replicate by concentration interaction will be treated as random effects. The analysis of variance fit will incorporate weights. The weight for responses in each concentration group will be based on the average of the DPMs across all the replicates and repetitions within replicates associated with that concentration group. The weight for each concentration group will be 1000/[Average DPM].

Normal probability plots will be prepared to identify outlying replicates or repetitions. Deviations of average within replicate from average across replicates within that concentration group will be ordered and plotted on a normal probability scale. The differences will be normalized by $[\text{Average DPM}]^{1/2}$ for their concentration group to adjust for differing variability across concentration groups. Deviations of repetitions from average across repetitions within replicate and concentration group will be ordered and plotted on a normal probability scale. The differences will be normalized by $[\text{Average DPM}]^{1/2}$ for their concentration group to adjust for differing variability across concentration groups.

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

6.6 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 (e.g. outlying laboratories), the extent of laboratory-to-laboratory variation, and overall consensus estimates among the laboratories.

The results of the intra-laboratory analyses will be concentration response curve fits associated with the positive control inhibitor 4-OH-ASDN. For each inhibitor compound they will also characterize variability among replicates and variability among repetitions within replicates.

The inter-laboratory analysis will be based on the IC_{50} and slope parameters of the concentration response curve fits and the replicate-to-replicate and repetition within replicate components of variation. The objectives of the inter-laboratory statistical analysis are to:

- Determine the average values and variability among laboratories with respect to the within-laboratory parameters mentioned above
- Determine the coefficient of variation among laboratories for each of the within-laboratory parameters mentioned above
- Estimate the ratio of within laboratory variation to among laboratory variation for each of the parameters
- Identify outlying laboratories, if any
- Assess the extent of variation across the inhibitor compounds of the coefficients of variation among laboratories for each of the inhibitor compounds.

For each endpoint a one-way mixed effects analysis of variance with heterogeneous variances among the participating laboratories will be fitted to the summary responses within laboratories. Laboratory will be treated as a random effect. Weights will incorporate laboratory-to-laboratory variation and within laboratory variation. The within laboratory variation will be the square of the standard error reported by each laboratory. The analysis of variance will provide an estimated weighted average effect across all laboratories and its associated standard error as well as an estimate of the laboratory-to-laboratory component of variation. The mixed effects analysis of variance will be carried out using PROC MIXED in the SAS statistical analysis system.

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 will be prepared for this study. This study will be conducted in compliance with the Federal Register, 40 CFR Part 160, Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) Good Laboratory Practice Standards.

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
- Quality Assurance Project Plan (QAPP) and any Amendments
- List of any QAPP Deviations

Appendix D

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

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
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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

Quality Assurance Statement

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

Sponsor: Battelle Memorial Institute

Study Code: An05-928

Protocol Number: RTI-928-AN

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

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

K. Collier
Quality Assurance Specialist

9/28/2005
Date

Approval:

Carrie Ingalls
Quality Assurance Assistant Manager

09/28/2005
Date

Introduction

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

Materials and Methods

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

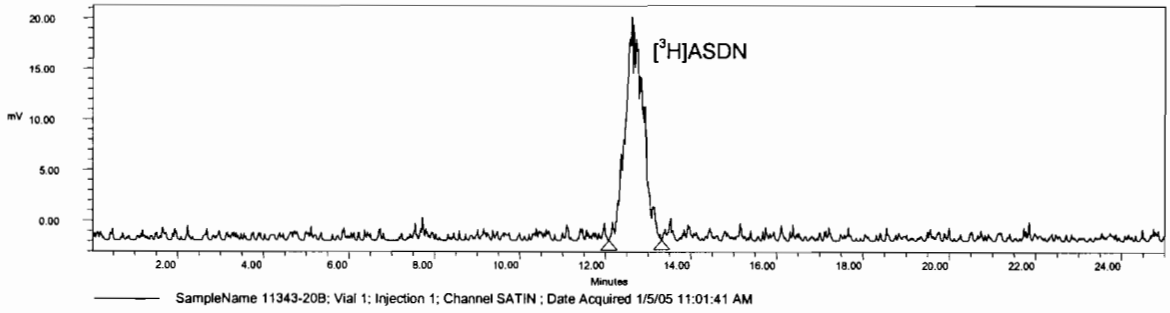
The radiochemical purity of the [^{3}H]ASDN (1:100 dilution in ethanol) was determined using high performance liquid chromatography (HPLC) and liquid scintillation counting. The HPLC system consists of a Waters 2690 Separations Module, a Waters 2487 Dual λ Absorbance Detector and a β-RAM Model 3 flow-through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

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

Results

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

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



Conclusion

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

Appendix E

Chemistry Reports (Sponsor-Provided Data)

Battelle
The Business *of* Innovation

ANALYTICAL CHEMISTRY ACTIVITIES REPORT

4-HYDROXYANDROSTENEDIONE (4-OH ASDN)

CAS No.: 566-48-3	Lot No.: 063K4069 (Sigma Aldrich)
Receipt Date: 10/22/04	Amount Received: 3.1 g
Appearance: Solid	Vendor Purity: 99% by TLC
Storage Conditions (@ Battelle): Refrigerated (~5°C)	

STRUCTURE:	Mol. Wt.:	Mol. Formula:
	302.41 g/mol	$C_{19}H_{26}O_3$

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 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
Dispensing*	12/ 2/2004	12/ 2/2004
Formulation analysis*	12/ 2/2004	12/ 2/2004
Formulation preparation*	12/ 2/2004	12/ 2/2004
Audit analytical report	7/26/2005	7/26/2005
Audit study file	7/26/2005	7/26/2005
Audit analytical report		

* 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 Date

EXECUTIVE SUMMARY

The title compound, 4-hydroxyandrostenedione, was analyzed in support of the 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 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 20-mL amber glass bottle of 4-hydroxyandrostenedione, 063K4069, was received from the repository at Battelle's Marine Science 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).

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	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

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-hydroxyandrostenedione (0.30200 ± 0.0.03020 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 ~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 ± 0.0.00302 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 ~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).

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, 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 1 minutes, increase at 15°C/minute to 320°C
Detector Type	Flame Ionization
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 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 of benzophenone was added to a 25-mL volumetric flask. 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 25 ± 1.0 mg of 4-hydroxyandrostenedione (4-OH ASDN) each into individual 25-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 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 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 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 correct 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 at each concentration.

4.3.4 Results

Specificity is shown by representative overlaid chromatograms from high and low 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.

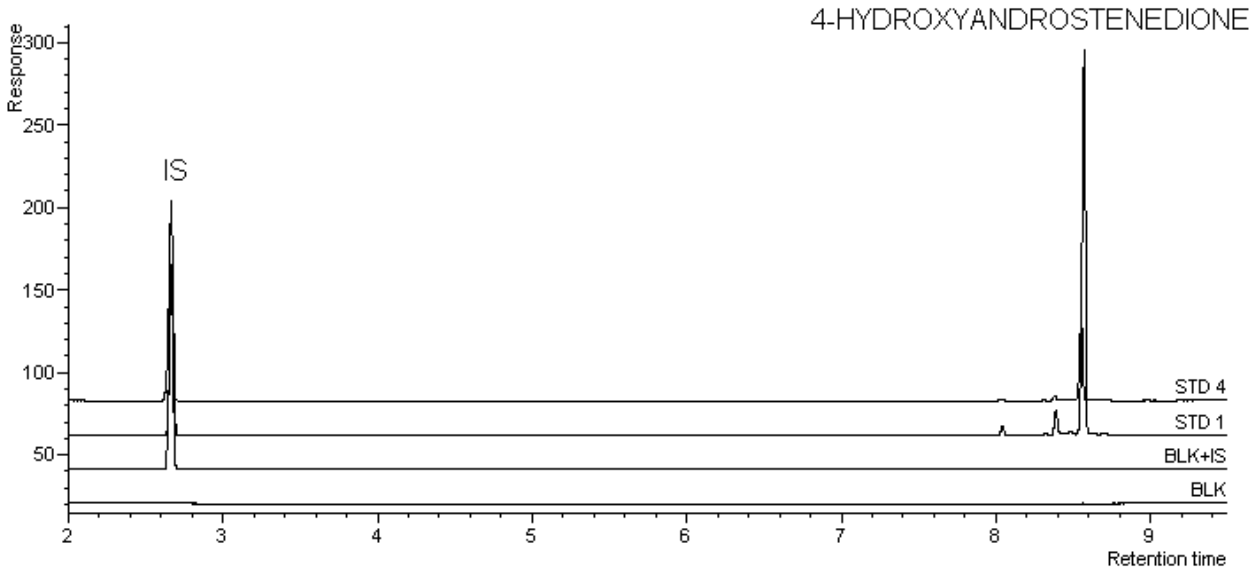


Figure 2 – Representative Overlaid Chromatograms from a High and Low Vehicle/Calibration Standard, Blank with Internal Standard, and Blank from the Validation (Shown Top to Bottom)

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	298.4	NA	NA	NA	-2.9	NA
202.5	198.8	NA	NA	NA	-1.9	NA
99.38	100.7	100.4	0.4	0.4	1.3	1.0
	99.98				0.5	
	100.5				1.1	

The method validation sensitivity was 1.2 µg/mL, the limit of detection (LOD), which is defined as three times the standard deviation of the low vehicle/calibration standard. This is equivalent to a formulation concentration of 12 µg/mL when a formulation is diluted 1 to 10 for analysis. The limit of quantitation (LOQ), was 4.2 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), Day 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 mintues and allowed to cool to room temperature. 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 flask was diluted to ~80% volume with 95% ethanol, sealed and mixed well. The flask was 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.

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

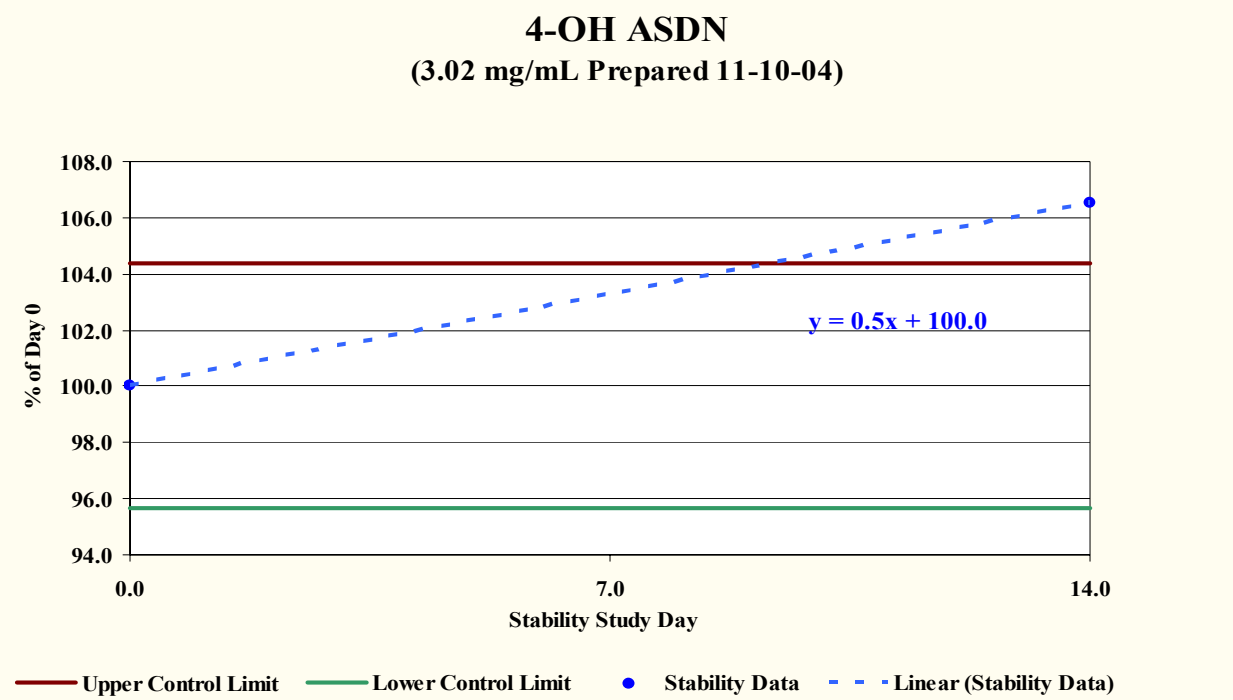
The results from the storage stability study are shown in Table 5 and presented in control chart format in Figure 3.

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±0.3
11/10/04	11/24/04	14	3.080	3.085	3.149	3.080±0.071	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	3.216	3.125±0.095	103.8±3.1
12/2/04	5/24/05	173	3.126	3.142	3.129	3.133±0.008	104.1±0.03

For the sample prepared 11/10/04, the pooled relative standard deviation 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 12/2/04, the pooled relative standard deviation 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.



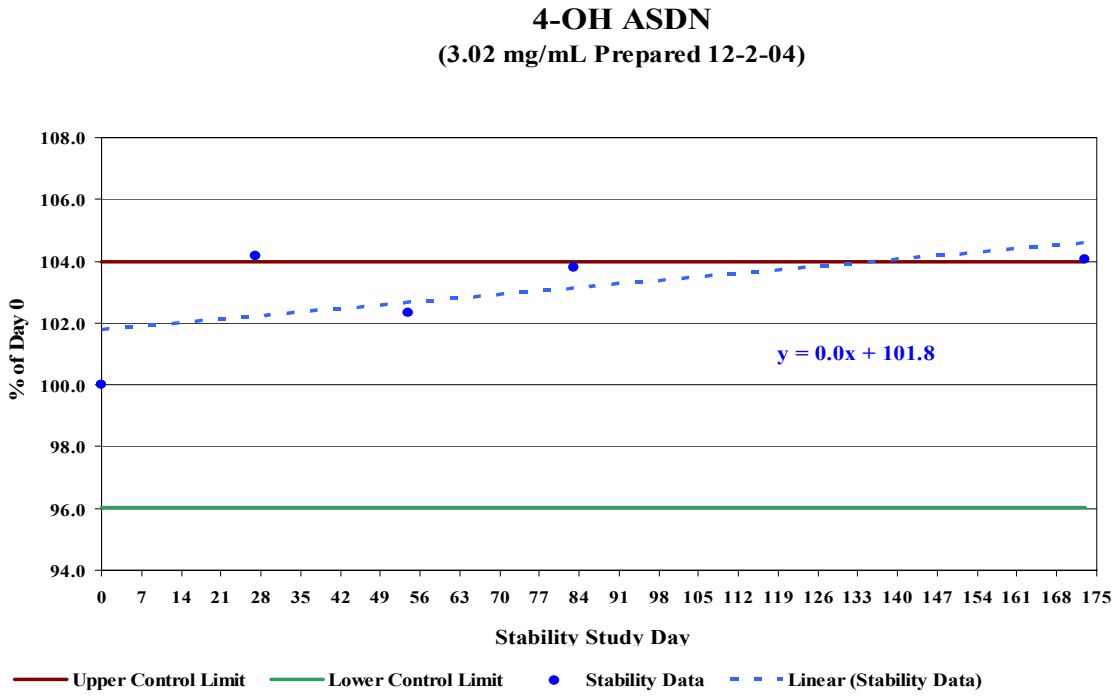


Figure 3 – Control Charts for the Storage Stability Studies

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 11/10/04). Concentrations for Day 54 and 83 samples were within the upper and lower significance levels and Day 27 and Day 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 12/2/04, 1/25/05, 3/21/05 and 6/27/05 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 flask was diluted to ~80% volume with 95% ethanol, sealed and mixed well. The flask was 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

Auto injector 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 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 relative error 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 relative error 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 relative standard deviation 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 internal standard and a blank presented in Figure 4.

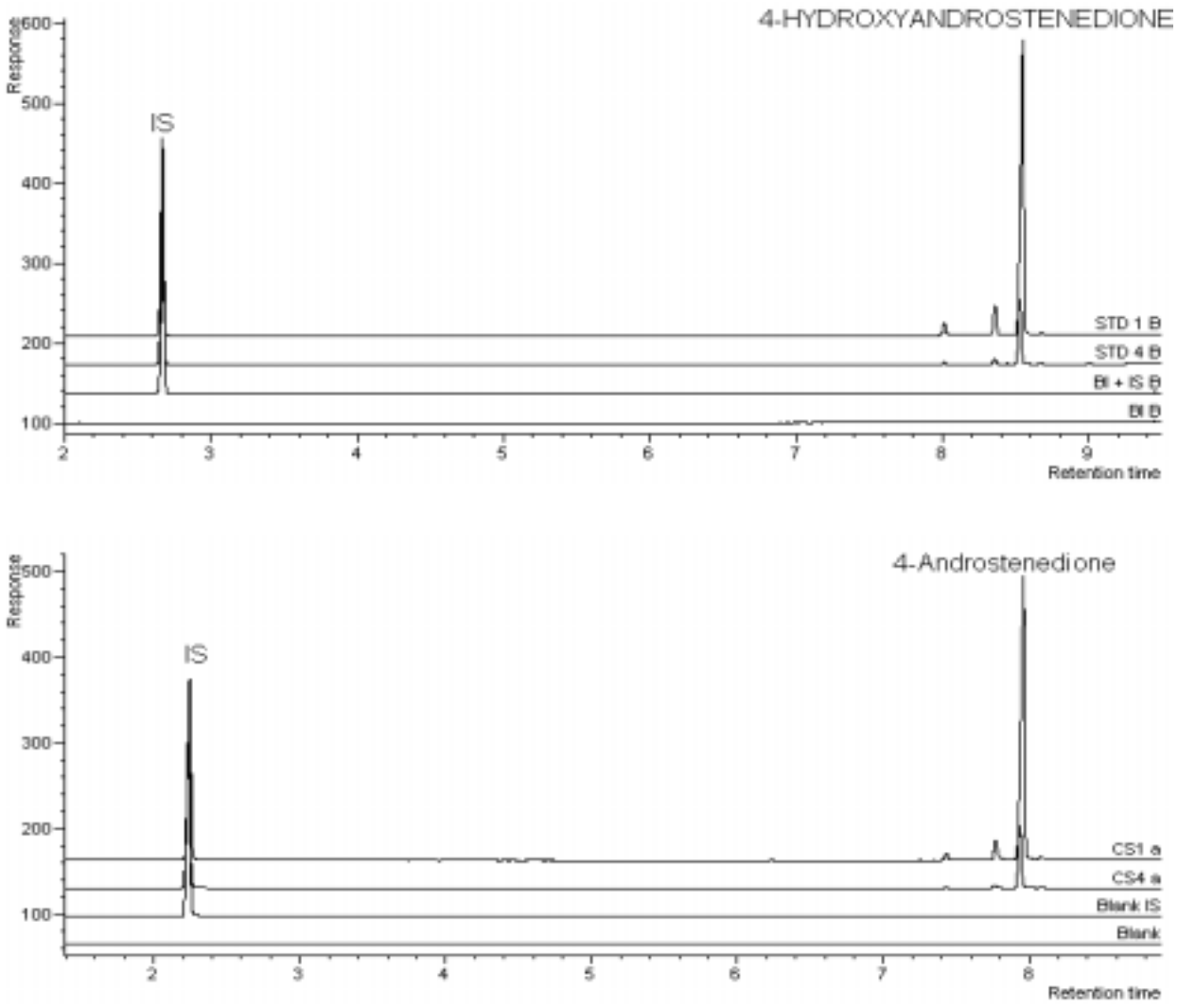


Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from Formulation Analysis Batch 1-ASDN and Batch 2-ASDN (Shown Top to Bottom)

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	1.5	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 relative standard deviation were within acceptance criteria. Therefore the formulations were suitable for use.

7 ACKNOWLEDGMENTS

Analytical support for this work was provided by Sandy Runyon, Chris 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. Assessment of the overall quality of the data and report was performed by Hillary Flory.

Appendix F

Individual Replicate Spreadsheets (1, 2 and 3)

Aromatase Assay Spreadsheet

Assay Date	12/13/04	Test Chemical ID	4-OH ASDN	# Concentrations tested	6		
Technician ID	JG	Replicate #	1	Microsome type	placental	Microsome ID	MET-0255A Vial 2

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.		
1	0.0196	31564	1610408		
2	0.0197	32489.06	1649191		
3	0.0198	32964.07	1664852		
4	0.0198	32794.91	1656309		
5	0.0198	33385.82	1686153		
			Average DPM/g soln		1653382
			SD		27741
			CV		1.68
			µCi/g soln		0.745

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	8.167 g
Mass of dilution B used in substrate prep	4.5964 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.579686 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00843 µg/g soln.
µg/g soln.

a. µCi/g soln	0.745
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.

= 0.579686 + 0.00843
= 0.588116 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.266 µCi/µg ASDN

805162 dpm/nmol

Assay Date	12/13/04	Test Chemical ID	4-OH ASDN	# Concentrations tested		6				
Technician ID	JG	Replicate #	1	Microsome type		placental	Microsome ID	MET-0255A Vial 2		
								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	1.42	0.95	0.71	0.47	0.24	0.12	0	23.649	10	
	0.3869	0.2942	0.2258	0.1655	0.0953	0.0614	0.0265			
	0.4076	0.2945	0.2341	0.1737	0.0974	0.0662	0.0272			
	0.4129	0.2961	0.2360	0.1775	0.1020	0.0612	0.0238			
Samples:										
		0.1577	0.1066							
		0.1714	0.1004							
		0.1555	0.1010							

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
1.42	60	100	0.00142	25	0.0355	0.402	0.377	0.0345
0.95	40	100	0.00095	25	0.0236	0.295	0.269	0.0243
0.71	30	100	0.00071	25	0.0177	0.232	0.206	0.0183
0.47	20	100	0.00047	25	0.0118	0.172	0.146	0.0126
0.24	10	100	0.00024	25	0.0059	0.098	0.072	0.0056
0.12	5.2	100	0.00012	25	0.0031	0.063	0.037	0.0023

Variables	Regression results	
m, b	0.095	-0.001
se_m, se_b	0.003	0.001
r^2, se_y	0.996	0.001
F, df	933	4
ss_{reg}, ss_{resid}	0.001	0.000

Regression results are calculated using the function LINEST

Blank 0.026

r^2= 0.996
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
				100	5000			
				100	5000			
				100	5000			
0.158	0.132	0.011	25	1	1	0.000	0.000	0.465
0.171	0.146	0.013	25	1	1	0.001		
0.156	0.130	0.011	25	1	1	0.000		
0.107	0.081	0.006	25	100	5000	0.013	0.012	12.092
0.100	0.075	0.006	25	100	5000	0.012		
0.101	0.075	0.006	25	100	5000	0.012		

Assay Date	12/13/04	Test Chemical ID	4-OH ASDN		# Concentrations tested		6		
Technician ID	JG	Replicate #		1	Microsome type	placental	Microsome ID	MET-0255A Vial 2	

								Protein stock (mg/10 mL)	Protein stock ID
Standards:	1.5	1	0.75	0.5	0.25	0.13	Blk		
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	12/13/04	Test Chemical ID	4-OH ASDN	# Concentrations tested	6	Microsome type	placental	Microsome ID	MET-0255A	Technician ID	JG	Replicate #	1

Microsome Dilution Details

Dilution A	0.08	mL microsome Stock used
	3.98	mL total volume
	49.75	dilution factor
Dilution B	3	mL microsome Dilution A used
	30	mL total volume
	10	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	497.5	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	12.092
Protein Concentration (dilution added to assay, mg/mL):	0.024306

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	12/13/04	Test Chemical ID	4-OH ASDN	# Concentrations tested	6	Microsome type	placental	Microsome ID	MET-0255A Vial 2	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	4886.21	9772.42	9193.94	18387.88	0.1	165338	11.12	18275	0.0227	1	0.012	15	0.0623
			0.5	2	4307.73	8615.46			0.1					1		15	
	2	2	0.5	1	6204.92	12409.84	12621.29	25242.58	0.1	165338	15.27	25130	0.0312	1	0.012	15	0.0856
			0.5	2	6416.37	12832.74			0.1					1		15	
	3	2	0.5	1	6494.33	12988.66	12835.81	25671.62	0.1	165338	15.53	25559	0.0317	1	0.012	15	0.0871
			0.5	2	6341.48	12682.96			0.1					1		15	
	4	2	0.5	1	6072.04	12144.08	12377.77	24755.54	0.1	165338	14.97	24643	0.0306	1	0.012	15	0.0839
			0.5	2	6305.73	12611.46			0.1					1		15	
Background control	1	2	0.5	1	23.65	47.3	57.13	114.26	0.1	165338	0.07	2	0.0000	1	0.012	15	0.0000
			0.5	2	33.48	66.96			0.1					1		15	
	2	2	0.5	1	26.44	52.88	50.76	101.52	0.1	165338	0.06	-11	0.0000	1	0.012	15	0.0000
			0.5	2	24.32	48.64			0.1					1		15	
	3	2	0.5	1	31.34	62.68	67.23	134.46	0.1	165338	0.08	22	0.0000	1	0.012	15	0.0001
			0.5	2	35.89	71.78			0.1					1		15	
	4	2	0.5	1	24.06	48.12	49.79	99.58	0.1	165338	0.06	-13	0.0000	1	0.012	15	0.0000
			0.5	2	25.73	51.46			0.1					1		15	
Positive control	1	2	0.5	1					0.1	165338		#VALUE!	#VALUE!	1	0.012	15	#VALUE!
			0.5	2					0.1					1		15	
	2	2	0.5	1					0.1	165338		#VALUE!	#VALUE!	1	0.012	15	#VALUE!
			0.5	2					0.1					1		15	
	3	2	0.5	1					0.1	165338		#VALUE!	#VALUE!	1	0.012	15	#VALUE!
			0.5	2					0.1					1		15	
	4	2	0.5	1					0.1	165338		#VALUE!	#VALUE!	1	0.012	15	#VALUE!
			0.5	2					0.1					1		15	
Negative Control	1	2	0.5	1					0.1	165338		#VALUE!	#VALUE!	1	0.012	15	#VALUE!
			0.5	2					0.1					1		15	
	2	2	0.5	1					0.1	165338		#VALUE!	#VALUE!	1	0.012	15	#VALUE!
			0.5	2					0.1					1		15	
	3	2	0.5	1					0.1	165338		#VALUE!	#VALUE!	1	0.012	15	#VALUE!
			0.5	2					0.1					1		15	
	4	2	0.5	1					0.1	165338		#VALUE!	#VALUE!	1	0.012	15	#VALUE!
			0.5	2					0.1					1		15	
4-OH ASDN	1-1	2	0.5	1	308.36	616.72	610.89	1221.78	0.1	165338	0.74	1109	0.0014	1	0.012	15	0.0038
			0.5	2	302.53	605.06			0.1					1		15	
	1-2	2	0.5	1	310.18	620.36	630.96	1261.92	0.1	165338	0.76	1149	0.0014	1	0.012	15	0.0039
			0.5	2	320.78	641.56			0.1					1		15	
	1-3	2	0.5	1	331.39	662.78	658.9	1317.8	0.1	165338	0.80	1205	0.0015	1	0.012	15	0.0041
			0.5	2	327.51	655.02			0.1					1		15	
	2-1	2	0.5	1	1871.61	3743.22	3764.37	7528.74	0.1	165338	4.55	7416	0.0092	1	0.012	15	0.0253
			0.5	2	1892.76	3785.52			0.1					1		15	
	2-2	2	0.5	1	1885.61	3771.22	3839.1	7678.2	0.1	165338	4.64	7566	0.0094	1	0.012	15	0.0258
			0.5	2	1953.49	3906.98			0.1					1		15	
	2-3	2	0.5	1	1949.88	3899.76	3923.29	7846.58	0.1	165338	4.75	7734	0.0096	1	0.012	15	0.0263
			0.5	2	1973.41	3946.82			0.1					1		15	
	3-1	2	0.5	1	2910.51	5821.02	5960.58	11921.16	0.1	165338	7.21	11809	0.0147	1	0.012	15	0.0402
			0.5	2	3050.07	6100.14			0.1					1		15	
	3-2	2	0.5	1	2961.84	5923.68	6062.74	12125.48	0.1	165338	7.33	12013	0.0149	1	0.012	15	0.0409
			0.5	2	3100.9	6201.8			0.1					1		15	
	3-3	2	0.5	1	2739.66	5479.32	5593.27	11186.54	0.1	165338	6.77	11074	0.0138	1	0.012	15	0.0377
			0.5	2	2853.61	5707.22			0.1					1		15	
	4-1	2	0.5	1	3992.44	7984.88	8045.59	16091.18	0.1	165338	9.73	15979	0.0198	1	0.012	15	0.0544
			0.5	2	4053.15	8106.3			0.1					1		15	
	4-2	2	0.5	1	4002.54	8005.08	8234.44	16468.88	0.1	165338	9.96	16356	0.0203	1	0.012	15	0.0557
			0.5	2	4231.9	8463.8			0.1					1		15	
	4-3	2	0.5	1	3941.99	7883.98	8120.92	16241.84	0.1	165338	9.82	16129	0.0200	1	0.012	15	0.0549
			0.5	2	4178.93	8357.86			0.1					1		15	
	5-1	2	0.5	1	5086.87	10173.74	10384.56	20769.12	0.1	165338	12.56	20657	0.0257	1	0.012	15	0.0704
			0.5	2	5297.69	10595.38			0.1					1		15	
	5-2	2	0.5	1	5230.47	10460.94	10549.24	21098.48	0.1	165338	12.76	20986	0.0261	1	0.012	15	0.0715
			0.5	2	5318.77	10637.54			0.1					1		15	
	5-3	2	0.5	1	4945.76	9891.52	10216.74	20433.48	0.1	165338	12.36	20321	0.0252	1	0.012	15	0.0692
			0.5	2	5270.98	10541.96			0.1					1		15	
	6-1	2	0.5	1	5968.02	11936.04	12103.54	24207.08	0.1	165338	14.64	24095	0.0299	1	0.012	15	0.0821
			0.5	2	6135.52	12271.04			0.1					1		15	
	6-2	2	0.5	1	6159.54	12319.08	12446.38	24892.76	0.1	165338	15.06	24780	0.0308	1	0.012	15	0.0844
			0.5	2	6286.84	12573.68			0.1					1		15	
	6-3	2	0.5	1	6111.52	12223.04	12453.15	24906.3	0.1	165338	15.06	24794	0.0308	1	0.012	15	0.0845
			0.5	2	6341.63	12683.26			0.1								

Assay Date	12/13/04	Test Chemical ID	4-OH ASDN	# Concentrations tested	6	Microsome type	placental	Microsome ID	MET-0255A Via	Technician ID	JG	Replicate #	1

Control Type	Portion	Average	SD
Full activity	Beginning	0.0739	0.0165
Full activity	End	0.0855	0.0022
Full activity	Overall	0.0797	0.0117
Background	Beginning	0.0000	3.06885E-05
Background	End	0.0000	8.40199E-05
Background	Overall	0.0000	5.46762E-05
Positive	Beginning	#VALUE!	#VALUE!
Positive	End	#VALUE!	#VALUE!
Positive	Overall	#VALUE!	#VALUE!
Negative	Beginning	#VALUE!	#VALUE!
Negative	End	#VALUE!	#VALUE!
Negative	Overall	#VALUE!	#VALUE!

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
4-OH ASDN	1	1	1.00E-06	-6.00	0.0038
4-OH ASDN	1	2	1.00E-06	-6.00	0.0039
4-OH ASDN	1	3	1.00E-06	-6.00	0.0041
4-OH ASDN	2	1	1.00E-07	-7.00	0.0253
4-OH ASDN	2	2	1.00E-07	-7.00	0.0258
4-OH ASDN	2	3	1.00E-07	-7.00	0.0263
4-OH ASDN	3	1	5.00E-08	-7.30	0.0402
4-OH ASDN	3	2	5.00E-08	-7.30	0.0409
4-OH ASDN	3	3	5.00E-08	-7.30	0.0377
4-OH ASDN	4	1	2.50E-08	-7.60	0.0544
4-OH ASDN	4	2	2.50E-08	-7.60	0.0557
4-OH ASDN	4	3	2.50E-08	-7.60	0.0549
4-OH ASDN	5	1	1.00E-08	-8.00	0.0704
4-OH ASDN	5	2	1.00E-08	-8.00	0.0715
4-OH ASDN	5	3	1.00E-08	-8.00	0.0692
4-OH ASDN	6	1	1.00E-09	-9.00	0.0821
4-OH ASDN	6	2	1.00E-09	-9.00	0.0844
4-OH ASDN	6	3	1.00E-09	-9.00	0.0845

Percent of control values				
	Log[test	Replicate		
Level	substance]	1	2	3
1	-6.00	4.74	4.91	5.15
2	-7.00	31.69	32.33	33.05
3	-7.30	50.46	51.33	47.32
4	-7.60	68.28	69.89	68.92
5	-8.00	88.27	89.68	86.83
6	-9.00	102.96	105.89	105.95

Aromatase Assay Spreadsheet

Assay Date	12/17/04	Test Chemical ID	4-OH ASDI	# Concentrations tested	6		
Technician ID	JG	Replicate #	2	Microsome type	placental	Microsome ID	MET-0255A Vial 4

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.		
1	0.0194	32295.11	1664696		
2	0.0200	32744.5	1637225		
3	0.0198	33439.01	1688839		
4	0.0196	33782.2	1723582		
5	0.0198	33050	1669192		
			Average DPM/g soln		1676707
			SD		32031
			CV		1.91
			µCi/g soln		0.755

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	8.1367	g
Mass of dilution B used in substrate prep	4.551	g
Concentration of nonradiolabeled ASDN in substrate soln.	0.570504	µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00855 µg/g soln.
µg/g soln.

a. µCi/g soln 0.755
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.

= 0.570504 + 0.00855
= 0.579054 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.304 µCi/µg ASDN

829299 dpm/nmol

Assay Date	12/17/04	Test Chemical ID	4-OH ASDN	# Concentrations tested	6					
Technician ID	JG	Replicate #	2	Microsome type	placental	Microsome ID	MET-0255A Vial 4			
								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	1.42	0.95	0.71	0.47	0.24	0.12	0			
	0.3976	0.2964	0.2276	0.1621	0.1005	0.0613	0.0226	23.649	10	
	0.4271	0.3132	0.2329	0.1657	0.1055	0.0666	0.0233			
	0.4124	0.3106	0.2407	0.1651	0.1093	0.0734	0.0234			
Samples:										
		0.1595	0.1126							
		0.1598	0.1033							
		0.1599	0.1017							

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	
1.42	60	100	0.00142	25	0.0355	0.412	0.389	0.0346	m, b	0.093	-0.002
0.95	40	100	0.00095	25	0.0236	0.307	0.284	0.0248	se_m, se_b	0.003	0.001
0.71	30	100	0.00071	25	0.0177	0.234	0.211	0.0180	r^2, se_y	0.997	0.001
0.47	20	100	0.00047	25	0.0118	0.164	0.141	0.0116	F, df	1150	4
0.24	10	100	0.00024	25	0.0059	0.105	0.082	0.0061	ss_{reg}, ss_{resid}	0.001	0.000
0.12	5.2	100	0.00012	25	0.0031	0.067	0.044	0.0026			

Regression results are calculated using the function LINEST

Blank 0.023

r^2= 0.997
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
				100	5000			
				100	5000			
				100	5000			
0.160	0.136	0.011	25	1	1	0.000	0.000	0.447
0.160	0.137	0.011	25	1	1	0.000		
0.160	0.137	0.011	25	1	1	0.000		
0.113	0.090	0.007	25	100	5000	0.014	0.012	12.343
0.103	0.080	0.006	25	100	5000	0.012		
0.102	0.079	0.006	25	100	5000	0.012		

Assay Date	12/17/04	Test Chemical ID	4-OH ASDN		# Concentrations tested	6			
Technician ID	JG	Replicate #	2		Microsome type	placental	Microsome ID	MET-0255A Vial 4	
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	12/17/04	Test Chemical ID	4-OH ASDN	# Concentrations tested	6	Microsome type	placental	Microsome ID	MET-0255	Technician ID	JG	Replicate #	2

Microsome Dilution Details

Dilution A	0.08	mL microsome Stock used
	3.98	mL total volume
	49.75	dilution factor
Dilution B	3	mL microsome Dilution A used
	30	mL total volume
	10	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	497.5	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	12.343
Protein Concentration (dilution added to assay, mg/mL):	0.02481

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	12/17/04	Test Chemical ID	4-OH ASDN	# Concentrations tested	6	Microsome type	placental	Microsome ID	MET-0255A Vial 4	Technician ID	JG	Replicate #	2

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate nmol ^3H_2O formed				Incubation time (min)	Aromatase activity (nmol estrogen formed/mg protein/min
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)		
Full activity control	1	2	0.5	1	5600.53	11201.06	11020.58	22041.16	0.1	167671	13.15	21963	0.0265	1	0.012	15	0.0712
			0.5	2	5420.05	10840.1			0.1					1		15	
	2	2	0.5	1	6240.39	12480.78	12695.78	25391.56	0.1	167671	15.14	25313	0.0305	1	0.012	15	0.0820
			0.5	2	6455.39	12910.78			0.1					1		15	
	3	2	0.5	1	5816.69	11633.38	11716.4	23432.8	0.1	167671	13.98	23354	0.0282	1	0.012	15	0.0757
			0.5	2	5899.71	11799.42			0.1					1		15	
	4	2	0.5	1	6251.38	12502.76	12311.38	24622.76	0.1	167671	14.69	24544	0.0296	1	0.012	15	0.0795
			0.5	2	6060	12120			0.1					1		15	
Background control	1	2	0.5	1	22.13	44.26	42.5	85	0.1	167671	0.05	6	0.0000	1	0.012	15	0.0000
			0.5	2	20.37	40.74			0.1					1		15	
	2	2	0.5	1	16.65	33.3	40.78	81.56	0.1	167671	0.05	3	0.0000	1	0.012	15	0.0000
			0.5	2	24.13	48.26			0.1					1		15	
	3	2	0.5	1	20.44	40.88	35.53	71.06	0.1	167671	0.04	-7	0.0000	1	0.012	15	0.0000
			0.5	2	15.09	30.18			0.1					1		15	
	4	2	0.5	1	19.63	39.26	38.25	76.5	0.1	167671	0.05	-2	0.0000	1	0.012	15	0.0000
			0.5	2	18.62	37.24			0.1					1		15	
Positive control	1	2	0.5	1					0.1	167671		#VALUE!	#VALUE!	1	0.012	15	#VALUE!
			0.5	2					0.1					1		15	
	2	2	0.5	1					0.1	167671		#VALUE!	#VALUE!	1	0.012	15	#VALUE!
			0.5	2					0.1					1		15	
	3	2	0.5	1					0.1	167671		#VALUE!	#VALUE!	1	0.012	15	#VALUE!
			0.5	2					0.1					1		15	
	4	2	0.5	1					0.1	167671		#VALUE!	#VALUE!	1	0.012	15	#VALUE!
			0.5	2					0.1					1		15	
Negative Control	1	2	0.5	1					0.1	167671		#VALUE!	#VALUE!	1	0.012	15	#VALUE!
			0.5	2					0.1					1		15	
	2	2	0.5	1					0.1	167671		#VALUE!	#VALUE!	1	0.012	15	#VALUE!
			0.5	2					0.1					1		15	
	3	2	0.5	1					0.1	167671		#VALUE!	#VALUE!	1	0.012	15	#VALUE!
			0.5	2					0.1					1		15	
	4	2	0.5	1					0.1	167671		#VALUE!	#VALUE!	1	0.012	15	#VALUE!
			0.5	2					0.1					1		15	
4-OH ASDN	1-1	2	0.5	1	306.46	612.92	602.42	1204.84	0.1	167671	0.72	1126	0.0014	1	0.012	15	0.0036
			0.5	2	295.96	591.92			0.1					1		15	
	1-2	2	0.5	1	285.73	571.46	573.15	1146.3	0.1	167671	0.68	1068	0.0013	1	0.012	15	0.0035
			0.5	2	287.42	574.84			0.1					1		15	
	1-3	2	0.5	1	294.06	588.12	594.97	1189.94	0.1	167671	0.71	1111	0.0013	1	0.012	15	0.0036
			0.5	2	300.91	601.82			0.1					1		15	
	2-1	2	0.5	1	1894.81	3789.62	3839.12	7678.24	0.1	167671	4.58	7600	0.0092	1	0.012	15	0.0246
			0.5	2	1944.31	3888.62			0.1					1		15	
	2-2	2	0.5	1	1842.6	3685.2	3710.65	7421.3	0.1	167671	4.43	7343	0.0089	1	0.012	15	0.0238
			0.5	2	1868.05	3736.1			0.1					1		15	
	2-3	2	0.5	1	1839.35	3678.7	3656.88	7313.76	0.1	167671	4.36	7235	0.0087	1	0.012	15	0.0234
			0.5	2	1817.53	3635.06			0.1					1		15	
	3-1	2	0.5	1	2815.77	5631.54	5677.04	11354.08	0.1	167671	6.77	11276	0.0136	1	0.012	15	0.0365
			0.5	2	2861.27	5722.54			0.1					1		15	
	3-2	2	0.5	1	2815.96	5631.92	5607.41	11214.82	0.1	167671	6.69	11136	0.0134	1	0.012	15	0.0361
			0.5	2	2791.45	5582.9			0.1					1		15	
	3-3	2	0.5	1	2814.14	5628.28	5609.87	11219.74	0.1	167671	6.69	11141	0.0134	1	0.012	15	0.0361
			0.5	2	2795.73	5591.46			0.1					1		15	
	4-1	2	0.5	1	3946.39	7892.78	7942.7	15885.4	0.1	167671	9.47	15807	0.0191	1	0.012	15	0.0512
			0.5	2	3996.31	7992.62			0.1					1		15	
	4-2	2	0.5	1	3960.16	7920.32	7917.06	15834.12	0.1	167671	9.44	15756	0.0190	1	0.012	15	0.0511
			0.5	2	3956.9	7913.8			0.1					1		15	
	4-3	2	0.5	1	3992.99	7985.98	7974.18	15948.36	0.1	167671	9.51	15870	0.0191	1	0.012	15	0.0514
			0.5	2	3981.19	7962.38			0.1					1		15	
	5-1	2	0.5	1	5153.67	10307.34	10527.54	21055.08	0.1	167671	12.56	20977	0.0253	1	0.012	15	0.0680
			0.5	2	5373.87	10747.74			0.1					1		15	
	5-2	2	0.5	1	5248.74	10497.48	10459.68	20919.36	0.1	167671	12.48	20841	0.0251	1	0.012	15	0.0675
			0.5	2	5210.94	10421.88			0.1					1		15	
	5-3	2	0.5	1	5126.55	10253.1	10310.75	20621.5	0.1	167671	12.30	20543	0.0248	1	0.012	15	0.0666
			0.5	2	5184.2	10368.4			0.1					1		15	
	6-1	2	0.5	1	6032.26	12064.52	12223.91	24447.82	0.1	167671	14.58	24369	0.0294	1	0.012	15	0.0790
			0.5	2	6191.65	12383.3			0.1					1		15	
	6-2	2	0.5	1	6040.19	12080.38	12216.36	24432.72	0.1	167671	14.57	24354	0.0294	1	0.012	15	0.0789
			0.5	2	6176.17	12352.34			0.1					1		15	
	6-3	2	0.5	1	6103.31	12206.62	12117.56	24235.12	0.1	167671	14.45	24157	0.0291	1	0.012	15	0.0783
			0.5	2	6014.25	12028.5			0.1					1		15	

Assay Date	12/17/04	Test Chemical ID	4-OH ASDN	# Concentrations tested	6	Microsome type	placental	Microsome ID	MET-0255A Vi	Technician ID	JG	Replicate #	2

Control Type	Portion	Average	SD
Full activity	Beginning	0.0766	0.0077
Full activity	End	0.0776	0.0027
Full activity	Overall	0.0771	0.0047
Background	Beginning	0.0000	7.88158E-06
Background	End	0.0000	1.24639E-05
Background	Overall	0.0000	1.9706E-05
Positive	Beginning	#VALUE!	#VALUE!
Positive	End	#VALUE!	#VALUE!
Positive	Overall	#VALUE!	#VALUE!
Negative	Beginning	#VALUE!	#VALUE!
Negative	End	#VALUE!	#VALUE!
Negative	Overall	#VALUE!	#VALUE!

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
4-OH ASDN	1	1	1.00E-06	-6.00	0.0036
4-OH ASDN	1	2	1.00E-06	-6.00	0.0035
4-OH ASDN	1	3	1.00E-06	-6.00	0.0036
4-OH ASDN	2	1	1.00E-07	-7.00	0.0246
4-OH ASDN	2	2	1.00E-07	-7.00	0.0238
4-OH ASDN	2	3	1.00E-07	-7.00	0.0234
4-OH ASDN	3	1	5.00E-08	-7.30	0.0365
4-OH ASDN	3	2	5.00E-08	-7.30	0.0361
4-OH ASDN	3	3	5.00E-08	-7.30	0.0361
4-OH ASDN	4	1	2.50E-08	-7.60	0.0512
4-OH ASDN	4	2	2.50E-08	-7.60	0.0511
4-OH ASDN	4	3	2.50E-08	-7.60	0.0514
4-OH ASDN	5	1	1.00E-08	-8.00	0.0680
4-OH ASDN	5	2	1.00E-08	-8.00	0.0675
4-OH ASDN	5	3	1.00E-08	-8.00	0.0666
4-OH ASDN	6	1	1.00E-09	-9.00	0.0790
4-OH ASDN	6	2	1.00E-09	-9.00	0.0789
4-OH ASDN	6	3	1.00E-09	-9.00	0.0783

Percent of control values

Level	Log[test substance]	Replicate 1	Replicate 2	Replicate 3
1	-6.00	4.73	4.49	4.67
2	-7.00	31.94	30.86	30.41
3	-7.30	47.39	46.80	46.82
4	-7.60	66.43	66.22	66.70
5	-8.00	88.16	87.59	86.34
6	-9.00	102.42	102.36	101.53

Aromatase Assay Spreadsheet

Assay Date	12/27/04	Test Chemical ID	4-OH ASDI	# Concentrations tested	6		
Technician ID	JG	Replicate #	3	Microsome type	placental	Microsome ID	MET-0255A Vial 5

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0195	33736.97	1730101	
2	0.0194	34202.98	1763040	
3	0.0196	34408.23	1755522	
4	0.0197	35101.15	1781784	
5	0.0199	35290.88	1773411	
			Average DPM/g soln	1760772
			SD	19841
			CV	1.13
			µCi/g soln	0.793

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	8.1892	g
Mass of dilution B used in substrate prep	4.6174	g
Concentration of nonradiolabeled ASDN in substrate soln.	0.580755	µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00898 µg/g soln.
µg/g soln.

a. µCi/g soln 0.793
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.

= 0.580755 + 0.00898
= 0.589734 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.345 µCi/µg ASDN

855106 dpm/nmol

Assay Date	12/27/04	Test Chemical ID	4-OH ASDN	# Concentrations tested	6					
Technician ID	JG	Replicate #	3	Microsome type	placental	Microsome ID	MET-0255A Vial 5			
								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	1.42	0.95	0.71	0.47	0.24	0.12	0	23.649	10	
	0.4104	0.2873	0.2284	0.1650	0.0965	0.0458	0.0295			
	0.4349	0.3113	0.2405	0.1751	0.0999	0.0487	0.0241			
	0.3993	0.3103	0.2464	0.1789	0.1015	0.0482	0.0245			
Samples:										
		0.1570	0.1495							
		0.1652	0.1091							
		0.1664	0.0947							

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	
1.42	60	100	0.00142	25	0.0355	0.415	0.389	0.0342	m, b	0.089	0.000
0.95	40	100	0.00095	25	0.0236	0.303	0.277	0.0243	se_m, se_b	0.004	0.001
0.71	30	100	0.00071	25	0.0177	0.238	0.212	0.0186	r^2, se_y	0.992	0.001
0.47	20	100	0.00047	25	0.0118	0.173	0.147	0.0128	F, df	504	4
0.24	10	100	0.00024	25	0.0059	0.099	0.073	0.0062	ss_{reg}, ss_{resid}	0.001	0.000
0.12	5.2	100	0.00012	25	0.0031	0.048	0.022	0.0016			

Regression results are calculated using the function LINEST

Blank 0.026

r^2= 0.992
m= 0.089
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
				100	5000			
				100	5000			
				100	5000			
0.157	0.131	0.011	25	1	1	0.000	0.000	0.474
0.165	0.139	0.012	25	1	1	0.000		
0.166	0.140	0.012	25	1	1	0.000		
0.150	0.123	0.011	25	100	5000	0.021	0.016	15.696
0.109	0.083	0.007	25	100	5000	0.014		
0.095	0.069	0.006	25	100	5000	0.012		

Assay Date	12/27/04	Test Chemical ID	4-OH ASDN		# Concentrations tested	6			
Technician ID	JG	Replicate #	3		Microsome type	placental	Microsome ID	MET-0255A Vial 5	

								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	12/27/04	Test Chemical ID	4-OH ASDN	# Concentrations tested	6	Microsome type	placental	Microsome ID	MET-0255	Technician ID	JG	Replicate #	3

Microsome Dilution Details		
Dilution A	0.08	mL microsome Stock used
	3.98	mL total volume
	49.75	dilution factor
Dilution B	3	mL microsome Dilution A used
	30	mL total volume
	10	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	497.5	total dilution factor

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

Protein Concentration (stock microsomes, mg/mL):	15.696
Protein Concentration (dilution added to assay, mg/mL):	0.03155

Assay Date	12/27/04	Test Chemical ID	4-OH ASDN	# Concentrations tested	6	Microsome type	placental	Microsome ID	MET-0255A Vial 5	Technician ID	JG	Replicate #	3

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate nmol ^3H_2O formed				Incubation time (min)	Aromatase activity (nmol estrogen formed/mg protein/min
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)		
Full activity control	1	2	0.5	1	5952	11903.2	12063.41	24126.82	0.1	176077	13.70	24007	0.0281	1	0.016	15	0.0593
			0.5	2	6112	12223.62			0.1					1		15	
	2	2	0.5	1	6525	13049.78	13034.62	26069.24	0.1	176077	14.81	25949	0.0303	1	0.016	15	0.0641
			0.5	2	6510	13019.46			0.1					1		15	
	3	2	0.5	1	5878	11755.3	11820.19	23640.38	0.1	176077	13.43	23520	0.0275	1	0.016	15	0.0581
			0.5	2	5943	11885.08			0.1					1		15	
	4	2	0.5	1	5462	10924.12	10921.88	21843.76	0.1	176077	12.41	21724	0.0254	1	0.016	15	0.0537
			0.5	2	5460	10919.64			0.1					1		15	
Background control	1	2	0.5	1	28	56.16	53.79	107.58	0.1	176077	0.06	-13	0.0000	1	0.016	15	0.0000
			0.5	2	26	51.42			0.1					1		15	
	2	2	0.5	1	27	53.26	64.95	129.9	0.1	176077	0.07	10	0.0000	1	0.016	15	0.0000
			0.5	2	38	76.64			0.1					1		15	
	3	2	0.5	1	33	65.86	60.08	120.16	0.1	176077	0.07	0	0.0000	1	0.016	15	0.0000
			0.5	2	27	54.3			0.1					1		15	
	4	2	0.5	1	31	61.76	61.57	123.14	0.1	176077	0.07	3	0.0000	1	0.016	15	0.0000
			0.5	2	31	61.38			0.1					1		15	
Positive control	1	2	0.5	1					0.1	176077		#VALUE!	#VALUE!	1	0.016	15	#VALUE!
			0.5	2					0.1					1		15	
	2	2	0.5	1					0.1	176077		#VALUE!	#VALUE!	1	0.016	15	#VALUE!
			0.5	2					0.1					1		15	
	3	2	0.5	1					0.1	176077		#VALUE!	#VALUE!	1	0.016	15	#VALUE!
			0.5	2					0.1					1		15	
	4	2	0.5	1					0.1	176077		#VALUE!	#VALUE!	1	0.016	15	#VALUE!
			0.5	2					0.1					1		15	
Negative Control	1	2	0.5	1					0.1	176077		#VALUE!	#VALUE!	1	0.016	15	#VALUE!
			0.5	2					0.1					1		15	
	2	2	0.5	1					0.1	176077		#VALUE!	#VALUE!	1	0.016	15	#VALUE!
			0.5	2					0.1					1		15	
	3	2	0.5	1					0.1	176077		#VALUE!	#VALUE!	1	0.016	15	#VALUE!
			0.5	2					0.1					1		15	
	4	2	0.5	1					0.1	176077		#VALUE!	#VALUE!	1	0.016	15	#VALUE!
			0.5	2					0.1					1		15	
4-OH ASDN	1-1	2	0.5	1	311	621.82	629.34	1258.68	0.1	176077	0.71	1138	0.0013	1	0.016	15	0.0028
			0.5	2	318	636.86			0.1					1		15	
	1-2	2	0.5	1	331	662.64	661.28	1322.56	0.1	176077	0.75	1202	0.0014	1	0.016	15	0.0030
			0.5	2	330	659.92			0.1					1		15	
	1-3	2	0.5	1	322	643.12	623.11	1246.22	0.1	176077	0.71	1126	0.0013	1	0.016	15	0.0028
			0.5	2	302	603.1			0.1					1		15	
	2-1	2	0.5	1	1860	3719.18	3821.15	7642.3	0.1	176077	4.34	7522	0.0088	1	0.016	15	0.0186
			0.5	2	1962	3923.12			0.1					1		15	
	2-2	2	0.5	1	1933	3866.46	3867.75	7735.5	0.1	176077	4.39	7615	0.0089	1	0.016	15	0.0188
			0.5	2	1935	3869.04			0.1					1		15	
	2-3	2	0.5	1	1918	3836.28	3859.9	7719.8	0.1	176077	4.38	7600	0.0089	1	0.016	15	0.0188
			0.5	2	1942	3883.52			0.1					1		15	
	3-1	2	0.5	1	2613	5225.64	5225.4	10450.8	0.1	176077	5.94	10331	0.0121	1	0.016	15	0.0255
			0.5	2	2613	5225.16			0.1					1		15	
	3-2	2	0.5	1	2734	5468.78	5537.44	11074.88	0.1	176077	6.29	10955	0.0128	1	0.016	15	0.0271
			0.5	2	2803	5606.1			0.1					1		15	
	3-3	2	0.5	1	2779	5557.74	5739.15	11478.3	0.1	176077	6.52	11358	0.0133	1	0.016	15	0.0281
			0.5	2	2960	5920.56			0.1					1		15	
	4-1	2	0.5	1	3786	7571.78	7555.1	15110.2	0.1	176077	8.58	14990	0.0175	1	0.016	15	0.0370
			0.5	2	3769	7538.42			0.1					1		15	
	4-2	2	0.5	1	3514	7028.64	7120.03	14240.06	0.1	176077	8.09	14120	0.0165	1	0.016	15	0.0349
			0.5	2	3606	7211.42			0.1					1		15	
	4-3	2	0.5	1	3704	7408.84	7538.77	15077.54	0.1	176077	8.56	14957	0.0175	1	0.016	15	0.0370
			0.5	2	3834	7668.7			0.1					1		15	
	5-1	2	0.5	1	5001	10002.8	10010.69	20021.38	0.1	176077	11.37	19901	0.0233	1	0.016	15	0.0492
			0.5	2	5009	10018.58			0.1					1		15	
	5-2	2	0.5	1	4894	9787.46	9724.27	19448.54	0.1	176077	11.05	19328	0.0226	1	0.016	15	0.0478
			0.5	2	4831	9661.08			0.1					1		15	
	5-3	2	0.5	1	4615	9230.12	9306.14	18612.28	0.1	176077	10.57	18492	0.0216	1	0.016	15	0.0457
			0.5	2	4691	9382.16			0.1					1		15	
	6-1	2	0.5	1	5566	11132.3	11282.05	22564.1	0.1	176077	12.81	22444	0.0262	1	0.016	15	0.0555
			0.5	2	5716	11431.8			0.1					1		15	
	6-2	2	0.5	1	5495	10989.26	11245.01	22490.02	0.1	176077	12.77	22370	0.0262	1	0.016	15	0.0553
			0.5	2	5750	11500.76			0.1					1		15	
	6-3	2	0.5	1	5684	11367.3	11245.02	22490.04	0.1	176077	12.77	22370	0.0262	1	0.016	15	0.0553
			0.5	2	5561	11122.74			0.1					1		15	

Assay Date	12/27/04	Test Chemical ID	4-OH ASDN	# Concentrations tested	6	Microsome type	placental	Microsome ID	MET-0255A Vi	Technician ID	JG	Replicate #	3

Control Type	Portion	Average	SD
Full activity	Beginning	0.0617	0.0034
Full activity	End	0.0559	0.0031
Full activity	Overall	0.0588	0.0043
Background	Beginning	0.0000	3.90007E-05
Background	End	0.0000	5.20708E-06
Background	Overall	0.0000	2.30931E-05
Positive	Beginning	#VALUE!	#VALUE!
Positive	End	#VALUE!	#VALUE!
Positive	Overall	#VALUE!	#VALUE!
Negative	Beginning	#VALUE!	#VALUE!
Negative	End	#VALUE!	#VALUE!
Negative	Overall	#VALUE!	#VALUE!

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
4-OH ASDN	1	1	1.00E-06	-6.00	0.0028
4-OH ASDN	1	2	1.00E-06	-6.00	0.0030
4-OH ASDN	1	3	1.00E-06	-6.00	0.0028
4-OH ASDN	2	1	1.00E-07	-7.00	0.0186
4-OH ASDN	2	2	1.00E-07	-7.00	0.0188
4-OH ASDN	2	3	1.00E-07	-7.00	0.0188
4-OH ASDN	3	1	5.00E-08	-7.30	0.0255
4-OH ASDN	3	2	5.00E-08	-7.30	0.0271
4-OH ASDN	3	3	5.00E-08	-7.30	0.0281
4-OH ASDN	4	1	2.50E-08	-7.60	0.0370
4-OH ASDN	4	2	2.50E-08	-7.60	0.0349
4-OH ASDN	4	3	2.50E-08	-7.60	0.0370
4-OH ASDN	5	1	1.00E-08	-8.00	0.0492
4-OH ASDN	5	2	1.00E-08	-8.00	0.0478
4-OH ASDN	5	3	1.00E-08	-8.00	0.0457
4-OH ASDN	6	1	1.00E-09	-9.00	0.0555
4-OH ASDN	6	2	1.00E-09	-9.00	0.0553
4-OH ASDN	6	3	1.00E-09	-9.00	0.0553

Percent of control values

Level	Log[test substance]	Replicate 1	Replicate 2	Replicate 3
1	-6.00	4.78	5.05	4.73
2	-7.00	31.61	32.00	31.93
3	-7.30	43.41	46.03	47.72
4	-7.60	62.98	59.33	62.85
5	-8.00	83.62	81.21	77.70
6	-9.00	94.30	93.99	93.99

Appendix G

Unsuccessful Replicate Spreadsheets (12/9/04 And 12/14/04)

Aromatase Assay Spreadsheet

Assay Date	12/09/04	Test Chemical ID	4-OH ASDI	# Concentrations tested	6		
Technician ID	JG	Replicate #	N/A	Microsome type	Placental	Microsome ID	

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.		
1	0.0195	33194.22	1702268		
2	0.0195	34166.27	1752116		
3	0.0199	34210.37	1719114		
4	0.0197	34828.63	1767951		
5	0.0198	34673.5	1751187		
			Average DPM/g soln		1738527
			SD		26937
			CV		1.55
			µCi/g soln		0.783

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	10		1000.00
Dilution A			100	10.00
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.2256	g
Mass of dilution B used in substrate prep	4.6176	g
Concentration of nonradiolabeled ASDN in substrate soln.	0.561369	µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00887 µg/g soln.
µg/g soln.

a. µCi/g soln 0.783
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.

= 0.561369 + 0.00887
= 0.570234 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.373 µCi/µg ASDN

873175 dpm/nmol

Assay Date	12/09/04	Test Chemical ID	4-OH ASDN	# Concentrations tested	6				
Technician ID	JG	Replicate #	N/A	Microsome type	Placental	Microsome ID	0		

Standards:	0.00142	0.00095	0.00071	0.00047	0.00024	0.00012	0	Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
	0.389	0.286	0.214	0.164	0.098	0.050	0.023	23.649	10	
	0.377	0.300	0.231	0.169	0.098	0.051	0.024			
	0.389	0.305	0.226	0.170	0.094	0.057	0.021			

Samples:	BSA Control	Microsomes
	0.163	0.119
	0.173	0.115
	0.175	0.105

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	
0.00142	60	100	0.00142	25	0.0355	0.385	0.362	0.0337	m, b	0.096	-0.001
0.00095	40	100	0.00095	25	0.0236	0.297	0.274	0.0253	se_m, se_b	0.005	0.001
0.00071	30	100	0.00071	25	0.0177	0.223	0.201	0.0182	r^2, se_y	0.988	0.001
0.00047	20	100	0.00047	25	0.0118	0.168	0.145	0.0128	F, df	334	4
0.00024	10	100	0.00024	25	0.0059	0.097	0.074	0.0060	ss_{reg}, ss_{resid}	0.001	0.000
0.00012	5.2	100	0.00012	25	0.0031	0.053	0.030	0.0017			

Regression results are calculated using the function LINEST

Blank	0.023	r^2=	0.988
		m=	0.096
		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
BSA Control	0.163	0.141	0.012	25	1	1	0.000	0.001	0.522
BSA Control	0.173	0.150	0.013	25	1	1	0.001		
BSA Control	0.175	0.152	0.013	25	1	1	0.001		
Microsomes	0.119	0.096	0.008	25	100	5000	0.016	0.015	15.003
Microsomes	0.115	0.092	0.008	25	100	5000	0.015		
Microsomes	0.105	0.082	0.007	25	100	5000	0.013		

Assay Date	12/09/04	Test Chemical ID	4-OH ASDN	# Concentrations tested	6				
Technician ID	JG	Replicate #	N/A	Microsome type	Placental	Microsome ID	0		

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	12/09/04	Test Chemical ID	4-OH ASDN	# Concentrations tested	6				
Technician ID	JG	Replicate #	N/A	Microsome type	Placental	Microsome ID	0		

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	12/09/04	Test Chemical ID	4-OH ASDN	# Concentrations tested	6	Microsome type	Placental	Microsome ID	0	Technician ID	JG	Replicate #	N/A

Microsome Dilution Details		
Dilution A	0.08	mL microsome Stock used
	3.98	mL total volume
	49.75	dilution factor
Dilution B	3	mL microsome Dilution A used
	30	mL total volume
	10	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	497.5	total dilution factor

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

Protein Concentration (stock microsomes, mg/mL):	15.003
Protein Concentration (dilution added to assay, mg/mL):	0.030157

Assay Date	12/09/04	Test Chemical ID	4-OH ASDN	# Concentrations tested	6	Microsome type	Placental	Microsome ID	0	Technician ID	JG	Replicate #	N/A

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate nmol ^3H_2O formed				Incubation time (min)	Aromatase activity (nmol estrogen formed/mg protein/min
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)		
Full activity control	1	2	0.5	1	6047.88	12095.76	12378.03	24756.06	1	1738527	1.42	24669	0.0283	1	0.015	15	0.0625
			0.5	2	6330.15	12660.3			1					1		15	
	2	2	0.5	1	5995.97	11991.94	11904.35	23808.7	1	1738527	1.37	23721	0.0272	1	0.015	15	0.0601
			0.5	2	5908.38	11816.76			1					1		15	
	3	2	0.5	1	4073.78	8147.56	8008.09	16016.18	1	1738527	0.92	15929	0.0182	1	0.015	15	0.0403
			0.5	2	3934.31	7868.62			1					1		15	
	4	2	0.5	1	4339.84	8679.68	8878.18	17756.36	1	1738527	1.02	17669	0.0202	1	0.015	15	0.0447
			0.5	2	4538.34	9076.68			1					1		15	
Background control	1	2	0.5	1	26.05	52.1	49.08	98.16	1	1738527	0.01	11	0.0000	1	0.015	15	0.0000
			0.5	2	23.03	46.06			1					1		15	
	2	2	0.5	1	19.85	39.7	36.95	73.9	1	1738527	0.00	-13	0.0000	1	0.015	15	0.0000
			0.5	2	17.1	34.2			1					1		15	
	3	2	0.5	1	19.64	39.28	41.24	82.48	1	1738527	0.00	-5	0.0000	1	0.015	15	0.0000
			0.5	2	21.6	43.2			1					1		15	
	4	2	0.5	1	26.37	52.74	47.49	94.98	1	1738527	0.01	8	0.0000	1	0.015	15	0.0000
			0.5	2	21.12	42.24			1					1		15	
Positive control	1	2	0.5	1					1	1738527		#VALUE!	#VALUE!	1	0.015	15	#VALUE!
			0.5	2					1					1		15	
	2	2	0.5	1					1	1738527		#VALUE!	#VALUE!	1	0.015	15	#VALUE!
			0.5	2					1					1		15	
	3	2	0.5	1					1	1738527		#VALUE!	#VALUE!	1	0.015	15	#VALUE!
			0.5	2					1					1		15	
	4	2	0.5	1					1	1738527		#VALUE!	#VALUE!	1	0.015	15	#VALUE!
			0.5	2					1					1		15	
Negative Control	1	2	0.5	1					1	1738527		#VALUE!	#VALUE!	1	0.015	15	#VALUE!
			0.5	2					1					1		15	
	2	2	0.5	1					1	1738527		#VALUE!	#VALUE!	1	0.015	15	#VALUE!
			0.5	2					1					1		15	
	3	2	0.5	1					1	1738527		#VALUE!	#VALUE!	1	0.015	15	#VALUE!
			0.5	2					1					1		15	
	4	2	0.5	1					1	1738527		#VALUE!	#VALUE!	1	0.015	15	#VALUE!
			0.5	2					1					1		15	
4-OH ASDN	1-1	2	0.5	1	253.26	506.52	499.54	999.08	1	1738527	0.06	912	0.0010	1	0.015	15	0.0023
			0.5	2	246.28	492.56			1					1		15	
	1-2	2	0.5	1	229.23	458.46	474.09	948.18	1	1738527	0.05	861	0.0010	1	0.015	15	0.0022
			0.5	2	244.86	489.72			1					1		15	
	1-3	2	0.5	1	251.19	502.38	519.14	1038.28	1	1738527	0.06	951	0.0011	1	0.015	15	0.0024
			0.5	2	267.95	535.9			1					1		15	
	2-1	2	0.5	1	1483.49	2966.98	2970.36	5940.72	1	1738527	0.34	5853	0.0067	1	0.015	15	0.0148
			0.5	2	1486.87	2973.74			1					1		15	
	2-2	2	0.5	1	1333.52	2667.04	2721.14	5442.28	1	1738527	0.31	5355	0.0061	1	0.015	15	0.0136
			0.5	2	1387.62	2775.24			1					1		15	
	2-3	2	0.5	1	1244.87	2489.74	2493.03	4986.06	1	1738527	0.29	4899	0.0056	1	0.015	15	0.0124
			0.5	2	1248.16	2496.32			1					1		15	
	3-1	2	0.5	1	1737.98	3475.96	3454.93	6909.86	1	1738527	0.40	6822	0.0078	1	0.015	15	0.0173
			0.5	2	1716.95	3433.9			1					1		15	
	3-2	2	0.5	1	1366.8	2733.6	2968.1	5936.2	1	1738527	0.34	5849	0.0067	1	0.015	15	0.0148
			0.5	2	1601.3	3202.6			1					1		15	
	3-3	2	0.5	1	1432.34	2864.68	2839.02	5678.04	1	1738527	0.33	5591	0.0064	1	0.015	15	0.0142
			0.5	2	1406.68	2813.36			1					1		15	
	4-1	2	0.5	1	1773.37	3546.74	3676.31	7352.62	1	1738527	0.42	7265	0.0083	1	0.015	15	0.0184
			0.5	2	1902.94	3805.88			1					1		15	
	4-2	2	0.5	1	1303.18	2606.36	2741.22	5482.44	1	1738527	0.32	5395	0.0062	1	0.015	15	0.0137
			0.5	2	1438.04	2876.08			1					1		15	
	4-3	2	0.5	1	1352.37	2704.74	2780.6	5561.2	1	1738527	0.32	5474	0.0063	1	0.015	15	0.0139
			0.5	2	1428.23	2856.46			1					1		15	
	5-1	2	0.5	1	1643.94	3287.88	3331.27	6662.54	1	1738527	0.38	6575	0.0075	1	0.015	15	0.0166
			0.5	2	1687.33	3374.66			1					1		15	
	5-2	2	0.5	1	3955.77	7911.54	8175.32	16350.64	1	1738527	0.94	16263	0.0186	1	0.015	15	0.0412
			0.5	2	4219.55	8439.1			1					1		15	
	5-3	2	0.5	1	3941.68	7883.36	8079.84	16159.68	1	1738527	0.93	16072	0.0184	1	0.015	15	0.0407
			0.5	2	4138.16	8276.32			1					1		15	
	6-1	2	0.5	1	4238.02	8476.04	8722.48	17444.96	1	1738527	1.00	17358	0.0199	1	0.015	15	0.0439
			0.5	2	4484.46	8968.92			1					1		15	
	6-2	2	0.5	1	4779.81	9559.62	9570.09	19140.18	1	1738527	1.10	19053	0.0218	1	0.015	15	0.0482
			0.5	2	4790.28	9580.56			1					1		15	
	6-3	2	0.5	1	4367.28	8734.56	8780.41	17560.82	1	1738527	1.01	17473	0.0200	1	0.015	15	0.0442
			0.5	2	4413.13	8826.26			1								

		Test Chemical			Microsome						Replicate		
Assay Date	12/09/04	ID	4-OH ASDN	# Concentrations tested	6 type	Placental	Microsome ID	0	Technician ID	JG	#	N/A	

Control Type	Portion	Average	SD
Full activity	Beginning	0.0613	0.0017
Full activity	End	0.0425	0.0031
Full activity	Overall	0.0519	0.0110
Background	Beginning	0.0000	4.34309E-05
Background	End	0.0000	2.23778E-05
Background	Overall	0.0000	2.84824E-05
Positive	Beginning	#VALUE!	#VALUE!
Positive	End	#VALUE!	#VALUE!
Positive	Overall	#VALUE!	#VALUE!
Negative	Beginning	#VALUE!	#VALUE!
Negative	End	#VALUE!	#VALUE!
Negative	Overall	#VALUE!	#VALUE!

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
4-OH ASDN	1	1	1.00E-06	-6.00	0.0023
4-OH ASDN	1	2	1.00E-06	-6.00	0.0022
4-OH ASDN	1	3	1.00E-06	-6.00	0.0024
4-OH ASDN	2	1	1.00E-07	-7.00	0.0148
4-OH ASDN	2	2	1.00E-07	-7.00	0.0136
4-OH ASDN	2	3	1.00E-07	-7.00	0.0124
4-OH ASDN	3	1	5.00E-08	-7.30	0.0173
4-OH ASDN	3	2	5.00E-08	-7.30	0.0148
4-OH ASDN	3	3	5.00E-08	-7.30	0.0142
4-OH ASDN	4	1	2.50E-08	-7.60	0.0184
4-OH ASDN	4	2	2.50E-08	-7.60	0.0137
4-OH ASDN	4	3	2.50E-08	-7.60	0.0139
4-OH ASDN	5	1	1.00E-08	-8.00	0.0166
4-OH ASDN	5	2	1.00E-08	-8.00	0.0412
4-OH ASDN	5	3	1.00E-08	-8.00	0.0407
4-OH ASDN	6	1	1.00E-09	-9.00	0.0439
4-OH ASDN	6	2	1.00E-09	-9.00	0.0482
4-OH ASDN	6	3	1.00E-09	-9.00	0.0442

Percent of control values

Level	Log[test substance]	Replicate 1	Replicate 2	Replicate 3
1	-6.00	4.45	4.20	4.64
2	-7.00	28.56	26.13	23.90
3	-7.30	33.29	28.54	27.28
4	-7.60	35.45	26.32	26.71
5	-8.00	32.08	79.34	78.41
6	-9.00	84.68	92.95	85.25

Aromatase Assay Spreadsheet

Assay Date	12/14/04	Test Chemical ID	4-OH ASDI	# Concentrations tested	6		
Technician ID	JG	Replicate #	N/A	Microsome type	Placental	Microsome ID	

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.		
1	0.0196	27678.36	1412161		
2	0.0199	27783.58	1396160		
3	0.0197	28129.63	1427900		
4	0.0197	28689.39	1456314		
5	0.0200	28579.63	1428982		
			Average DPM/g soln		1424303
			SD		22355
			CV		1.57
			µCi/g soln		0.642

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	10		1000.00
Dilution A			100	10.00
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.114	g
Mass of dilution B used in substrate prep	4.5632	g
Concentration of nonradiolabeled ASDN in substrate soln.	0.562386	µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00726 µg/g soln.
µg/g soln.

a. µCi/g soln 0.642
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.

= 0.562386 + 0.00726
= 0.569649 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.126 µCi/µg ASDN

716091 dpm/nmol

Assay Date	12/14/04	Test Chemical ID	4-OH ASDN	# Concentrations tested	6				
Technician ID	JG	Replicate #	N/A	Microsome type	Placental	Microsome ID	0		

Standards:	0.00142	0.00095	0.00071	0.00047	0.00024	0.00012	0	Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
	0.3944	0.2763	0.2347	0.1552	0.0945	0.0592	0.0236	23.649	10	
	0.4136	0.3087	0.2383	0.1626	0.0920	0.0541	0.0226			
	0.3703	0.3017	0.2373	0.1570	0.0970	0.0535	0.0218			

Samples:	BSA Control	Microsomes
	0.1487	0.1159
	0.1653	0.1120
	0.1594	0.1158

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	
0.00142	60	100	0.00142	25	0.0355	0.393	0.370	0.0339	m, b	0.094	-0.001
0.00095	40	100	0.00095	25	0.0236	0.296	0.273	0.0248	se_m, se_b	0.005	0.001
0.00071	30	100	0.00071	25	0.0177	0.237	0.214	0.0192	r^2, se_y	0.991	0.001
0.00047	20	100	0.00047	25	0.0118	0.158	0.136	0.0118	F, df	429	4
0.00024	10	100	0.00024	25	0.0059	0.095	0.072	0.0058	ss_{reg}, ss_{resid}	0.001	0.000
0.00012	5.2	100	0.00012	25	0.0031	0.056	0.033	0.0022			

Regression results are calculated using the function LINEST

Blank	0.023	r^2=	0.991	
		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
BSA Control	0.149	0.126	0.011	25	1	1	0.000	0.000	0.471
BSA Control	0.165	0.143	0.012	25	1	1	0.000		
BSA Control	0.159	0.137	0.012	25	1	1	0.000		
Microsomes	0.116	0.093	0.008	25	100	5000	0.016	0.015	15.417
Microsomes	0.112	0.089	0.007	25	100	5000	0.015		
Microsomes	0.116	0.093	0.008	25	100	5000	0.016		

Assay Date	12/14/04	Test Chemical ID	4-OH ASDN		# Concentrations tested	6			
Technician ID	JG	Replicate #	N/A		Microsome type	Placental	Microsome ID	0	

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	12/14/04	Test Chemical ID	4-OH ASDN	# Concentrations tested	6	Microsome type	Placental	Microsome ID	0	Technician ID	JG	Replicate #	N/A

Microsome Dilution Details		
Dilution A	0.08	mL microsome Stock used
	3.98	mL total volume
	49.75	dilution factor
Dilution B	3	mL microsome Dilution A used
	30	mL total volume
	10	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	497.5	total dilution factor

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

Protein Concentration (stock microsomes, mg/mL):	15.417
Protein Concentration (dilution added to assay, mg/mL):	0.030989

Assay Date	12/14/04	Test Chemical ID	4-OH ASDN	# Concentrations tested	6	Microsome type	Placental	Microsome ID	0	Technician ID	JG	Replicate #	N/A

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate nmol ^3H_2O formed				Incubation time (min)	Aromatase activity (nmol estrogen formed/mg protein/min
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)		
Full activity control	1	2	0.5	1	5457.29	10914.58	11034.66	22069.32	1	1424303	1.55	21952	0.0307	1	0.015	15	0.0659
			0.5	2	5577.37	11154.74			1					1		15	
	2	2	0.5	1	4498.41	8996.82	9030.09	18060.18	1	1424303	1.27	17943	0.0251	1	0.015	15	0.0539
			0.5	2	4531.68	9063.36			1					1		15	
	3	2	0.5	1	1174.59	2349.18	2399.53	4799.06	1	1424303	0.34	4682	0.0065	1	0.015	15	0.0141
			0.5	2	1224.94	2449.88			1					1		15	
	4	2	0.5	1	1068.4	2136.8	2125.75	4251.5	1	1424303	0.30	4134	0.0058	1	0.015	15	0.0124
			0.5	2	1057.35	2114.7			1					1		15	
Background control	1	2	0.5	1	39.29	78.58	77.45	154.9	1	1424303	0.01	37	0.0001	1	0.015	15	0.0001
			0.5	2	38.16	76.32			1					1		15	
	2	2	0.5	1	23.9	47.8	59.78	119.56	1	1424303	0.01	2	0.0000	1	0.015	15	0.0000
			0.5	2	35.88	71.76			1					1		15	
	3	2	0.5	1	14.4	28.8	43.26	86.52	1	1424303	0.01	-31	0.0000	1	0.015	15	-0.0001
			0.5	2	28.86	57.72			1					1		15	
	4	2	0.5	1	25.05	50.1	54.62	109.24	1	1424303	0.01	-8	0.0000	1	0.015	15	0.0000
			0.5	2	29.57	59.14			1					1		15	
Positive control	1	2	0.5	1					1	1424303		#VALUE!	#VALUE!	1	0.015	15	#VALUE!
			0.5	2					1					1		15	
	2	2	0.5	1					1	1424303		#VALUE!	#VALUE!	1	0.015	15	#VALUE!
			0.5	2					1					1		15	
	3	2	0.5	1					1	1424303		#VALUE!	#VALUE!	1	0.015	15	#VALUE!
			0.5	2					1					1		15	
	4	2	0.5	1					1	1424303		#VALUE!	#VALUE!	1	0.015	15	#VALUE!
			0.5	2					1					1		15	
Negative Control	1	2	0.5	1					1	1424303		#VALUE!	#VALUE!	1	0.015	15	#VALUE!
			0.5	2					1					1		15	
	2	2	0.5	1					1	1424303		#VALUE!	#VALUE!	1	0.015	15	#VALUE!
			0.5	2					1					1		15	
	3	2	0.5	1					1	1424303		#VALUE!	#VALUE!	1	0.015	15	#VALUE!
			0.5	2					1					1		15	
	4	2	0.5	1					1	1424303		#VALUE!	#VALUE!	1	0.015	15	#VALUE!
			0.5	2					1					1		15	
4-OH ASDN	1-1	2	0.5	1	159.42	318.84	314.78	629.56	1	1424303	0.04	512	0.0007	1	0.015	15	0.0015
			0.5	2	155.36	310.72			1					1		15	
	1-2	2	0.5	1	157.94	315.88	320.02	640.04	1	1424303	0.04	522	0.0007	1	0.015	15	0.0016
			0.5	2	162.08	324.16			1					1		15	
	1-3	2	0.5	1	131.72	263.44	269.36	538.72	1	1424303	0.04	421	0.0006	1	0.015	15	0.0013
			0.5	2	137.64	275.28			1					1		15	
	2-1	2	0.5	1	648.23	1296.46	1308.61	2617.22	1	1424303	0.18	2500	0.0035	1	0.015	15	0.0075
			0.5	2	660.38	1320.76			1					1		15	
	2-2	2	0.5	1	555.15	1110.3	1140.07	2280.14	1	1424303	0.16	2163	0.0030	1	0.015	15	0.0065
			0.5	2	584.92	1169.84			1					1		15	
	2-3	2	0.5	1	480.02	960.04	976.84	1953.68	1	1424303	0.14	1836	0.0026	1	0.015	15	0.0055
			0.5	2	496.82	993.64			1					1		15	
	3-1	2	0.5	1	681.98	1363.96	1393.35	2786.7	1	1424303	0.20	2669	0.0037	1	0.015	15	0.0080
			0.5	2	711.37	1422.74			1					1		15	
	3-2	2	0.5	1	623.05	1246.1	1257.18	2514.36	1	1424303	0.18	2397	0.0033	1	0.015	15	0.0072
			0.5	2	634.13	1268.26			1					1		15	
	3-3	2	0.5	1	561.64	1123.28	1130.4	2260.8	1	1424303	0.16	2143	0.0030	1	0.015	15	0.0064
			0.5	2	568.76	1137.52			1					1		15	
	4-1	2	0.5	1	680.69	1361.38	1346.59	2693.18	1	1424303	0.19	2576	0.0036	1	0.015	15	0.0077
			0.5	2	665.9	1331.8			1					1		15	
	4-2	2	0.5	1	486.54	973.08	985	1970	1	1424303	0.14	1852	0.0026	1	0.015	15	0.0056
			0.5	2	498.46	996.92			1					1		15	
	4-3	2	0.5	1	718.65	1437.3	1465.69	2931.38	1	1424303	0.21	2814	0.0039	1	0.015	15	0.0085
			0.5	2	747.04	1494.08			1					1		15	
	5-1	2	0.5	1	856.23	1712.46	1724.34	3448.68	1	1424303	0.24	3331	0.0047	1	0.015	15	0.0100
			0.5	2	868.11	1736.22			1					1		15	
	5-2	2	0.5	1	750.85	1501.7	1509.68	3019.36	1	1424303	0.21	2902	0.0041	1	0.015	15	0.0087
			0.5	2	758.83	1517.66			1					1		15	
	5-3	2	0.5	1	663.66	1327.32	1341.83	2683.66	1	1424303	0.19	2566	0.0036	1	0.015	15	0.0077
			0.5	2	678.17	1356.34			1					1		15	
	6-1	2	0.5	1	1727.08	3454.16	3487.81	6975.62	1	1424303	0.49	6858	0.0096	1	0.015	15	0.0206
			0.5	2	1760.73	3521.46			1					1		15	
	6-2	2	0.5	1	1450.84	2901.68	2965.54	5931.08	1	1424303	0.42	5814	0.0081	1	0.015	15	0.0175
			0.5	2	1514.7	3029.4			1					1		15	
	6-3	2	0.5	1	1239.83	2479.66	2563.71	5127.42	1	1424303	0.36	5010	0.0070	1	0.015	15	0.0151
			0.5	2	1323.88	2647.76			1								

		Test Chemical				Microsome						Replicate	
Assay Date	12/14/04	ID	4-OH ASDN	# Concentrations tested	6	type	Placental	Microsome ID	0	Technician ID	JG	#	N/A

Control Type	Portion	Average	SD
Full activity	Beginning	0.0599	0.0085
Full activity	End	0.0132	0.0012
Full activity	Overall	0.0366	0.0274
Background	Beginning	0.0001	7.50732E-05
Background	End	-0.0001	4.82644E-05
Background	Overall	0.0000	8.55192E-05
Positive	Beginning	#VALUE!	#VALUE!
Positive	End	#VALUE!	#VALUE!
Positive	Overall	#VALUE!	#VALUE!
Negative	Beginning	#VALUE!	#VALUE!
Negative	End	#VALUE!	#VALUE!
Negative	Overall	#VALUE!	#VALUE!

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
4-OH ASDN	1	1	1.00E-06	-6.00	0.0015
4-OH ASDN	1	2	1.00E-06	-6.00	0.0016
4-OH ASDN	1	3	1.00E-06	-6.00	0.0013
4-OH ASDN	2	1	1.00E-07	-7.00	0.0075
4-OH ASDN	2	2	1.00E-07	-7.00	0.0065
4-OH ASDN	2	3	1.00E-07	-7.00	0.0055
4-OH ASDN	3	1	5.00E-08	-7.30	0.0080
4-OH ASDN	3	2	5.00E-08	-7.30	0.0072
4-OH ASDN	3	3	5.00E-08	-7.30	0.0064
4-OH ASDN	4	1	2.50E-08	-7.60	0.0077
4-OH ASDN	4	2	2.50E-08	-7.60	0.0056
4-OH ASDN	4	3	2.50E-08	-7.60	0.0085
4-OH ASDN	5	1	1.00E-08	-8.00	0.0100
4-OH ASDN	5	2	1.00E-08	-8.00	0.0087
4-OH ASDN	5	3	1.00E-08	-8.00	0.0077
4-OH ASDN	6	1	1.00E-09	-9.00	0.0206
4-OH ASDN	6	2	1.00E-09	-9.00	0.0175
4-OH ASDN	6	3	1.00E-09	-9.00	0.0151

Percent of control values

Level	Log[test substance]	Replicate 1	Replicate 2	Replicate 3
1	-6.00	4.20	4.29	3.46
2	-7.00	20.53	17.76	15.08
3	-7.30	21.92	19.68	17.60
4	-7.60	21.15	15.21	23.11
5	-8.00	27.35	23.83	21.07
6	-9.00	56.32	47.74	41.14

Appendix H

Statistical Analysis Report (BioSTAT Consultants, Inc.)

Statistical Analysis Summary
WIL Research Laboratories, LLC
Study Protocol – 431006

Les Freshwater
BioSTAT Consultants, Inc.
3261 Lost Pine Way
Portage, MI 49024
(269) 329-7976

Date

BioSTAT Consultants, Inc.
Statement of Quality Control

This report was quality checked in accordance with BioSTAT Procedural Guideline 2.0 (Quality Control Process for Tables and Reports). The statistical methodology and results of inferential statistics were verified by an independent quality control statistician. Based on these documented quality control activities, it is concluded that the statistical results incorporated in this report accurately reflect the statistical analysis of data received by BioSTAT.

1 Statistical Methodology

1.1 Analysis Methodology

Analysis of Variance Comparisons Among Concentration Response Curve Fits

Slope (β) and log10IC50 (μ) were subjected to a random effects analysis of variance. Response variable was β or μ and replicates were treated as random effects. β and μ were estimated, separately within each replicate, and plotted along with the average across replicates (LSMean) and associated 95% confidence interval across replicates.

Full Enzyme Activity and Background Activity Percent of Control Values Across Replicates

For the full enzyme activity control tubes and the background activity tubes a two way analysis of variance was conducted. The response variable was percent of control aromatase activity. Fixed effect factors in the model included replicate, portion (beginning or end) and the replicate by portion interaction.

If the replicate by portion interaction was significant (at the 0.05 level) the nature of the effect was assessed by comparing the portion effect within each replicate to the portion effect averaged across replicates, adjusting for simultaneity by Bonferroni's method.

P-values for replicate, portion and replicate by portion are presented in the table. In addition, estimates for the LSMeans, standard errors and 95% confidence intervals are presented for percent of control aromatase activity within each replicate and averaged across replicates.

2 Results

Results of statistical analyses are summarized in Section 3.

References

SAS® Proprietary Software, Version 8.2; SAS Institute Inc.: Cary, NC, 1999-2001.

3 Statistical Analysis Summaries

Table 3.1: Analysis of Variance Comparisons Among Concentration Response Curve Fits

		Replicate			
Parameter	Statistic	1	2	3	Overall
Slope (ß)	Prism Best Fit Value (S.E.)	-1.048 (0.024)	-1.039 (0.023)	-0.934 (0.019)	--
	Prism 95% CI (lower, upper)	(-1.099, -0.997)	(-1.087, -0.991)	(-0.975, -0.894)	--
	LSM (LSM s.e.)	--	--	--	-1.007 (0.036)
	95% CI (lower, upper)	--	--	--	(-1.164, -0.850)
log10IC50 (µ)	Prism Best Fit Value (S.E.)	-7.286 (0.011)	-7.323 (0.010)	-7.370 (0.011)	--
	Prism 95% CI (lower, upper)	(-7.309, -7.263)	(-7.344, -7.301)	(-7.392, -7.347)	--
	LSM (LSM s.e.)	--	--	--	-7.326 (0.024)
	95% CI (lower, upper)	--	--	--	(-7.431, -7.222)

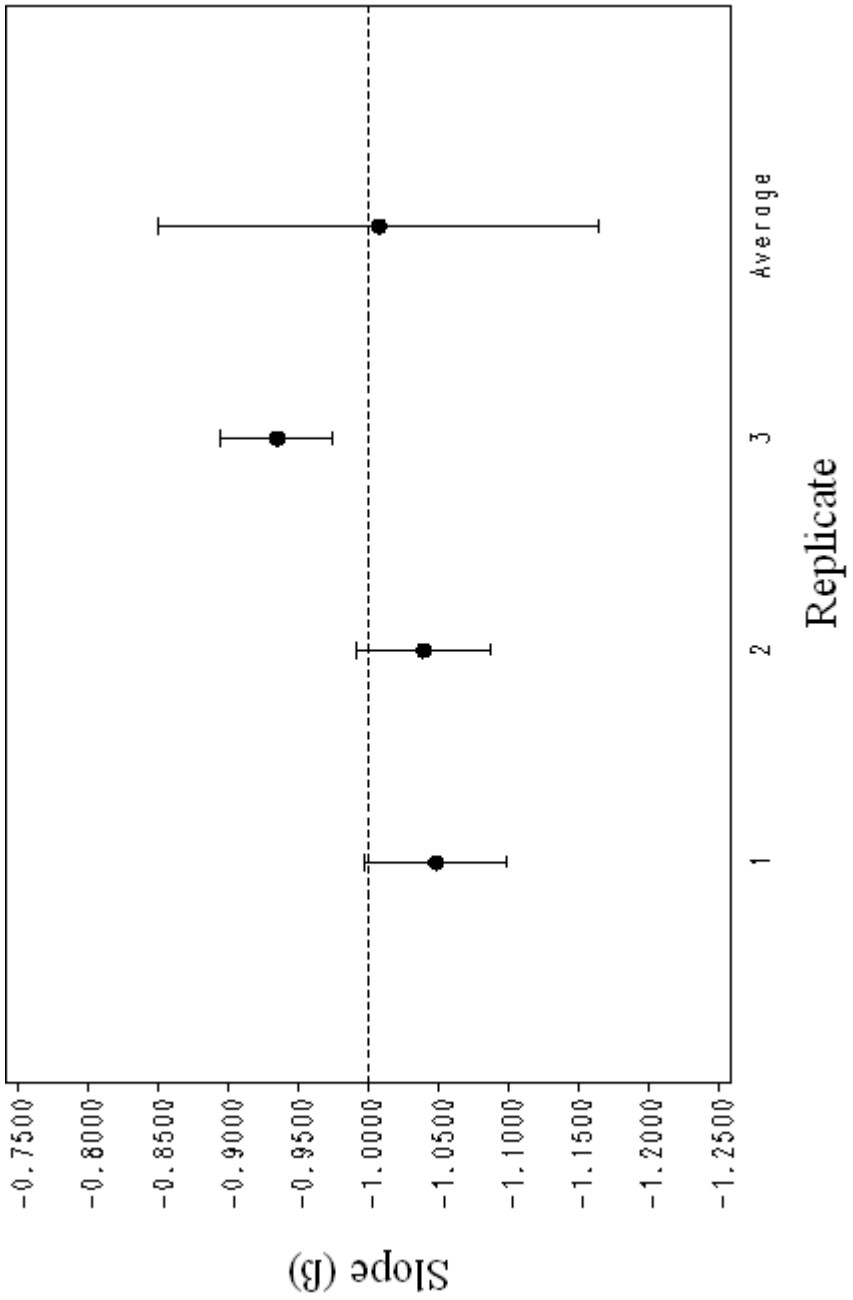


Figure 3.2: Estimates for Slope and Confidence Intervals for all Replicates

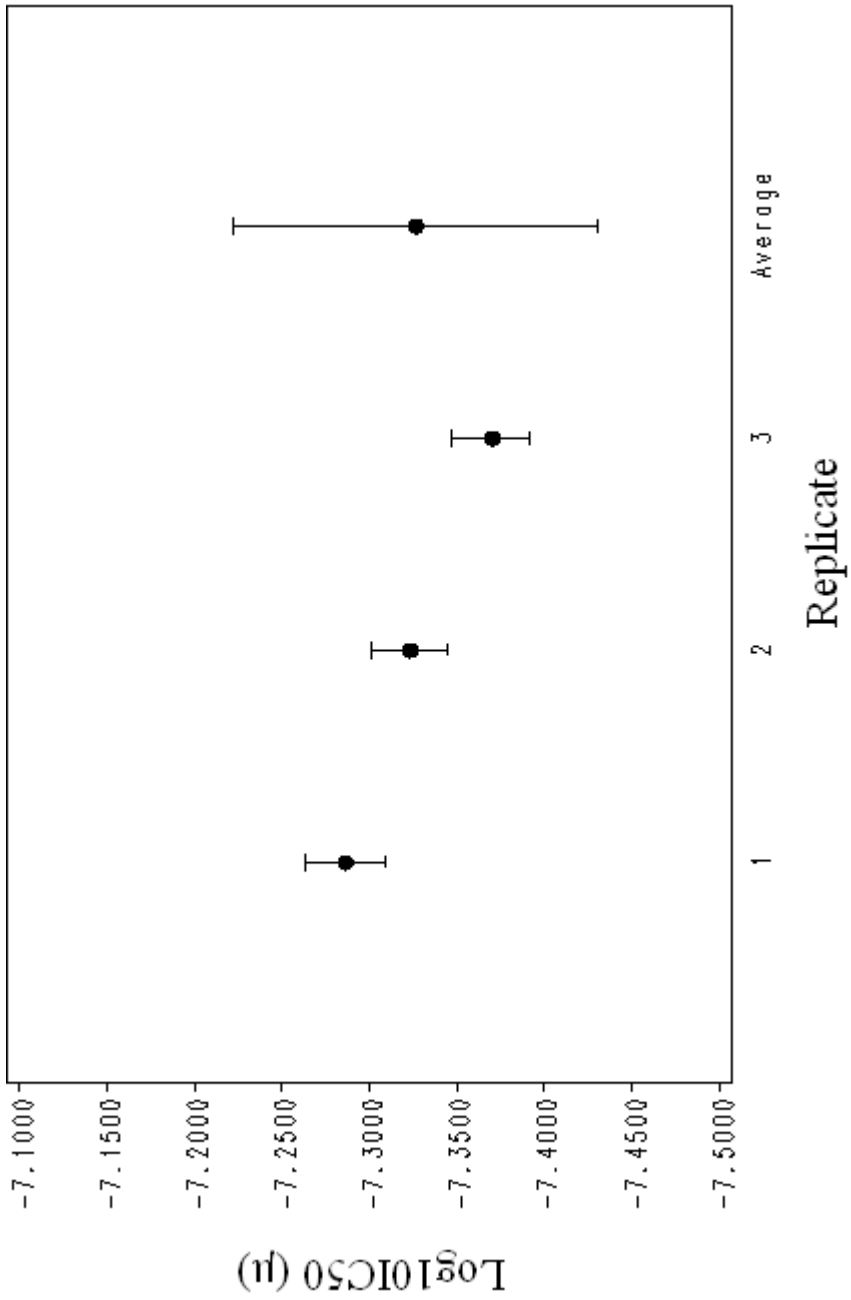


Figure 3.3: Estimates for Log10IC50 and Confidence Intervals for all Replicates

Table 3.4: Full Enzyme Activity and Background Activity Percent of Control Values Across Replicates

Activity	Portion	Statistic	Replicate 1	Replicate 2	Replicate 3	Overall
Background	Beginning	LSM (LSM s.e.)	-0.020 (0.038)	0.020 (0.038)	-0.006 (0.038)	-0.002 (0.022)
		95% CI (lower, upper)	(-0.113, 0.074)	(-0.073, 0.113)	(-0.099, 0.087)	(-0.056, 0.052)
	End	LSM (LSM s.e.)	0.020 (0.038)	-0.020 (0.038)	0.006 (0.038)	0.002 (0.022)
		95% CI (lower, upper)	(-0.074, 0.113)	(-0.113, 0.073)	(-0.087, 0.099)	(-0.052, 0.056)
	Overall	LSM (LSM s.e.)	0.000 (0.027)	0.000 (0.027)	0.000 (0.027)	
		95% CI (lower, upper)	(-0.066, 0.066)	(-0.066, 0.066)	(-0.066, 0.066)	
	ANOVA P-Values	Replicate = 1.0000 Portion = 0.9075 Portion*Replicate = 0.6011				
Full Enzyme	Beginning	LSM (LSM s.e.)	92.739 (7.130)	99.346 (7.130)	104.949 (7.130)	99.011 (4.117)
		95% CI (lower, upper)	(75.292, 110.186)	(81.898, 116.793)	(87.502, 122.397)	(88.938, 109.084)
	End	LSM (LSM s.e.)	107.261 (7.130)	100.654 (7.130)	95.050 (7.130)	100.989 (4.117)
		95% CI (lower, upper)	(89.814, 124.708)	(83.207, 118.102)	(77.603, 112.498)	(90.915, 111.062)
	Overall	LSM (LSM s.e.)	100.000 (5.042)	100.000 (5.042)	100.000 (5.042)	
		95% CI (lower, upper)	(87.663, 112.337)	(87.663, 112.337)	(87.663, 112.337)	
	ANOVA P-Values	Replicate = 1.0000 Portion = 0.7457 Portion*Replicate = 0.3024				