# HUMAN RECOMBINANT MICROSOMAL AROMATASE ASSAY VALIDATION STUDY: POSITIVE CONTROL STUDY

## WA 4-17, Task 3

## OVERALL TASK DRAFT FINAL REPORT

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**Study Initiation Date:**

September 8, 2004 (Work Plan Submission)

**Task Initiation Date:**

December 7, 2004 (QAPP Approved)

**Experimental Performance Dates:**

December 28, 2004 to April 8, 2005

**Overall Draft Final Report Date:**

January 27, 2006

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**Title:** Human Recombinant Microsomal Aromatase Assay Validation Study: Positive Control Study


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## EXECUTIVE SUMMARY

The aromatase assay using human recombinant microsomes was conducted by staff from three participating laboratories (Battelle, In Vitro Technologies, and WIL Research Laboratories). The identities of the laboratories were coded as Labs A, B, and C for presentation of the results (order of labs above is a different order than listed). A positive control study design was followed in which three repetitions at each of six concentrations of a known aromatase inhibitor, 4-hydroxyandrostenedione (4-OH ASDN), were tested in at least three independent replicates. The objectives of this task were to evaluate the responsiveness of the human recombinant microsomal aromatase assay when performed by the participating laboratories and to obtain intralaboratory and interlaboratory values for aromatase enzyme activity and aromatase inhibition (IC50).

The human recombinant microsomal protein concentration (mean ± SEM, % CV) was determined to be 4.77 ± 0.55 (20.0 %), 6.64 ± 0.41 (10.7 %), and 5.68 ± 0.26 (9.2 %) mg/mL for Labs A, B, and C, respectively. The overall task mean ± SEM protein concentration was 5.70 ± 0.54 mg/mL (% CV = 16.4).

The human recombinant aromatase activity (mean ± SEM, % CV) was determined to be 0.216 ± 0.038 (30.1 %), 0.284 ± 0.022 (13.2 %), and 0.187 ± 0.007 (7.6 %) nmol/mg protein/min for Labs A, B, and C, respectively. The overall task mean ± SEM aromatase activity was 0.229 ± 0.029 nmol/mg protein/min (% CV = 21.7).

Increasing concentrations of 4-OH ASDN decreased the activity (as a percent of control) of the human recombinant microsomal aromatase activity and the decrease was dose-dependent. At $10^{-6}$ M 4-OH ASDN, aromatase inhibition was almost complete; the laboratory percent of control values ranged from 7 to 9 percent. At $10^{-9}$ M 4-OH ASDN, there was little to no aromatase inhibition; the laboratory percent of control values ranged from 93 to 100 percent. Overall task mean ± SEM percent of control values at $10^{-6}$ and $10^{-9}$ M 4-OH ASDN were 8.47 ± 0.85 and 95.3 ± 2.3 percent, respectively. The overall percent CV values over all six concentrations by laboratory were less than 6, 7, and 16 percent for laboratories A, B, and C, respectively. The overall task percent CV values ranged from 2 to 17 percent.

The 4-OH ASDN $IC_{50}$ values (mean ± SEM, % CV) were determined to be 89.3 ± 3.8 (7.3 %), 65.5 ± 3.8 (10.1 %), and 83.9 ± 6.7 (16.1 %) for Laboratories A, B, and C, respectively. The overall task mean ± SEM $IC_{50}$ value was 79.5 ± 7.2 (% CV = 15.7).

The principal results of the interlaboratory analysis are summarized below.

a) The estimates for $\log_{10}IC_{50}$ were consistent among the three laboratories. The coefficient of variation among laboratories was about 4% whether replicate 1 in Laboratory C was included or excluded. The averages and associated 95% CIs were about same including and excluding replicate 1 in Laboratory C. The variance among laboratories was about 1.4 times higher than the average within laboratory variance when replicate 1 in Laboratory C was included, and was about 0.8 times the average within laboratory variance when replicate 1 in Laboratory C was excluded.

b) The estimated slope for Laboratory B was slightly smaller than those for Laboratories A and C, whether replicate 1 in Laboratory C was included or excluded. The coefficient of variation among laboratories was less than 5% whether replicate 1 in Laboratory C was included or excluded. The variance among laboratories was about four times higher than the average within laboratory variance when replicate 1 in Laboratory C was included and was about three times higher than the average within laboratory variance when replicate 1 in Laboratory C was excluded.

c) Average background activity control was not significantly different at the end and at the beginning of each replicate, for each laboratory or across the three laboratories. The estimated variance among the laboratories was zero or near zero.

d) Averaged across replicates, full enzyme activity control was significantly lower at the end than at the beginning in Laboratories A and C whether replicate 1 in Laboratory C was included or excluded. Full enzyme activity control was not significantly lower at the end than at the beginning in Laboratory B or combined across the three laboratories. The estimated variance among the laboratories was nearly three times of the average within laboratory variance when replicate 1 in Laboratory C was included and was nearly two times of the average within laboratory variance when replicate 1 in Laboratory C was excluded.

In conclusion, the results from this task indicated that the human recombinant aromatase assay was sufficiently responsive for the laboratories to demonstrate the effects of a known aromatase inhibitor using the provided assay procedure. Also, this task provided information about the assay's intralaboratory and interlaboratory variability.

# 1.0 INTRODUCTION

## 1.1 Background

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

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

Aromatase is a cytochrome $P450_{arom}$ enzyme complex responsible for estrogen biosynthesis and converts androgens, such as testosterone and androstenedione, into the estrogens estradiol and estrone. Aromatase is present in the ovary, placenta, uterus, testis, brain, and extraglandular adipose tissues. Two proteins, cytochrome $P450_{arom}$ and NADPH-cytochrome P450 reductase, are necessary for enzymatic activity, and the enzyme complex is localized in the smooth endoplasmic reticulum. The aromatase gene, designated CYP19, encodes the cytochrome $P450_{arom}$ and consists of ten exons, with the exact size of the gene exceeding 70 kilobases. Aromatase is found in breast tissue, and the importance of intratumoral aromatase and local estrogen production is being unraveled. Effective aromatase inhibitors have been developed as therapeutic agents for estrogen-dependent breast cancer to reduce the growth stimulatory effects of estrogens in breast cancer. Investigations on the development of aromatase inhibitors began in the 1970's and have expanded greatly in the past three decades.

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

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

The human recombinant 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 human recombinant aromatase (WA 2-24) were conducted to optimize the microsomal aromatase assay protocol for human recombinant microsomes, demonstrate the utility of the microsomal assay to detect known aromatase inhibitors, and compare the performance of a human recombinant assay system and the placental microsomal assays.

## 1.2 Task Description and Objectives

In this task, the aromatase assay was conducted by staff from three participating laboratories (Battelle, In Vitro Technologies, and WIL Research Laboratories) but not by the lead laboratory (Research Triangle Institute, RTI). Each of the participating laboratories conducted at least three independent replicates of the aromatase assay by following a positive control study design. The positive control was the known aromatase inhibitor 4-hydroxyandrostenedione (4-OH ASDN). The human recombinant microsomes were procurred and distributed to the participating laboratories by RTI. In the positive control study design, three repetitions at each of six concentrations of the 4-OH ASDN were tested for each of the three independent replicates. Reagents and assay solutions were made fresh for each replicate so that the replicates were truly independent. The 4-OH ASDN was prepared and analyzed at a central laboratory (Chemical Repository at Battelle) before it was distributed to the participating laboratories, where this stock formulation was used to prepare the working dilutions used in the conduct of the assay.

The objectives of this task were to evaluate the responsiveness of the human recombinant microsomal aromatase assay when performed by three participating laboratories that had the capabilities but only limited experience with this assay and to obtain intralaboratory and

interlaboratory values for aromatase enzyme activity and aromatase inhibition ($IC_{50}$) when using the known aromatase inhibitor 4-OH ASDN as a positive control.

### 1.3 Overall Report Content and Format

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

The laboratories that conducted the experiments of this task are coded in those sections of the overall report where laboratory performance is described or data are presented. Coded presentation is used so that the data could be evaluated in an unbiased manner and, whatever the outcome of the study, there would be no connotation, favorable or otherwise, put on the laboratories.

## 2.0 MATERIALS AND METHODS

### 2.1 Chemistry

#### 2.1.1 Substrate – Androstenedione (ASDN)

The substrate for the assay was androstenedione (ASDN). Non-radiolabeled and radiolabeled ASDN were used. The non-radiolabeled ASDN had a reported purity of 100%. The radiolabeled androstenedione ([1β-$^3$H]-androstenedione, [$^3$H]ASDN) had a reported specific activity of 25.3 Ci/mmol. Radiochemical purity was reported by the supplier to be > 97%. Radiochemical purity was assessed by high performance liquid chromatography (HPLC) by the lead laboratory and the results are included in the individual laboratory report appendices.

#### 2.1.2 Test Substance – 4-Hydroxyandrostenedione (4-OH ASDN)

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

**Table 1. Chemistry Information for 4-OH ASDN**

| Chemical Name | Chemical Code | Mfr. Purity | CAS No. | Molecular Formula | Molecular Weight (g/mol) |
|---|---|---|---|---|---|
| 4-hydroxyandrostenedione | 4-OH ASDN | 99% | 566-48-3 | $C_{19}H_{26}O_3$ | 302.4 |

The 4-OH ASDN stock formulation was prepared by the Chemical Repository as a 0.01 M solution in ethanol. The participating laboratories prepared fresh dilutions of the stock formulation using ethanol (supplied by the CR) according to the procedure described in Table 2.

**Table 2. Preparation of 4-OH ASDN Dilutions**

| 4-OH ASDN Stock Formulation Concentrations (mM) | | Volume of Stock (µL) | Volume of Ethanol (µL) | Dilution Number & Concentrations (mM) | | Final Concentration in the Assay (M) |
|---|---|---|---|---|---|---|
| CR Stock[a] | 10 | 100 | 900 | A | 1.0 | N/A[b] |
| Working Stock #1 | 1.0 | 100 | 900 | 1 | 0.1 | $1 \times 10^{-6}$ |
| Working Stock #2 | 0.1 | 100 | 900 | 2 | 0.01 | $1 \times 10^{-7}$ |
| | | 50 | 950 | 3 | 0.005 | $5 \times 10^{-8}$ |
| | | 25 | 975 | 4 | 0.0025 | $2.5 \times 10^{-8}$ |
| Working Stock #3 | 0.01 | 100 | 900 | 5 | 0.001 | $1 \times 10^{-8}$ |
| Working Stock #4 | 0.001 | 100 | 900 | 6 | 0.0001 | $1 \times 10^{-9}$ |

a. Chemical Respository stock formulation.
b. Not applicable.

## 2.2 Human Recombinant Microsomes

Human recombinant microsomes were provided by RTI, who procured the microsomes from Gentest (Human CYP19 + P450 Reductasee SUPERSOMES, Woburn, MA). The microsomes were stored at approximately -70°C until the time of the assay. On the day of use, the microsomes were thawed rapidly in a 37 ± 1°C water bath, rehomogenized using a Potter-Elvejhem homogenizer and then kept on ice until used. The reported protein concentration was approximately 4.9 mg/mL. For use in the assay, the microsomes were diluted in the assay buffer in two serial dilutions in order to achieve the desired final working stock concentration of approximately 0.008 mg/mL. The final target protein concentration in the incubation mixture was approximately 0.004 mg/mL.

## 2.3 Other Assay Components

Information about the other assay components is provided in Table 3. The Chemical Repository obtained the NADPH (β-Nicotinamide Adenine Dinucleotide Phosphate, reduced form) and ethanol. The NADPH used was from the same supplier and lot for the participating laboratories, whereas the ethanol used was from two different suppliers for at least one of the laboratories.

**Table 3. Other Assay Components**

| Component | Supplier | | |
|---|---|---|---|
| | Battelle | In Vitro | WIL |
| NADPH (co-factor)[a] | Sigma-Aldrich | Sigma-Aldrich | Sigma-Aldrich |
| Propylene glycol | Spectrum Chemical | J. T. Baker | J. T. Baker |
| Sodium phosphate dibasic (buffer) | Sigma-Aldrich | J. T. Baker | J. T. Baker |
| Sodium phosphate monobasic (buffer) | Sigma-Aldrich | J. T. Baker | J. T. Baker |

a. Supplied by the EDSP Chemical Repository at Battelle.

## 2.4 Protein Determination

The microsomal protein concentration was determined on each day the microsomes were used in the assay. A DC Protein Assay kit from Bio-Rad (Hercules, CA) was used for protein determination. The 6-point standard curve was prepared using bovine serum albumin (BSA) reconstituted in Milli-Q water. The standard curve range was from 0.12 to 1.5 mg protein/mL (varied slightly for each laboratory). 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.

## 2.5 Aromatase Assay Procedure

Details of how the assay was actually performed by each participating laboratory are presented in the individual laboratory reports. The general procedure is presented as follows. The assays were performed in test tubes maintained at 37 ± 1°C in a shaking water bath. Propylene glycol, [$^3$H]ASDN, NADPH, and assay buffer were combined in the test tubes with or without inhibitor to the total volume of 1.0 mL. The final concentrations for the assay major components are presented in Table 4. The tubes and the microsomal suspension were placed at 37 ± 1°C in the water bath for approximately 5 minutes prior to initiation of the assay by the addition of 1 mL of the diluted microsomal suspension.

**Table 4. Aromatase Assay Conditions using Human Recombinant Microsomes**

| Assay Components | Component Volume Added to the Assay | Final Concentration in the Assay |
|---|---|---|
| Microsomes | 1.0 mL | 0.004 mg/mL |
| NADPH | 100 µL | 0.3 mM |
| [$^3$H]ASDN | 100 µL | 100 nM |
| Propylene glycol | 100 µL | 5% (v/v) |
| 4-OH ASDN or vehicle (control) | 20 µL | $10^{-6}$ to $10^{-9}$ M |
| Buffer | 680 µL | Not Applicable |

The total assay volume was 2.0 mL and the tubes were incubated for 15 minutes at 37 ± 1°C. The incubations were stopped by the addition of methylene chloride (2 mL); the tubes were vortex-mixed for ca. 5 seconds and placed on ice. The tubes were then vortex-mixed an

additional 20-25 seconds to extract unreacted ASDN, then centrifuged for 10 minutes to facilitate separation of the organic and aqueous layers. The methylene chloride layer was removed and discarded; the aqueous layers were extracted two more times, each time with 2 mL of methylene chloride. The aqueous layers were transferred to vials and duplicate aliquots (0.5 mL) were transferred to 20 mL liquid scintillation counting vials. Liquid scintillation cocktail was added to each counting vial and the vials shaken to mix.

Analysis of the samples was performed using liquid scintillation spectrometry (LSS). Radioactivity found in the aqueous fractions 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 was determined by dividing the total amount of $^3H_2O$ formed by the specific activity of the [$^3$H]ASDN substrate (expressed in dpm/nmol). Results are presented as the activity (velocity) of the enzyme reaction and expressed in nmol (mg protein)$^{-1}$min$^{-1}$.

Each laboratory performed at least three independent replicates and, for a given replicate, each inhibitor concentration was performed in triplicate. In each replicate, full enzyme activity and background activity control samples were included. Full enzyme activity control samples contained substrate ([$^3$H]-ASDN), NADPH, propylene glycol, buffer, vehicle used for preparation of 4-OH ASDN solutions, and microsomes. Background activity controls contained the same components as for the full enzyme activity control samples except for the NADPH. Four full enzyme activity and four background activity controls were included with each replicate of the assay run and were processed in the same manner as the other samples. The controls sets were split, so that two tubes (for each full and background activity control set) were run at the beginning, and two at the end of each assay. The study design is summarized in Table 5.

**Table 5. Positive Control Study Design**

| Sample Type | Repetitions (Test Tubes) | Description | Final 4-OH ASDN Concentration (M) |
|---|---|---|---|
| Full Enzyme Activity Control | 4 | Complete assay[a] with inhibitor vehicle control | N/A |
| Background Activity Control | 4 | Complete assay with inhibitor vehicle control omitting NADPH | N/A |
| 4-OH ASDN Concentration 1 | 3 | Complete assay with 4-OH ASDN added | $1 \times 10^{-6}$ |
| 4-OH ASDN Concentration 2 | 3 | Complete assay with 4-OH ASDN added | $1 \times 10^{-7}$ |
| 4-OH ASDN Concentration 3 | 3 | Complete assay with 4-OH ASDN added | $5 \times 10^{-8}$ |
| 4-OH ASDN Concentration 4 | 3 | Complete assay with 4-OH ASDN added | $2.5 \times 10^{-8}$ |
| 4-OH ASDN Concentration 5 | 3 | Complete assay with 4-OH ASDN added | $1 \times 10^{-8}$ |
| 4-OH ASDN Concentration 6 | 3 | Complete assay with 4-OH ASDN added | $1 \times 10^{-9}$ |

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

## 2.6 Data Analysis

Each participating laboratory analyzed their data using a spreadsheet developed and validated by RTI and Battelle. The spreadsheet was provided to the participating laboratories for processing the relevant data into final data (aromatase activity and percent of control), which could then be used for evaluating the results. The final spreadsheets are included in the appendices of the individual laboratory reports. Data recorded included the assay date and run number, technician, chemical and log chemical concentration, total dpm-background dpm and percent activity, as well as many other intermediate calculations. The individual calculation steps used to arrive at the enzyme activity and percent of control values are described in the appendices of the individual laboratory reports. The final values were used to calculate the averages, standard deviations, standard errors of the mean, and coefficient of variance in order to assess the variation among repetitions (within a single replicate) and between replicates.

$IC_{50}$ was calculated using Prism (version 4.0 or higher, GraphPad, San Diego, CA). Percent of control activity data was exported to Prism for curve fitting of the percent of control activity versus log of 4-OH ASDN concentration data using the following equation:

$$Y=100/(1+10^{((Log_{10}IC_{50}-X)*HillSlope)})$$

Where: X is the logarithm of 4-OH ASDN concentration (M)
Y is the percent activity.

The software incorporated a weighting factor for the percent of activity values of 1/Y. As shown in the equation, the curve fitting equation uses the fixed value of 100 as the numerator. 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%. Concentration response fits were carried out for each replicate. The resultant $\mu$ (log $IC_{50}$) and slope were analyzed using a one-way random effects analysis of variance model. For each replicate the estimated $\log_{10}IC_{50}$ ($\mu$), within replicate standard error of $\mu$, $IC_{50}$, slope ($\beta$), within replicate standard error of $\beta$, and the "Status" of each response curve were determined. Other details of the intralaboratory data and statistical analysis are described in the individual participating laboratory reports included in the appendices.

## 2.7 Statistical Analysis

### 2.7.1 Intralaboratory Statistical Analysis

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

Intralaboratory statistical analyses were carried out on the "percent of control" responses. Percent of control is defined as the ratio of the background adjusted aromatase activity in the tube under consideration to the average background adjusted aromatase activity among the four full enzyme activity control tubes within the replicate, times 100. The average percent of control among the four full enzyme activity control tubes is necessarily 100 percent within each replicate. The average percent of control among the four background activity control tubes is necessarily 0 percent.

Nominally for an inhibitor the percent of control activity values vary between approximately 0% near the high inhibition concentrations and approximately 100% near the low inhibition concentrations, but this may vary with the inhibitor.

Intralaboratory statistical analyses were performed based on a common analysis plan. The following results were reported in each intralaboratory analysis.

1. Concentration curve fits within each replicate to describe the trend in the percent of control activity across varying inhibitor concentrations of test substance 4-OH ASDN.

2. Estimates of the $\log_{10}IC_{50}$ concentration, slope, and associated standard errors within each replicate.

3. Average $\log_{10}IC_{50}$ concentration, average slope, and associated standard errors across replicates.

4. Comparisons between the full enzyme activity and background activity controls obtained at the beginning and those obtained at the end of each replicate.

Laboratories A and B reported results based on three replicates. Laboratory C reported results based on replicates 1 to 4 as well as results based on replicates 2 to 4. The reported standard errors of the average results across replicates for Laboratories A and C incorporated the among replicate component of variation. The reported standard error for laboratory B did not.

### 2.7.2 Interlaboratory Statistical Analysis

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

The "interlaboratory" statistical analysis combines summary values developed in each of the intralaboratory analyses to assess relationships among the results at each laboratory, the extent of laboratory-to-laboratory variation, and overall consensus estimates among the laboratories with associated variability estimates (incorporating laboratory-to-laboratory variability). The interlaboratory analysis is based on the average $\log_{10}IC_{50}$ and slope parameters of the concentration response curve fits determined by each of the test laboratories, as reported in the intralaboratory analyses. The interlaboratory analysis also compares among laboratories the full enzyme activity control results and the background activity control results obtained at the end of each replicate with those obtained at the beginning.

The objectives of the interlaboratory statistical analysis are to:

- Determine the average values and the variabilities among laboratories for the parameters mentioned above.
- Determine the coefficients of variation among laboratories for the $\log_{10}IC_{50}$ and the slope parameters.
- Estimate the ratio of the among laboratory variation to the average within laboratory variation for the parameters mentioned above.

The interlaboratory analyses were performed on two versions of the data:

- Including all the data
- Excluding replicate 1 from laboratory C.

Statistical analyses were carried out for each of the four endpoints discussed above in the Test Organization section: $\log_{10}IC_{50}$, slope, portion effect (beginning minus end) for background activity control, and portion effect for full enzyme activity control.

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

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

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

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

It should be noted that when calculating the within laboratory mean $\log_{10}IC_{50}$ and slope across replicates, Laboratories A and C incorporated the replicate-to-replicate component of variation in the standard errors of the averages, while laboratory B did not. Also Laboratories A and C calculated the differences between beginning and end (beginning minus end) when

comparing the full enzyme activity and background activity controls obtained at the beginning and those obtained at the end of each replicate, whereas laboratory B reported only beginning and end values. The sums of beginning and end values must be equal to 0 for the background activity controls and 200 for full enzyme activity controls. Therefore, for laboratory B the differences were calculated as $-2 \times$ (End values) for the background activity controls and as 200 $-$ 2 x (End values) for the full enzyme activity controls. The associated standard errors for these differences were calculated as $2 \times$ (Standard errors associated with the end values).

To describe the variability among the individual laboratory values relative to the overall average value, coefficients of variation (CV) and their associated 95% confidence intervals (95% CIs) were calculated for the $\log_{10}IC_{50}$ and the slope parameters. The coefficient of variation is defined as the standard deviation of the effect response divided by its mean. The methods for calculating the CV and the associated 95% CI were different depending on the underlying assumption about the distribution of the endpoint parameter.

For $\log_{10}IC_{50}$, the measurements were assumed to be approximately log normally distributed. The CV therefore is expressed as

$$CV = [\exp(S^2)-1]^{1/2}$$

where $S^2$ is the total variance among the three laboratories. $S^2$ is approximated by $3(se)^2$ where se is the standard error of the pooled mean estimate. This would be exact if the within laboratory variances were equal across laboratories.

The 95% confidence interval is based on the chi square distribution and is calculated as

$$[(\exp(df*S^2/(\chi^2_{df,\,0.975}))- 1)^{1/2}, (\exp(df*S^2/(\chi^2_{df,\,0.025}))- 1)^{1/2}]$$

where df is the estimated degree of freedom among the three laboratories.

For slope ($\beta$), the measurements are assumed to be approximately normal. The CV therefore is expressed as

$$CV=S/\beta_{avg}$$

where $S^2$ is the total variance among the three laboratories, defined as above and $S = \sqrt{S^2}$. The endpoints of the confidence interval for CV are based on the noncentral t distribution (Lehman, 1986).

To describe the variability among laboratories relative to variability within laboratories the ratio of the variance between laboratories to the average variance within laboratories was calculated as

$$R=S^2_{lab}\,[1/3(s_1^2 + s_2^2 + s_3^2)]$$

where $S^2_{lab}$ is the variance among the three laboratories and ($s_1^2$, $s_2^2$, $s_3^2$) are the squares of the within laboratory standard errors at the three laboratories. A confidence interval for this ratio is based on the F-distribution with ($\nu_{lab}$, $\nu_{wi}$) degree of freedom

$$[R/F^{-1}(0.975), R/F^{-1}(0.025)]$$

where $\nu_{lab}$=2 and $\nu_{wi}$ is based on Satterthwaite's approximation

$$\nu_{wi} \approx [(s_1^2 + s_2^2 + s_3^2)^2]/[s_1^4/\nu_1 + s_2^4/\nu_2 + s_3^4/\nu_3].$$

This ratio is calculated for each of the four endpoint parameters.

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

### 2.8 Good Laboratory Practices

The toxicology laboratories at Battelle and WIL Research Laboratories are operated in compliance with the U.S. EPA FIFRA Good Laboratory Practices Standards, whereas In Vitro Technologies follows the U.S. FDA Good Laboratory Practices Standards. Thus, these studies were conducted in compliance with EPA FIFRA or FDA Regulations for GLPs.

### 2.9 Personnel

A complete list of the personnel involved in the conduct of this task is included in their respective laboratory reports (see appendices). The study directors for this study at each of the laboratories were:

- Dr. Bozena Lusiak – Battelle Memorial Institute
- Dr. Neil Jensen – In Vitro Technologies, Inc.
- Dr. Christopher Bowman – WIL Research Laboratories, LLC

## 3.0 RESULTS

### 3.1 [$^3$H]-ASDN Radiochemical Purity

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

### 3.2 4-OH ASDN Analysis

The actual 4-OH ASDN stock formulation concentration was within 5 percent of the target concentration (3.02 mg/mL, 0.01 M). The formulation was determined to be stable when stored refrigerated for at 173 days (25 weeks). The chemistry report is included as an appendix of the individual laboratory reports.

## 3.3 Microsomal Protein Analysis

The microsomal protein concentration was determined on the day that the microsomes were used in the assay. Two of the laboratories reported analysis results for three replicates whereas the third laboratory reported analysis results for four replicates (Table 6). The overall task mean ± SEM protein concentration was 5.70 ± 0.54 mg/mL with a percent CV of 16.4 percent. According to the lead laboratory, the human recombinant microsomes protein concentration was approximately 4.9 mg/mL. The overall mean from the laboratories was compared to the lead laboratory value (assumed to be the standard), which resulted in a percent relative error (%RE) of +16.3 percent.

**Table 6. Protein Concentration[a]**

| Replicate | Protein Concentration (mg/mL) | | |
|---|---|---|---|
| | Lab A | Lab B | Lab C |
| 1 | 5.07 | 6.76 | 6.46 |
| 2 | 5.53 | 7.28 | 5.42 |
| 3 | 3.70 | 5.88 | 5.44 |
| 4 | -- | -- | 5.40 |
| Average | 4.77 | 6.64 | 5.68 |
| Sd | 0.95 | 0.71 | 0.52 |
| SEM | 0.55 | 0.41 | 0.26 |
| % CV | 20.0 | 10.7 | 9.2 |

a. For each laboratory, the number of replicates actually used in the analysis of the task was included.

## 3.4 Human Recombinant Microsomal Aromatase Activity

Full enzyme activity controls were conducted in duplicate repetitions at the beginning and end of each replicate of the assay (a total of four tubes/replicate). Two of the laboratories reported analysis results for three replicates whereas the third laboratory reported analysis results for four replicates. The average full aromatase activity control values for all four repetitions of a given replicate are shown in Table 7 for each laboratory. The overall task mean ± SEM full enzyme activity control value was 0.229 ± 0.029 nmol/mg protein/min with a percent CV of 21.7 percent.

**Table 7. Full (Uninhibited) Aromatase Activity[a]**

| Replicate | Aromatase Activity (nmol/mg protein/min) | | |
|---|---|---|---|
| | Lab A | Lab B | Lab C |
| 1 | 0.177 | 0.272 | 0.186 |
| 2 | 0.180 | 0.254 | 0.170 |
| 3 | 0.291 | 0.326 | 0.188 |
| 4 | | | 0.205 |
| Average | 0.216 | 0.284 | 0.187 |
| Sd | 0.065 | 0.037 | 0.014 |
| SEM | 0.038 | 0.022 | 0.007 |
| % CV | 30.1 | 13.2 | 7.6 |

a. For each laboratory, the number of replicates actually used in the analysis of the task was included.

### 3.4.1 Intralaboratory Statistical Analysis for Full Aromatase Activity Controls

For Laboratory A, the average percent of control responses at the end were lower than at the beginning for all three replicates. When averaged across replicates, there was a highly significant difference between the beginning and the end portions. The end was lower than the beginning. The variation of portion (end vs. beginning) effects among replicates was estimated to be zero.

For Laboratory B, replicate by portion (beginning and end) interactions were not significant.

For Laboratory C, the average percent of control responses at the end was lower than at the beginning for each replicate. The ends were about 5 to 17 percent lower than the beginnings. The average across replicates was highly significantly lower at the end portion than at the beginning (whether or not replicate #1 was included). The variation of portion (end vs. beginning) effects among replicates was zero or near zero.

### 3.4.2 Interlaboratory Statistical Analysis for Full Aromatase Activity Controls

Interlaboratory statistical analysis values are presented in tables and graphs that were placed at the end of the results section in order to keep this information together for the purpose of providing convenience to the reader since so much of this information is interrelated. Tables and figures will be called out in the text to enable the reader to easily find the relevant information.

Full enzyme activity control was significantly higher at the beginning than at the end for Laboratories A and C but not for Laboratory B or combined across the three laboratories (Table 11). This is the case whether replicate 1 in Laboratory C was included or was excluded. The estimated variance among the laboratories was slightly less than three times the average within laboratory variance when replicate 1 in Laboratory C was included and slightly less than twice the average within laboratory variance when replicate 1 in Laboratory C was excluded.

## 3.5 Background Activity

Background enzyme activity controls were conducted in duplicate repetitions at the beginning and end of each replicate of the assay (a total of four tubes/replicate). For all laboratories the aromatase activity in these control samples was negligible, indicating that there was no background activity that interfered with the interpretation of the results.

### 3.5.1 Intralaboratory Statistical Analysis for Background Activity Controls

For Laboratory A, the measurements at the end were less than those at the beginning, in replicates 1 and 2. When averaged across replicates there were not significant differences between the beginning and the end portions.

For Laboratory B, there were no significant differences between the beginning and the end.

For Laboratory C, the measurements at the end were similar to those at the beginning for all replicates except replicate 2. Replicate 2 had a higher average at the beginning due to the higher measurement in a repetition at the beginning. Without this data, the average at the beginning would be similar to that at the end for replicate 2. When averaged across replicates there was not a significant difference between the beginning and the end portions whether or not replicate 1 was included in the analysis.

### 3.5.2 Interlaboratory Statistical Analysis for Background Activity Controls

No significant differences (beginning minus end) existed for the background activity controls, for any laboratory or across the three laboratories combined (Table 11). The estimated variance among the laboratories was negligible (Table 12).

## 3.6 4-OH ASDN Inhibition of Aromatase Activity

The effect of increasing the concentrations of 4-OH ASDN on aromatase activity was determined and the results were expressed as a percent of the control aromatase activity. The individual replicate percent of control results for each laboratory can be found in the appendices. The overall percent of control results by laboratory and the overall percent of control results for the task are summarized in Table 8.

**Table 8. Effect of 4-OH ASDN on Aromatase Activity (Percent of Control)**

| Laboratory | Log 4-OH ASDN Conc (M) | Overall Percent of Control by Laboratory | | | |
|---|---|---|---|---|---|
| | | Mean | Sd | SEM | %CV |
| Laboratory A | -6.00 | 9.35 | 0.55 | 0.32 | 5.92 |
| | -7.00 | 45.23 | 0.61 | 0.35 | 1.35 |
| | -7.30 | 59.87 | 2.71 | 1.57 | 4.53 |
| | -7.60 | 73.14 | 1.23 | 0.71 | 1.69 |
| | -8.00 | 84.22 | 2.72 | 1.57 | 3.23 |
| | -9.00 | 93.52 | 2.74 | 1.58 | 2.93 |
| Laboratory B | -6.00 | 6.77 | 0.50 | 0.29 | 7.45 |
| | -7.00 | 38.96 | 2.06 | 1.19 | 5.28 |
| | -7.30 | 55.85 | 2.42 | 1.40 | 4.33 |
| | -7.60 | 71.99 | 2.23 | 1.29 | 3.10 |
| | -8.00 | 89.19 | 2.19 | 1.26 | 2.46 |
| | -9.00 | 99.83 | 2.05 | 1.18 | 2.05 |
| Laboratory C | -6.00 | 9.29 | 1.48 | 0.74 | 15.89 |
| | -7.00 | 45.83 | 4.95 | 2.48 | 10.81 |
| | -7.30 | 62.83 | 4.44 | 2.22 | 7.06 |
| | -7.60 | 75.30 | 1.69 | 0.85 | 2.25 |
| | -8.00 | 84.90 | 4.53 | 2.27 | 5.34 |
| | -9.00 | 92.61 | 5.04 | 2.52 | 5.44 |
| Overall Task | Log 4-OH ASDN Conc (M) | Overall Mean | Overall sd | Overall SEM | Overall %CV |
| | -6.00 | 8.47 | 1.47 | 0.85 | 17.4 |
| | -7.00 | 43.34 | 3.81 | 2.20 | 8.8 |
| | -7.30 | 59.52 | 3.50 | 2.02 | 5.9 |
| | -7.60 | 73.48 | 1.68 | 0.97 | 2.3 |
| | -8.00 | 86.10 | 2.69 | 1.56 | 3.1 |
| | -9.00 | 95.32 | 3.93 | 2.27 | 4.1 |

The individual 4-OH ASDN inhibition response curves by replicate for each laboratory are reported in the appendices. The overall inhibition response curves by laboratory are shown in Figure 1 and the overall task curve is shown in Figure 2. The curves in these figures are not fitted by the model but are representative of the curve as denoted by the symbols (mean data). For all three laboratories, increasing concentrations of 4-OH ASDN decreased the activity of the human recombinant microsomal aromatase activity and the decrease was dose-dependent. The shape of the enzyme activity vs 4-OH ASDN curve was sigmoidal. At a 4-OH ASDN concentration of $10^{-6}$ M, aromatase inhibition was almost complete; the laboratory percent of control values ranged from 7 to 9 percent. In contrast, at a 4-OH ASDN concentration of $10^{-9}$ M, there was little to no aromatase inhibition; the laboratory percent of control values ranged from 93 to 100 percent. Overall task mean ± SEM percent of control values at $10^{-6}$ and $10^{-9}$ M were 8.47 ± 0.85 and 95.3 ± 2.3 percent, respectively.

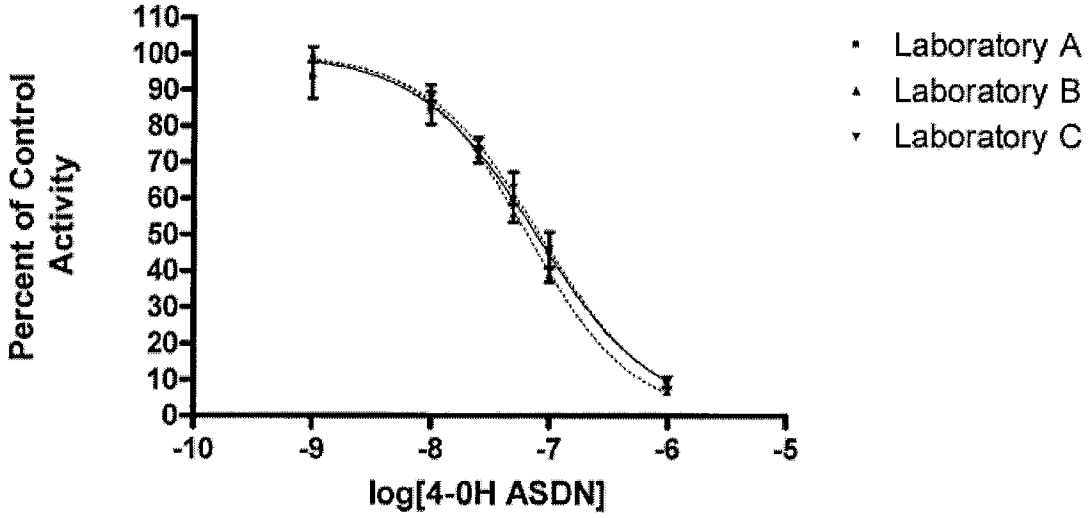


**Figure 1. Overall 4-OH ASDN Inhibition Response Curve by Laboratory**

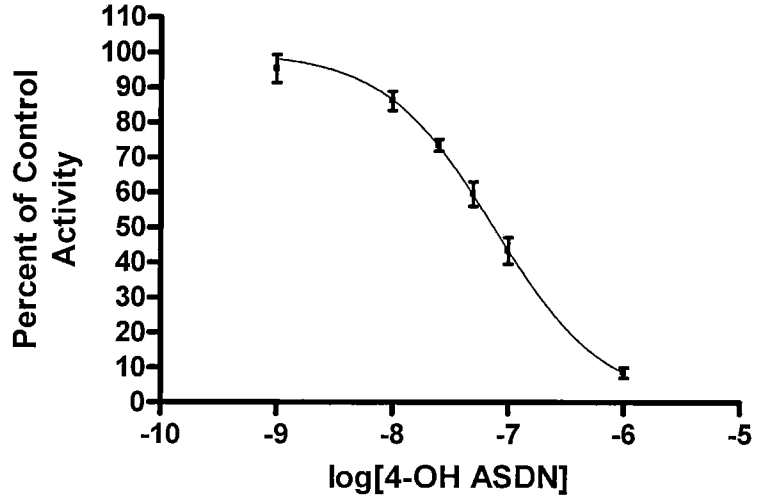


**Figure 2. Overall Task 4-OH ASDN Inhibition Response Curve**

The CV in percent of control for each replicate and laboratory are reported in the appendices. The overall percent CV values by laboratory (Table 8) were less than 6, 7, and 16 percent for laboratories A, B, and C, respectively. The overall task percent CV values ranged from 2 to 17 percent and showed a trend to decrease with decreasing concentrations of the inhibitor down to approximately $10^{-8}$ M.

### 3.7 $IC_{50}$ and Slope Determination

Based on the curve-fit of the percent of control aromatase activity values across six concentrations of 4-OH ASDN, the calculated $IC_{50}$ values by replicate and laboratory are summarized in Table 9. The average ± SEM $IC_{50}$ values for Laboratories A, B, and C were 89.3 ± 3.8, 65.5 ± 3.8, and 83.9 ± 6.7 nM; the percent CV values were 7.3, 10.1, and 16.1 percent, respectively. The overall task mean ± SEM $IC_{50}$ value was 79.5 ± 7.2 nM and the percent CV was 15.7 percent.

**Table 9. $IC_{50}$ Values[a]**

| Replicate | $IC_{50}$ Values (nM) | | |
|---|---|---|---|
| | Lab A | Lab B | Lab C |
| 1 | 88.1 | 73.1 | 86.8 |
| 2 | 96.3 | 61.2 | 65.6 |
| 3 | 83.4 | 62.1 | 84.9 |
| 4 | | | 98.1 |
| Average | 89.3 | 65.5 | 83.9 |
| Sd | 6.5 | 6.6 | 13.5 |
| SEM | 3.8 | 3.8 | 6.7 |
| % CV | 7.3 | 10.1 | 16.1 |

a. For each laboratory, the number of replicates actually used in the analysis of the task was included.

The slope determinations by replicate and laboratory are summarized in Table 10. The average ± SEM slope values for Laboratories A, B, and C were -0.9443 ± 0.0449, -0.9828 ± 0.0072, and -0.9065 ± 0.0236; the percent CV values were 8.2, 1.3, and 5.2 percent, respectively. The overall task mean ± SEM $IC_{50}$ value was -0.9445 ± 0.0220 and the percent CV was 4.0 percent.

**Table 10. Slope Values[a]**

| Replicate | Slope Values | | |
|---|---|---|---|
| | Lab A | Lab B | Lab C |
| 1 | -0.8874 | -0.9929 | -0.9022 |
| 2 | -0.9125 | -0.9689 | -0.9031 |
| 3 | -1.033 | -0.9866 | -0.9678 |
| 4 | | | -0.8528 |
| Average | -0.9443 | -0.9828 | -0.9065 |
| Sd | 0.0778 | 0.0124 | 0.0472 |
| SEM | 0.0449 | 0.0072 | 0.0236 |
| % CV | 8.2 | 1.3 | 5.2 |

a. For each laboratory, the number of replicates actually used in the analysis of the task was included.

### 3.7.1 Intralaboratory Statistical Analysis

For Laboratory A, the concentration response curves were similar across the three replicates. Replicate 2 had a slightly lower estimated $IC_{50}$ value than replicates 1 and 3. For the slope, the replicate-to-replicate variation was within the range of the individual replicate within-replicate variances.

For Laboratory B, there were no significant differences between the $IC_{50}$ or slope values.

For Laboratory C, replicate 2 had a lower estimated $IC_{50}$ than the other three replicates. Replicate 4 had a higher estimated $IC_{50}$ and a less negative slope than the other replicates. The variance for $IC_{50}$ among the replicates was high compared to the variances within replicates; it was at least near 8 times higher when only replicates 2 to 4 were considered or 5 times higher when all 4 replicates were considered.

### 3.7.2 Interlaboratory Statistical Analysis

Table 11 displays the estimated parameter values and the associated within laboratory 95% confidence intervals about these values. It also displays the overall mean values across laboratories and their associated 95% confidence intervals, incorporating among laboratory variation based on the random effects analysis of variance. The overall mean was calculated with and without replicate 1 for Laboratory C. These means and confidence intervals are shown in Figures 3 through 10. Each figure includes reference lines corresponding to the overall average. The estimated CVs and their associated 95% confidence intervals for the overall means for the $\log_{10}IC_{50}$ and slope parameters are also presented in Table 11.

Table 12 displays the within laboratory variances and their associated degrees of freedom for each laboratory. These are the squares of the within laboratory standard errors associated with the estimated parameter values. Table 12 also displays the laboratory to laboratory random variation and the squares of the standard errors of the overall mean values, as well as their associated degrees of freedom. The ratios of the among laboratory variances to the unweighted average within laboratory variances are also displayed, with their associated 95% confidence intervals.

The estimates for $\log_{10}IC_{50}$ were similar among the three laboratories, with or without replicate 1 in laboratory C (Table 11). For Laboratory C, the estimated variance without replicate 1 was about twice that with replicate 1 included (Table 12). The average values and the associated 95% CIs for $\log_{10}IC_{50}$ across the three laboratories were about same whether replicate 1 for Laboratory C was included or excluded. The variance among laboratories was about 1.4 times higher than the pooled average within laboratory variance when replicate 1 for Laboratory C was included and about 0.8 times the average within laboratory variance when replicate 1 for Laboratory C was excluded. The confidence intervals about these ratios were wide because of the small number of degrees of freedom (2) associated with the among laboratory variance. The coefficient of variation among laboratories was about 4%, whether replicate 1 in Laboratory C was included or excluded.

The estimated slope for Laboratory B was 7% to 11% smaller than those for Laboratories A and C whether replicate 1 in Laboratory C was included or was excluded. For Laboratory C, the estimated variance when replicate 1 was excluded was about twice that when replicate 1 was included. The average value and the associated 95% confidence interval for slope across the three laboratories remained about the same whether replicate 1 in Laboratory C was included or excluded. The among laboratory variance was about four times the average within laboratory variance when replicate 1 in Laboratory C was included and about three times the average within laboratory variance when replicate 1 in Laboratory C was excluded. The coefficient of variation among laboratories was slightly less than 5% whether replicate 1 in Laboratory C was included or excluded.

**Table 11. Parameter Estimate and the 95% Confidence Interval for the Percent of Control Responses for Human Recombinant Aromatase Assay**

| Parameter | Estimate and 95% Confidence Interval | | | | CV(%) and 95% CI[3] |
|---|---|---|---|---|---|
| | Lab A[1] | Lab B[1] | Lab C[1] | Average[2, 4] | |
| **Rep 1-4 for Lab C** | | | | | |
| $Log_{10}IC_{50}$ | -7.107 (-7.176, -7.038) | -7.185 (-7.293, -7.077) | -7.083 (-7.199, -6.967) | -7.127 (-7.203, -7.051) | 4.014 (2.245, 16.082) |
| Slope | -0.877 (-0.960, -0.794) | -0.977 (-1.020, -0.934) | -0.911 (-0.984, -0.839) | -0.926 (-1.002, -0.850) | 4.725 (2.459, 30.120) |
| Difference (Beginning Minus End) for Background Activity Control | 0.084 (-0.179, 0.346 ) | 0.026 (-0.112, 0.164) | 0.100 (-0.055, 0.255) | 0.041 (-0.019, 0.100) | |
| Difference (Beginning Minus End) for Full Enzyme Activity Control | 9.354 (3.426, 15.282) | 0.512 (-9.005, 10.030) | 10.844 (6.116, 15.572) | 6.827 (-1.578, 15.231) | |
| **Rep 2-4 for Lab C** | | | | | |
| $Log_{10}IC_{50}$ | -7.107 (-7.176, -7.038) | -7.185 (-7.293, -7.077) | -7.090 (-7.310, -6.870) | -7.132 (-7.201, -7.063) | 4.026 (2.342, 13.195) |
| Slope | -0.877 (-0.960, -0.794) | -0.977 (-1.020, -0.934) | -0.912 (-1.056, -0.768) | -0.927 (-1.004, -0.850) | 4.940 (2.570, 31.529) |
| Difference (Beginning Minus End) for Background Activity Control | 0.084 (-0.179, 0.346) | 0.026 (-0.112, 0.164) | 0.111 (-0.107, 0.328) | 0.037 (-0.024, 0.099) | |
| Difference (Beginning Minus End) for Full Enzyme Activity Control | 9.354 (3.426, 15.282) | 0.512 (-9.005, 10.030) | 8.247 (4.110, 12.383) | 5.975 (-1.270, 13.221) | |

1. The estimates and 95% confidence intervals are based on the intralaboratory analyses for the three participating laboratories. Laboratory C provided results separately for replicates 1 to 4 and for replicates 2 to 4.

2. The overall effects and confidence intervals in this table were estimated using a one-way random effects analysis of variance, with heterogeneous variances among the three laboratories. The variances for each laboratory were specified as the squares of the within laboratory standard errors.

3. CV was calculated for the average results for the $\log_{10}IC_{50}$ and the slope parameters.

4. Averages were calculated:
   - including all three replicates for laboratories A and B and replicates 1 to 4 for laboratory C
   - including all three replicates for laboratories A and B and replicates 2 to 4 for laboratory C

**Table 12. Variance Components and Ratio of Between and Within Laboratories Variances. Human Recombinant Aromatase Assay Percent of Control**

| Parameter | Within Laboratory Variance[1] | | | | Among Laboratory Variance[3] and (p-value) (df=2) | Mean Variance[4, 5] | Ratio and 95% CI[6] |
|---|---|---|---|---|---|---|---|
| | Lab A | Lab B | Lab C | Pooled Average[2] | | | |
| **Rep 1-4 for Lab C** | | | | | | | |
| $Log_{10}IC_{50}$ | 0.0002 /df=1.67 | 0.0006 /df=2 | 0.0013 /df=3.04 | 0.0007 /df=5.69 | 0.0010 (p=0.2329) | 0.0005 /df=2.82 | 1.4250 (0.1886, 56.0338) |
| Slope | 0.0005 /df=2.34 | 0.0001 /df=2 | 0.0005 /df=3.16 | 0.0004 /df=6.40 | 0.0016 (p=0.1478) | 0.0006 /df=3.32 | 4.1032 (0.5915, 161.427) |
| Difference (Beginning Minus End) for Background Activity Control | 0.0139 /df=10 | 0.0010 /df=2 | 0.0052 /df=14 | 0.0067 /df=18.66 | 1.61E-22 (p=1.0000) | 0.0008 /df=18.66 | 2.40E-20 (5.31E-21, 9.48E-19) |
| Difference (Beginning Minus End) for Full Enzyme Activity Control | 7.0783 /df=10 | 4.8929 /df=2 | 4.8585 /df=14 | 5.6099 /df=15.17 | 16.1907 (p=0.1757) | 7.2509 /df=3.11 | 2.8861 (0.6075, 113.804) |
| **Rep 2-4 for Lab C** | | | | | | | |
| $Log_{10}IC_{50}$ | 0.0002 /df=1.67 | 0.0006 /df=2 | 0.0026 /df=1.98 | 0.0011 /df=3.21 | 0.0009 (p=0.2366) | 0.0005 /df=3.44 | 0.8166 (0.0568, 32.0000) |
| Slope | 0.0005 /df=2.34 | 0.0001 /df=2 | 0.0010 /df=1.91 | 0.0005 /df=3.96 | 0.0016 (p=0.1510) | 0.0007 /df=3.57 | 3.0161 (0.2803, 118.369) |
| Difference (Beginning Minus End) for Background Activity Control | 0.0139 /df=10 | 0.0010 /df=2 | 0.0095 /df=10 | 0.0081 /df=20.68 | 0 (p=1.0000) | 0.0009 /df=20.68 | 0 (0,0) |
| Difference (Beginning Minus End) for Full Enzyme Activity Control | 7.0783 /df=10 | 4.8929 /df=2 | 3.4466 /df=10 | 5.1393 /df=13.08 | 10.1661 (p=0.2075) | 5.0542 /df=2.93 | 1.9781 (0.3992, 77.9803) |

1. The within laboratory variance for each laboratory is the square of the standard error associated with parameter estimate, which was reported in the intralaboratory analyses for each of the three participating laboratories. Laboratory C provided results for replicates 1 to 4 and for replicates 2 to 4.

2. Pooled average for the within laboratory variances is the unweighted average of the within laboratory variances among the three laboratories. Associated degrees of freedom were based on Satterthwaite's approximation.

3. Variance among laboratories is based on a one-way random effects analysis of variance model with heterogeneous variances among the three laboratories, equal to the squares of the within laboratory standard errors.

4. Mean variance is the square of the standard error of the pooled weighted mean value. It includes both within and among laboratory variation.

5. Degrees of freedom for the (mean) overall effect variance were estimated as $2*((1/K)*\sum(S_L^2 + S_i^2))^2/(var(S_L^2)+(2/K^2)*\sum(S_i^4/df_i))$, where $S_L^2$ is the among laboratory variance, $S_i^2$ and $df_i$ are the reported variance and degrees of freedom for laboratory i, $var(S_L^2)$ is the variance of $S_L^2$, and K is the number of laboratories (Hartung and Makambi, 2001).

6. Ratio of the among laboratory variance and the pooled average within laboratory variance.

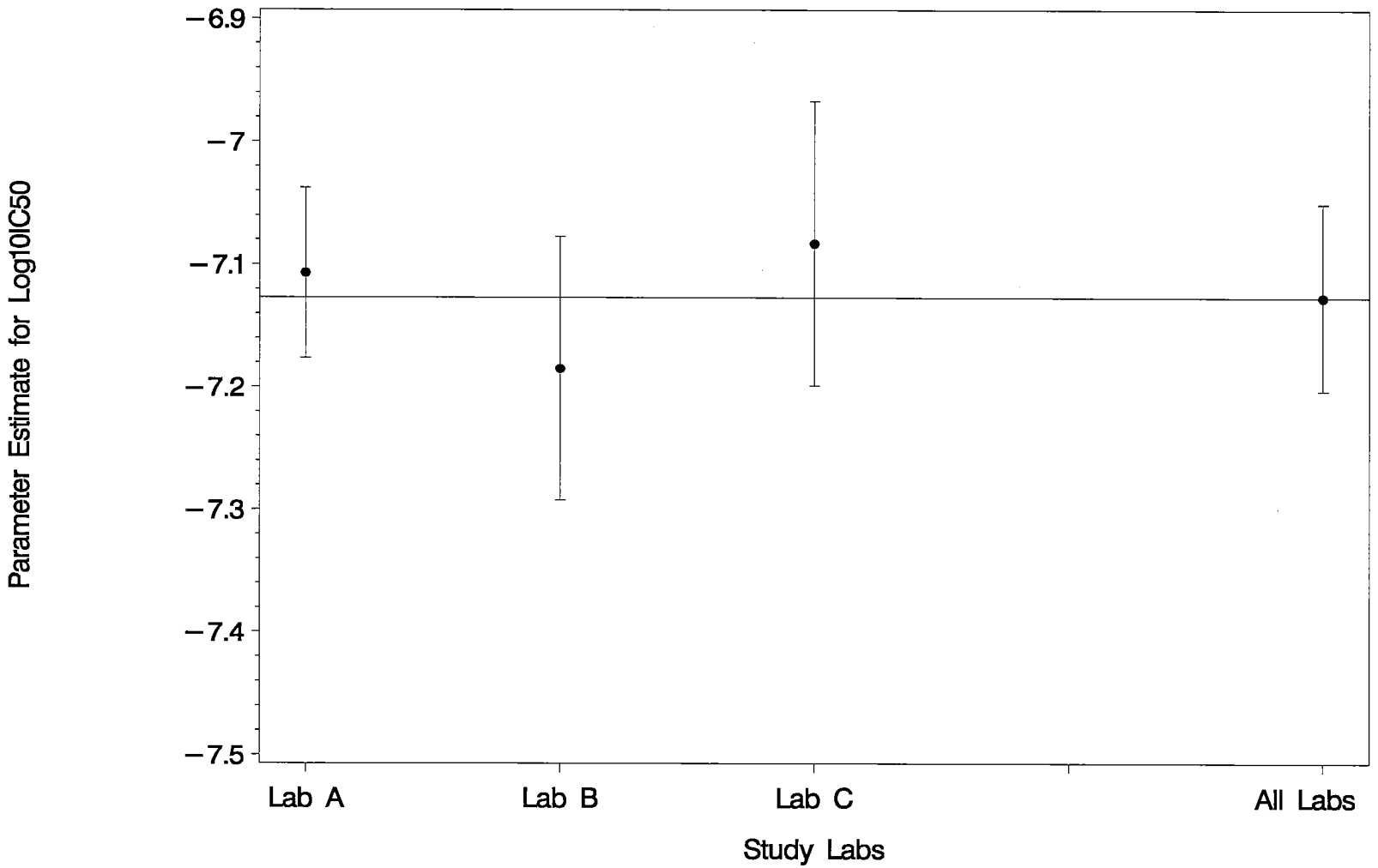


**Figure 3.** **Parameter Estimates and Their Associated 95% Confidence Intervals for $Log_{10}IC_{50}$ in Human Recombinant Aromatase Assay, Across Laboratories and by Each Laboratory. The Solid Reference Line Corresponds to the Average Across Laboratories. Laboratory C Includes Replicates 1-4.**

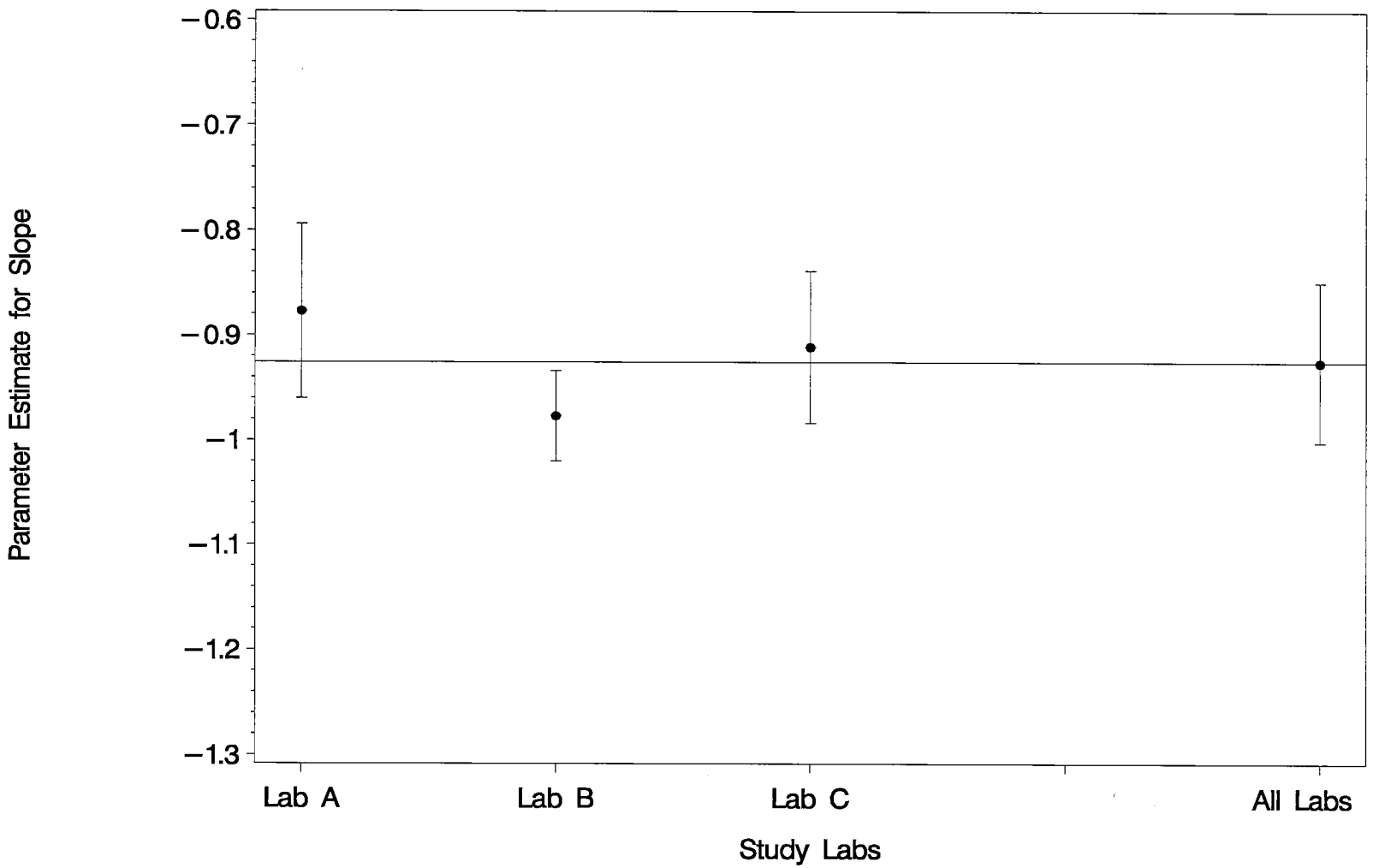


**Figure 4.** **Parameter Estimates and Their Associated 95% Confidence Intervals for Slope in the Human Recombinant Aromatase Assay, by Laboratory and Across Laboratories. The Solid Reference Line Corresponds to the Average Across Laboratories. Laboratory C Includes Replicates 1-4.**

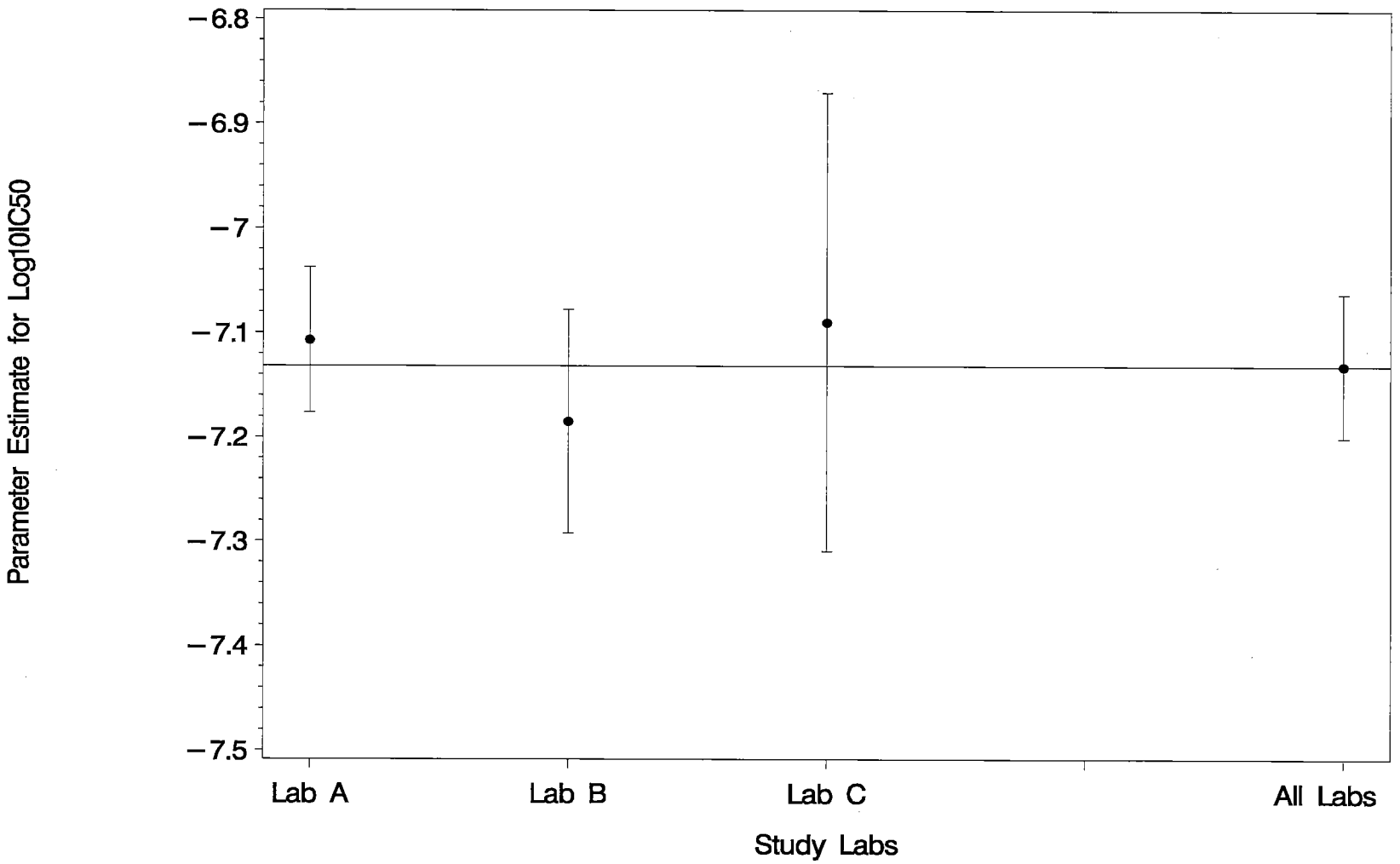


**Figure 5.** **Parameter Estimates and Their Associated 95% Confidence Intervals for $Log_{10}IC_{50}$ in Human Recombinant Aromatase Assay, Across Laboratories and by Each Laboratory. The Solid Reference Line Corresponds to the Average Across Laboratories. Laboratory C Includes Replicates 2-4.**

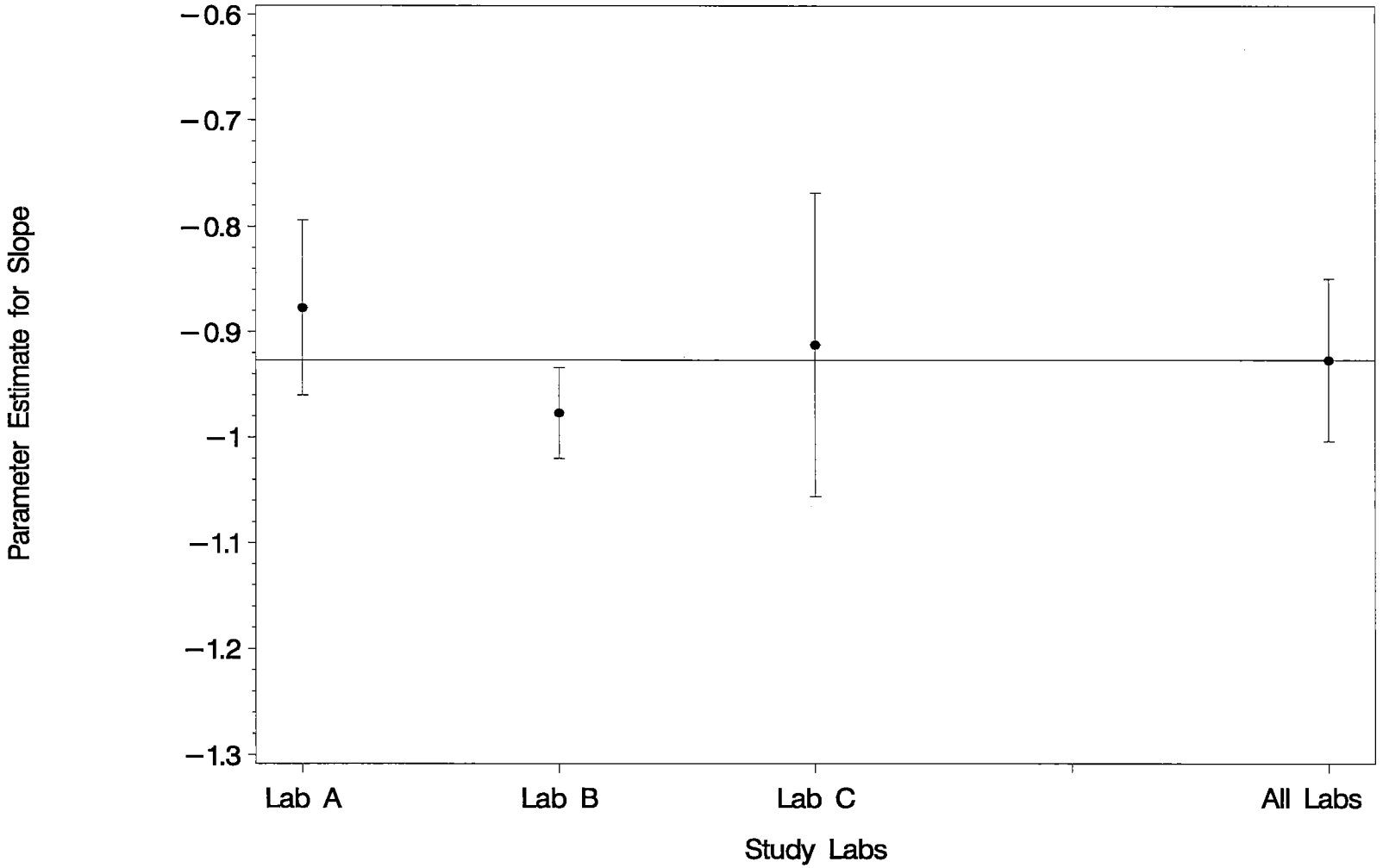


**Figure 6.** **Parameter Estimates and Their Associated 95% Confidence Intervals for Slope in the Human Recombinant Aromatase Assay, by Laboratory and Across Laboratories. The Solid Reference Line Corresponds to the Average Across Laboratories. Laboratory C Includes Replicates 2-4.**

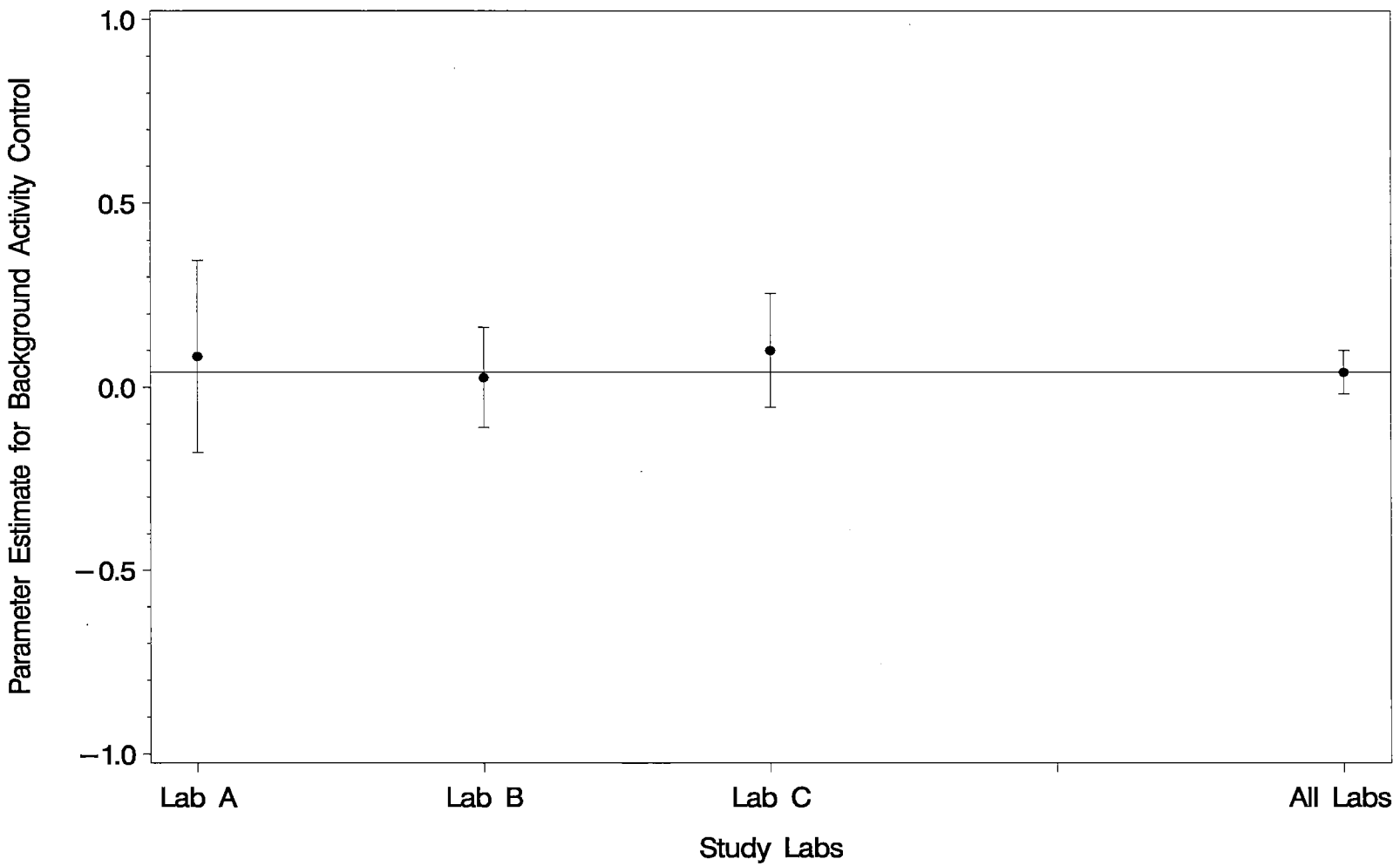


**Figure 7.** **Parameter Estimates and Their Associated 95% Confidence Intervals for Difference (Beginning Minus End) for Background Activity Controls in the Human Recombinant Aromatase Assay, by Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average Across Laboratories. Laboratory C Includes Replicates 1-4.**

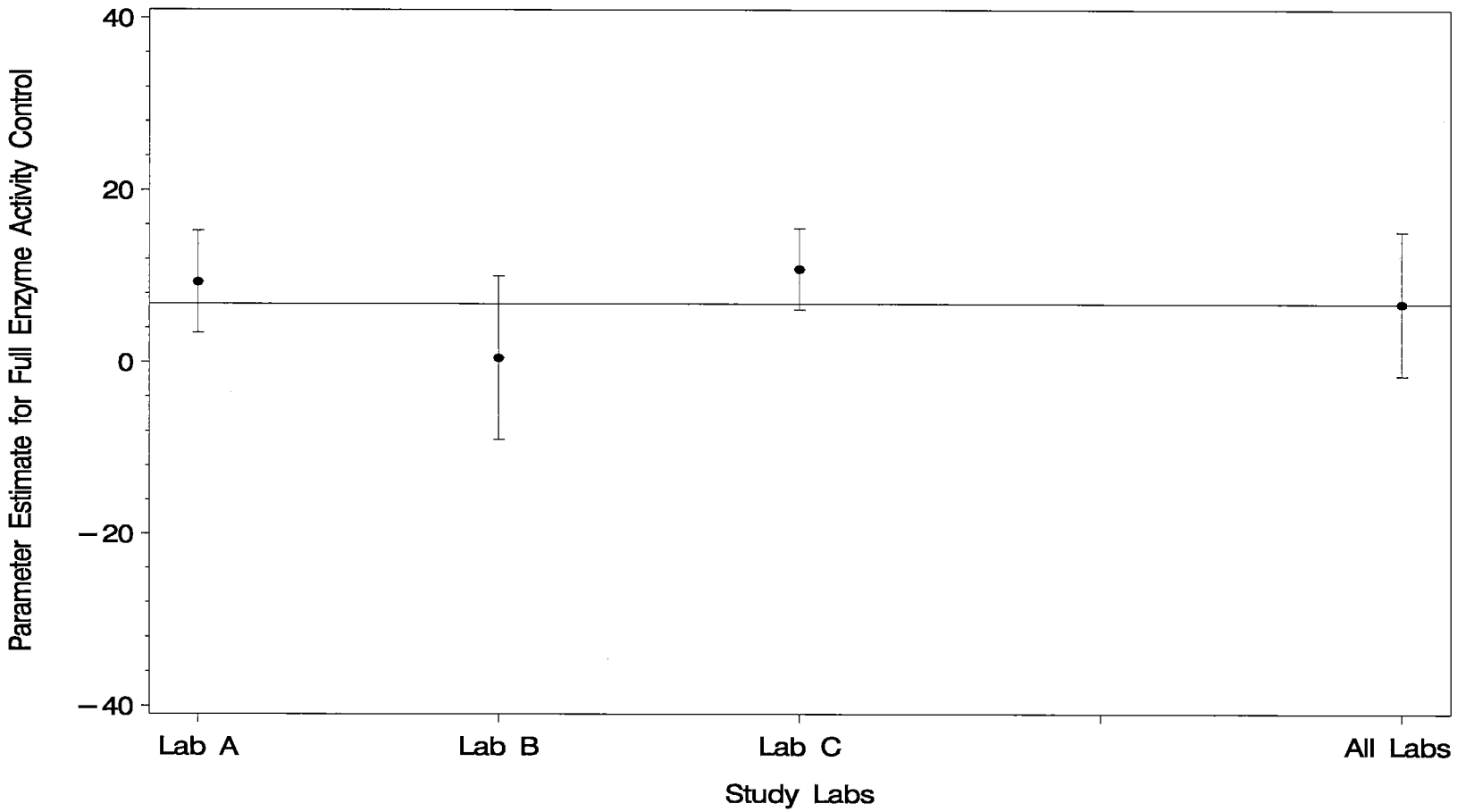


**Figure 8.** **Parameter Estimates and Their Associated 95% Confidence Intervals for Difference (Beginning Minus End) for Full Enzyme Activity Controls in the Human Recombinant Aromatase Assay, by Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average Across Laboratories. Laboratory C Includes Replicates 1-4.**

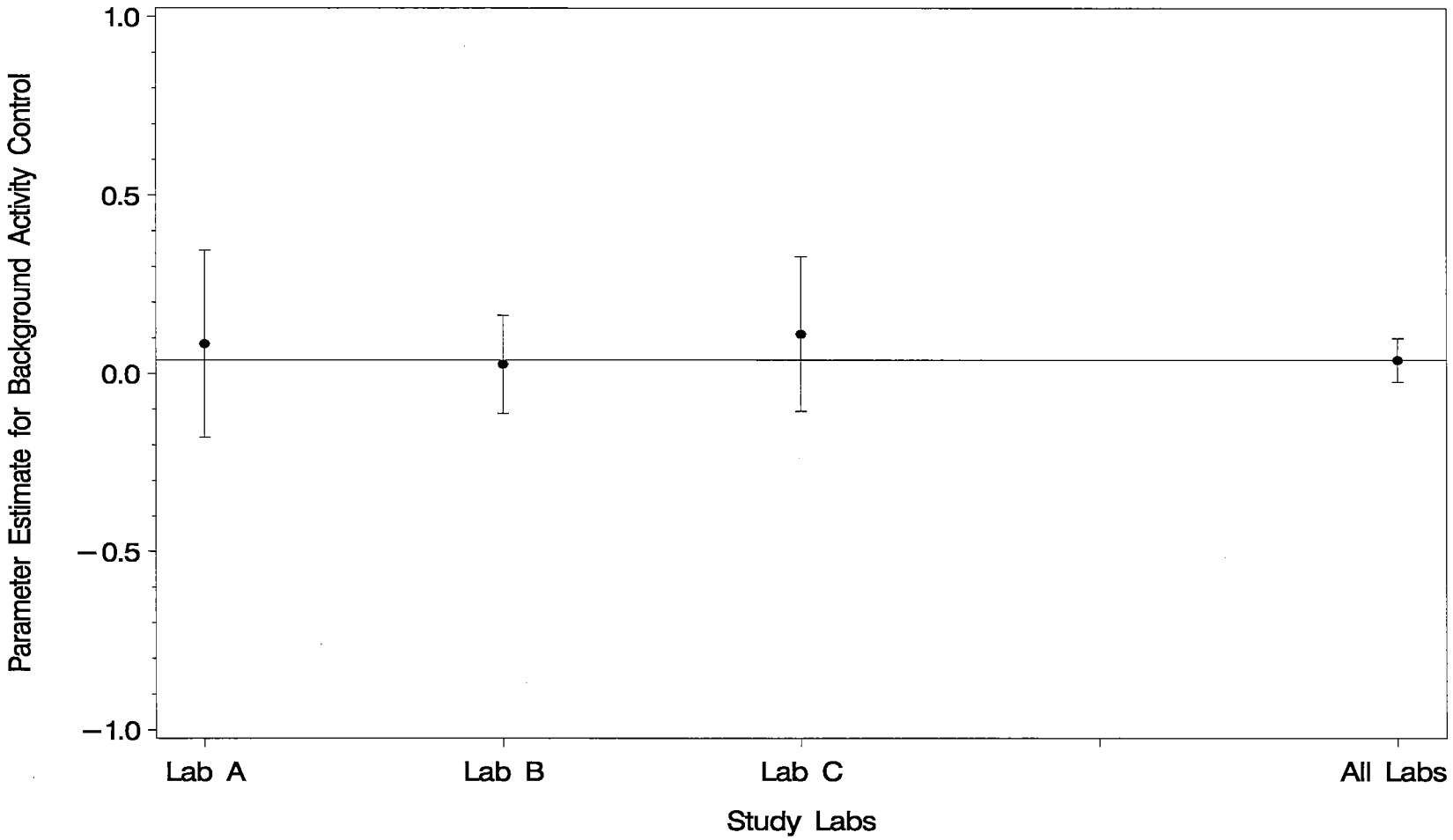


**Figure 9.** **Parameter Estimates and Their Associated 95% Confidence Intervals for Difference (Beginning Minus End) for Background Activity Controls in the Human Recombinant Aromatase Assay, by Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average Across Laboratories. Laboratory C Includes Replicates 2-4.**

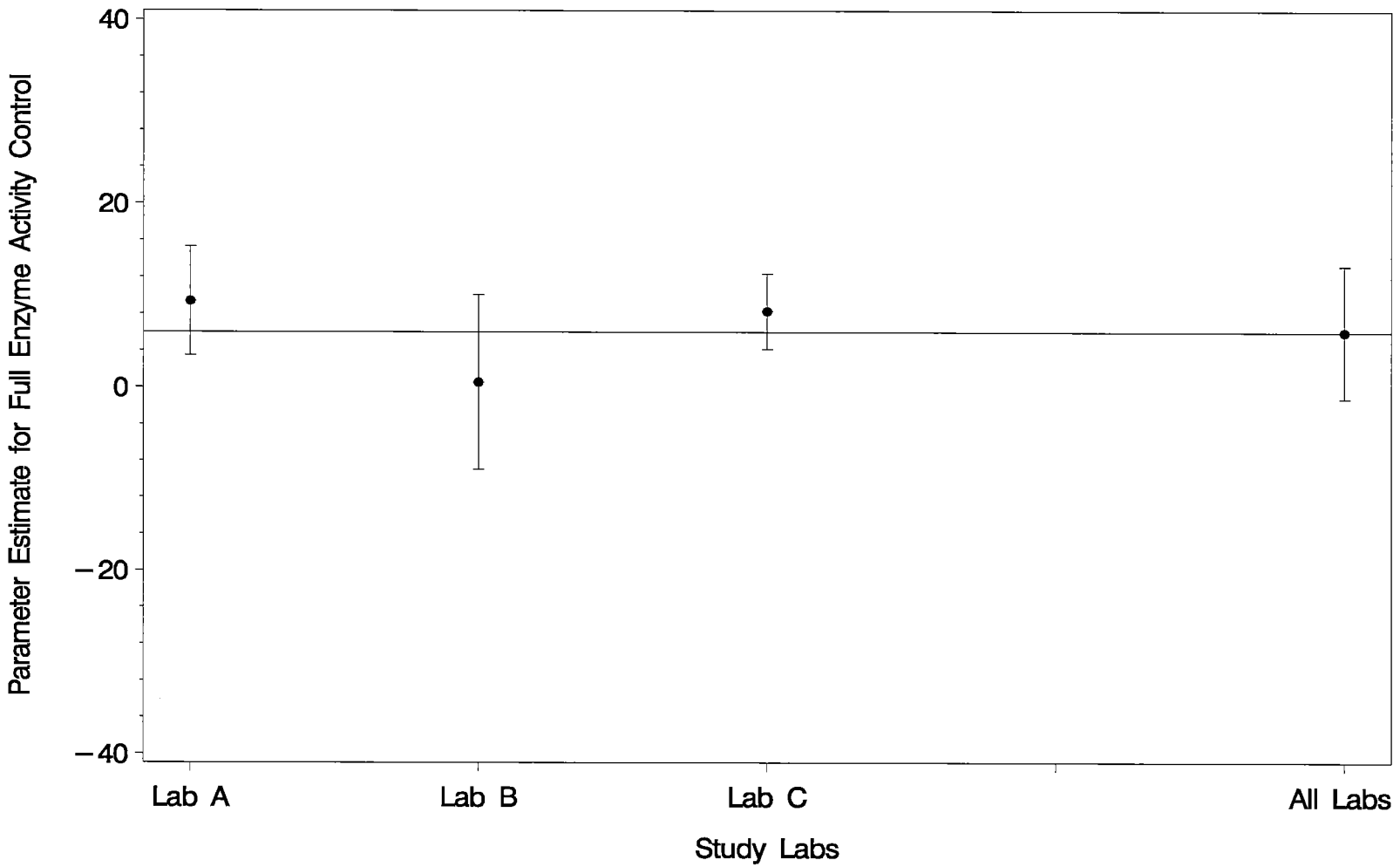


**Figure 10.** **Parameter Estimates and Their Associated 95% Confidence Intervals for Difference (Beginning Minus End) for Full Enzyme Activity Controls in the Human Recombinant Aromatase Assay, by Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average Across Laboratories. Laboratory C Includes Replicates 2-4.**

## 4.0 DISCUSSION

The results of this task showed that the participating laboratories were able to conduct the aromatase assay and obtain similar results as those reported by the lead laboratory. However, this task also demonstrated that there were difficulties that could be attributed to level of experience. For example, one laboratory (Lab C) repeated a replicate due to variability in the results. A second laboratory (Lab B) repeated all three replicates because the concentration of the microsomes in the protein assay were below the range of the standard curve, which resulted in unusually high full enzyme activity control values. Thus, the results of this task provided a measure of the intralaboratory and interlaboratory variability for this assay when performed by laboratory staff with a minimal level of training.

The results obtained by the laboratories in the present study were in good agreement with previous results reported by RTI (Work Assignment 4-10, Task 3 and Work Assignment 2-24) and in the literature. In the present study, the 4-OH ASDN $IC_{50}$ values ranged from 61.2 to 98.1 nM with an overall average (± SEM) $IC_{50}$ value of 79.5 ± 7.2 nM. In WA 4-10, Task 3, RTI reported an average (± sd) $IC_{50}$ value for 4-OH ASDN to be 65.2 ± 10.5 nM (range 54.7 – 83.5 nM). Literature citations have been found that reported the 4-OH ASDN $IC_{50}$ to range from approximately 30 – 50 nM (WA 2-24 protocol).

It is also interesting to compare the results from this study with those obtained using the human placental microsomes. Again, the results between the studies and different types of microsomes were similar. In WA 4-10, Task 3, RTI reported an average (± sd) $IC_{50}$ value for 4-OH ASDN of 56.0 ± 10.3 nM (range 44.5 – 68.9 nM). Also, in WA 4-16, Task 4 (multiple laboratories conducted this same positive control aromatase assay using human placental) the overall average (± SEM) $IC_{50}$ value was 62.1 ± 10.0 nM.

## 5.0 CONCLUSIONS

In conclusion, the results from this task indicated that the human recombinant microsomal aromatase assay was sufficiently responsive for the laboratories to demonstrate the effects of a known aromatase inhibitor using the provided assay procedure. Also, this task provided information about the assay's intralaboratory and interlaboratory variability.

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

Work Assignment 2-24 (2003). "Pre-Validation of the Aromatase Assay using Human, Bovine, and Porcine Placental Microsomes, and Human Recombinant Microsomes" EPA Contract Number 68-W-01-023.

# APPENDIX A

## Battelle Draft Final Report

Battelle Study Number G608317

**DRAFT REPORT**

# AROMATASE ASSAY VALIDATION
# POSITIVE CONTROL STUDY: RECOMBINANT MICROSOMES

*Prepared By:*
Battelle Memorial Institute
505 King Avenue
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*Prepared For:*
EPA Contract: 68-W-01-023
Work Assignment: WA 4-17, Task 3

**DRAFT REPORT**

| | |
|---|---|
| Title: | AROMATASE ASSAY VALIDATION POSITIVE CONTROL STUDY: RECOMBINANT MICROSOMES Task 3 |
| Author: | Bozena D. Lusiak, Ph.D. |
| Performing Laboratory: | Battelle Memorial Institute<br>505 King Avenue<br>Columbus, OH 43201-2693 |
| Sponsor: | Battelle Memorial Institute<br>505 King Avenue<br>Columbus, OH 43201-2693 |
| Sponsor's Representatives: | David Houchens, Ph.D.<br>EDSP Program Manager<br>Battelle<br><br>Jerry D. Johnson, Ph.D.<br>Diplomate, A.B.T.<br>Work Assignment Leader<br>Battelle |
| Study Initiation Date: | December 7, 2004 |
| Experimental Dates: | December 20, 21, 22, 2004 and March 11, 2005 |

Prepared By:

_____

Signature Date

Approved:

_____

Signature Date

## QUALITY ASSURANCE STATEMENT

The Quality Assurance Unit inspected this study and reports were submitted to the study director and management as follows:

| Phase Inspected | Inspection Date | Date Reported to Study Director /Management |
|---|---|---|
| Protocol review | 12/2/2004 | 12/2/2004 |
| Centrifugation | 12/22/2004 | 12/22/2004 |
| Dilution | 12/22/2004 | 12/22/2004 |
| Extraction | 12/22/2004 | 12/22/2004 |
| Sample homogenization | 12/22/2004 | 12/22/2004 |
| Solution preparation | 12/22/2004 | 12/22/2004 |
| Standard preparation | 12/22/2004 | 12/22/2004 |
| Test system preparation | 12/22/2004 | 12/22/2004 |
| Spectrophotometer absorbance readings | 12/22/2004 | 12/22/2004 |
| Sample preparation | 12/22/2004 | 12/22/2004 |
| Protein analysis | 12/22/2004 | 12/22/2004 |
| Audit study file | 1/3/2005 | 1/3/2005 |
| Protocol Amendment Review | 1/3/2005 | 1/3/2005 |
| Protocol Amendment Review | 1/24/2005 | 1/24/2005 |
| Audit study file | 5/25/2005 | 5/25/2005 |
| Audit draft report | 9/2/2005 | 9/2/2005 |
| Audit draft report | 11/18/2005 | 11/18/2005 |

This report provides an accurate record of the results obtained.

Quality Assurance Unit

Date

## COMPLIANCE STATEMENT

This study, Battelle Study Number G608317 was conducted in compliance with the United States Environmental Protection Agency (EPA) Good Laboratory Practice Standards (40 CFR Part 160), October 16, 1989; the standard operating procedures of Battelle Memorial Institute; and the protocol as approved by the Sponsor.

| | |
|---|---|
| Bozena D. Lusiak, Ph.D.<br>Study Director | Date |

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## 1.0 EXECUTIVE SUMMARY

The primary objective of this task was to demonstrate the responsiveness of the aromatase assay using the classical $^3H_2O$ method, 4-hydroxyandrostenedione (a known aromatase inhibitor), and recombinant microsomes. A secondary objective was to determine intralaboratory variability estimates for the assay and, as one of three laboratories conducting this assay, provide data that could be used to determine interlaboratory variability. Briefly, 4-hydroxyandrostenedione (4-OH ASDN), at six different concentrations, was incubated with human recombinant microsomes in the presence of $^3$H-androstenedione (substrate for aromatase), propylene glycol, and β-NADPH in a 0.1 M sodium phosphate buffer solution (pH 7.4) at 37 ± 1°C for 15 minutes. Control included conducting the assay with all assay components except 4-OH ASDN (full enzyme activity control) and β-NADPH (background activity control). Within each replicate three repetitions were run at each graded concentration of 4-OH ASDN. Additionally, two full enzyme activity control tubes and two background activity control tubes were analyzed at the beginning of each replicate and two full enzyme activity and two background activity controls were analyzed at the end. Concentration response curves were fitted within each replicate to describe the relation between 4-OH ASDN concentration and extent of inhibition. Four independent replicates of the assay were conducted.

For human recombinant microsomes, the overall mean (± sd, ± sem, and percent CV) full aromatase activity control value was 0.187 nmol/mg protein/min (± 0.014, ± 0.007, 7.52%). The background activity control value was <0.2% of the full enzyme activity control.

4-OH ASDN produced a concentration-dependent inhibition in aromatase activity. At the lowest ($10^{-9}$ M) and highest ($10^{-6}$ M) concentrations tested, the overall mean (± sem) percent of control aromatase activity values were 92.6 (± 2.52) and 9.29 (± 0.74) %, respectively. The overall mean (± sem) $IC_{50}$ value for 4-OH ASDN was 83.8 (± 6.74) nM.

Statistical analyses were carried out on the percent of control responses for aromatase activity in four independent replicates. Results were compared across replicates. In addition, full enzyme activity and background activity control tube responses were compared between beginning and end of each replicate to identify differences within replicates and differences across replicates. Statistical analysis showed: a) the concentration response curves were similar across the four replicates; b) replicate 2 had a lower estimated $IC_{50}$ than the other three replicates, replicate 3 had a more negative slope than the other replicates and replicate 4 had a higher estimated $IC_{50}$ and a less negative slope than the others replicates; c) the variance for $IC_{50}$ among the replicates was high compared to the variances within replicates; d) for the full enzyme activity controls the average percent of control responses at the end was lower than at the beginning for each replicate, the ends were about 5 to 17% lower than the beginnings; e) for the full enzyme activity controls the average across replicates was highly significantly lower at the end portion than at the beginning for all four replicates.

## 2.0 INTRODUCTION

### 2.1 Background

The Food Quality Protection Act of 1996 was enacted by Congress to authorize the Environmental Protection Agency (EPA) to implement a screening program on pesticides and other chemicals found in food or water sources for endocrine effects in humans. Thus, the U.S. EPA is implementing an Endocrine Disruptor Screening Program (EDSP).

In this program, comprehensive toxicological and ecotoxicological screens and tests are being developed for identifying and characterizing the endocrine effects of various environmental contaminants, industrial chemicals, and pesticides. The program's aim is to develop a two-tiered approach, e.g., a combination of *in vitro* and *in vivo* mammalian and ecotoxicological screens (Tier 1) and a set of *in vivo* tests (Tier 2) for identifying and characterizing endocrine effects of pesticides, industrial chemicals, and environmental contaminants. Validation of the individual screens and tests is required, and the Endocrine Disruptor Methods Validation Advisory Committee (EDMVAC) will provide advice and counsel on the validation assays.

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

Aromatase is a cytochrome $P450_{arom}$ enzyme complex responsible for estrogen biosynthesis and converts androgens, such as testosterone and androstenedione, into the estrogens estradiol and estrone. Aromatase is present in the ovary, placenta, uterus, testis, brain, and extraglandular adipose tissues. Two proteins, cytochrome $P450_{arom}$ and $\beta$-NADPH-cytochrome P450 reductase, are necessary for enzymatic activity, and the enzyme complex is localized in the smooth endoplasmic reticulum. The aromatase gene, designated CYP19, encodes the cytochrome $P450_{arom}$ and consists of ten exons, with the exact size of the gene exceeding 70 kilobases. Aromatase is found in breast tissue, and the importance of intratumoral aromatase and local estrogen production is being unraveled. Effective aromatase inhibitors have been developed as therapeutic agents for estrogen-dependent breast cancer to reduce the growth stimulatory effects of estrogens in breast cancer. Investigations on the development of aromatase inhibitors began in the 1970's and have expanded greatly in the past three decades.

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

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

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

## 2.2 Task Description and Objectives

Four independent replicates (in triplicate) of aromatase assay were performed using 4-hydroxyandrostenedione (4-OH ASDN) and recombinant microsomes. Six different concentrations of 4-OH ASDN were tested, and $IC_{50}$ for each replicate were calculated using Prism software as specified in the protocol.

The objective of the presented study was to conduct the aromatase assay using recombinant microsomes and 4-OH ASDN (known aromatase inhibitor) to demonstrate the responsiveness of the assay to aromatase inhibitors. Additional aim of the study was to use the optimized assay to obtain intralaboratory and interlaboratory assay variability estimates. The study protocol and Quality Assurance Project Plan (QAPP) can be found in Appendix A and B, respectively.

## 3.0 MATERIALS AND METHODS

### 3.1 Preparation of Substrate Solution

The substrate for the aromatase assay was androstenedione (ASDN). Non-radiolabeled and radiolabeled ASDN were used. The non-radiolabeled ASDN (Lot # 024K0809) was obtained from Sigma, St. Louis, MO by the Sponsor's Chemical Repository and was then distributed to the participating laboratories. It had a reported purity of 99%. The radiolabeled androstenedione ([1$\beta$-$^3$H]-androstenedione, [$^3$H]ASDN, Lot #3538-496) was obtained from PerkinElmer Life Sciences, Inc., Boston, MA and had a reported specific activity of 25.3 Ci/mmol. Radiochemical purity was reported by the supplier to be >97%. Radiochemical purity was assessed by high performance liquid chromatography (HPLC) by the lead laboratory. (See Results Section.)

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

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

### 3.2 Test Substance

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

#### 3.2.1 4-Hydroxyandrostenedione (4-OH ASDN)

Table 1 summarizes all information about test substance.

**Table 1. 4-Hydroxyandrostenedione (4-OH ASDN)**

| Substance Name | Substance Code | Mfr. Purity | CAS No. | Molecular Formula | Molecular Weight (g/mol) | Stock Solution ID | Vehicle | Storage Conditions (°C) |
|---|---|---|---|---|---|---|---|---|
| 4-hydroxyandrostenedione | 4-OH ASDN | 99% | 566-48-3 | $C_{19}H_{26}O_3$ | 302.4 | 1-ASDN-1 | 95% ethanol | 2-8 |

3.2.2 Preparation of the Working Solutions of 4-Hydroxyandrostenedione

Test substance stock solution was prepared (as described in Table 2) by the Chemical Repository as a 0.01 M solution in 95% ethanol. Subsequent dilutions of the stock solution were prepared in 95% ethanol (supplied by Chemical Repository) according to Table 2.

**Table 2. Preparation of 4-Hydroxyandrostenedione Dilutions**

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

3.3 Microsomes

Recombinant microsomes were obtained through RTI International (Research Triangle Park, NC), Lot No. 5, from GENTEST (Woburn, MA) and were stored at approximately -70°C until the time of assay. On the day of use, microsomes were thawed rapidly in a 37 ± 1°C water bath, rehomogenized using a Potter-Elvejhem homogenizer (about 5 to 10 passes) and then kept on ice until used (no longer than 2 hours).

The protein concentration in the stock microsomes was approximately 4.9 mg/mL. Microsomes were diluted in assay buffer in two serial dilutions. The first dilution (1:10) was achieved by gently mixing 0.1 mL of the microsomes stock suspension with 0.9 mL of buffer (total volume 1 mL). The second dilution (1:61.22) was obtained by gently mixing 0.49 mL of the first microsomes dilution with 29.51 mL of buffer (total volume 30 mL). The first dilution was kept on ice until the protein concentration was measured. In the second dilution, the target protein concentration was of ca. 0.008 mg/mL to achieve final protein concentration in the incubation mixture ca. 0.004 mg/mL. The second dilution was also kept on ice until it was placed in the water bath just prior to its addition to the incubation mixture to start the reaction.

3.4 Other Assay Components

The information about other assay components is provided in Table 3.

**Table 3. Assay Components**

| Chemical | Supplier | Lot Number |
|---|---|---|
| β-NADPH | Sigma | 103K7046 |
| Propylene glycol | Spectrum Chemical | SQ0397 |
| Sodium phosphate dibasic | Sigma | 083K0120 |
| Sodium phosphate monobasic | Sigma | 054K0144 and 100K0246 |
| Ethanol, 95% | Spectrum<br>Sigma | SW0045<br>04B10UB |

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

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

3.4.2 Assay Buffer

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

3.5 Protein Determination

The protein concentration in the microsomes was determined each day the microsomes were used with a DC Protein Assay kit from Bio-Rad (Hercules, CA). The 6-point standard curve was prepared using bovine serum albumin (BSA) reconstituted in Milli-Q water. The standard curve range was from 0.14 to 1.0 mg protein/mL. Briefly, to a 25 µL aliquot of microsomes solution (1:10 dilution in assay buffer) or 25 µL aliquot of each standard, 125 µL of BioRad DC Protein Kit Reagent A was added and mixed. Next, 1 mL of BioRad DC Protein Kit Reagent B was added to each standard and microsomes solution and gently mixed. The samples were incubated at room temperature for at least 15 minutes. Each sample (standard and microsomes) was transferred to disposable polystyrene cuvettes and the absorbance at 750 nm was measured using a spectrophotometer. The protein concentration of the microsomal sample was determined by interpolation, reading the protein concentration on the standard curve that corresponded to its absorbance.

3.6 Cytochrome P450 Aromatase (CYP19) Activity

The assays were performed in 13x100 mm test tubes maintained at 37 ± 1°C in a shaking water bath. Propylene glycol, [$^3$H]ASDN, β-NADPH, and assay buffer were combined in the test tubes with or without inhibitor (as described below) to the total volume of 1.0 mL. The final concentrations for the assay major components are presented in Table 4. The tubes and the microsomal suspension were placed at 37 ± 1°C in the water bath for approximately five minutes prior to initiation of the assay by the addition of 1 mL of the diluted microsomal suspension. See Table 5 for recombinant microsomes thaw time.

**Table 4. Aromatase Assay Conditions Using Recombinant Microsomes**

| Assay Components | Component Volume Added to the Assay | Final Concentration in the Assay |
|---|---|---|
| Microsomal protein | 1.0 mL | 0.004 mg/mL |
| β-NADPH | 100 µL | 0.3 mM |
| [$^3$H]ASDN | 100 µL | 100 nM |
| Propylene glycol | 100 µL | 5% (V/V) |
| 4-OH ASDN | 20 µL | Varies[a] |
| Assay buffer | 680 µL | ~ 0.094 M |

a. See Table 7 for details.

**Table 5. Microsomes Thaw Time**

| Replicate | Removed from Freezer | End Time of 5 Min. Incubation Prior to Assay Start | Time Last Assay Tube Quenched | Time Elapsed Between Removal from Freezer to the Quenching of the Last Assay Tube (min) |
|---|---|---|---|---|
| 1 | 11:20 AM | 12:00 PM | 12:22 PM | 62 |
| 2 | 12:10 AM | 12:45 PM | 1:10 PM | 60 |
| 3 | 10:30 AM | 11:05 AM | 11:30 AM | 60 |
| 4 | 11:50 AM | 12:20 PM | 12:45 PM | 55 |

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

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

Results are presented as the activity (velocity) of the enzyme (aromatase). The amount of the estrogen product formed was determined by dividing the total amount of $^3H_2O$ formed by the specific activity of the [$^3$H]ASDN substrate (expressed in dpm/nmol). The activity of the enzyme was expressed in nmol (mg protein)$^{-1}$min$^{-1}$ and was calculated by dividing the amount of estrogen formed by the amount of microsomal protein used (in mg) times the incubation time (15 minutes).

Four independent replicates (each in triplicate) of aromatase assay were performed as presented in Table 6. The fourth replicate was added to the study as per request of the Sponsor in order to evaluate possible disparity in one of the three replicates.

**Table 6. Summary of the Assays by Dates and Technician**

| Replicate Number | Date of Assay | Technician |
|---|---|---|
| 1 | 12-20-2004 | BDL/TD |
| 2 | 12-21-2004 | BDL/TD |
| 3 | 12-22-2004 | BDL/TD |
| 4 | 03-11-2005 | BDL/TD |

In each replicate/test run, full and background activity control samples were included. See Table 7 for a design of the assay groups. Full activity control contained substrate (ASDN), β-NADPH, propylene glycol, buffer, vehicle used for preparation of 4-OH ASDN solutions, and microsomes. Background activity controls contained all full activity control assay components except aromatase co-factor β-NADPH and served as assay blanks. Four full activity and four background activity controls were included with each assay run and were processed in the same manner as the other samples. The controls sets were split, so that two tubes (for each full and background activity control set) were run at the beginning and two at the end of each assay.

**Table 7. Positive Control Study Design**

| Sample type | Repetitions (Test Tubes) | Description | Final 4-OH ASDN Concentration (M) |
|---|---|---|---|
| Full Activity Control | 4 | Complete assay[a] with inhibitor vehicle control | N/A |
| Background Activity Control | 4 | Complete assay with inhibitor vehicle control omitting β-NADPH | N/A |
| 4-OH ASDN Concentration 1 | 3 | Complete assay with 4-OH ASDN added | $1 \times 10^{-6}$ |
| 4-OH ASDN Concentration 2 | 3 | Complete assay with 4-OH ASDN added | $1 \times 10^{-7}$ |
| 4-OH ASDN Concentration 3 | 3 | Complete assay with 4-OH ASDN added | $5 \times 10^{-8}$ |
| 4-OH ASDN Concentration 4 | 3 | Complete assay with 4-OH ASDN added | $2.5 \times 10^{-8}$ |
| 4-OH ASDN Concentration 5 | 3 | Complete assay with 4-OH ASDN added | $1 \times 10^{-8}$ |
| 4-OH ASDN Concentration 6 | 3 | Complete assay with 4-OH ASDN added | $1 \times 10^{-9}$ |

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

## 3.7 Data Analysis

The reported data include the following information: assay date and run number, technician, inhibitor, total dpm minus background dpm, and percent activity. The average background dpm values were subtracted from the assayed sample dpm values to provide dpm for specific aromatase activity. A spreadsheet developed by RTI International was used to process the data into a final form for analysis and evaluation.

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). Multiplication of the volume (mL) of substrate solution added to the incubation by the substrate solution radiochemical content (dpm/mL) yielded 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 yielded the percent of the substrate that was converted to product. The total dpm remaining in the aqueous portion after extraction was corrected for background by subtracting the average dpm present in the aqueous portion of the background activity control tubes (for that day/assay). This corrected dpm was then converted to nmol product formed by dividing by the substrate specific activity (dpm/nmol). The activity of the enzyme was expressed in nmol (mg protein)$^{-1}$min$^{-1}$ and was calculated by dividing the amount of estrogen formed (nmol) by the amount of microsomal protein used (in mg) times the incubation time (in min). Average activity in the full activity control samples for a given study was calculated. Percent of activity remaining in the presence of various inhibitor concentrations was calculated by dividing the aromatase activity at a given inhibitor concentration by the average full activity control and multiplying by 100.

$IC_{50}$ was calculated using Prism version 4.0 (GraphPad, San Diego, CA). Percent of control activity data was exported to Prism for curve fitting of the percent of control activity versus log of 4-OH ASDN concentration data using the following equation:

$$Y=100/(1+10^{((LogIC_{50}-X)*HillSlope)})$$

Where: X is the logarithm of 4-OH ASDN concentration (M)
Y is the percent activity

The software incorporated a weighting factor for the percent of activity values of 1/Y. Observed individual percent activity values above 100% were set to 99.5%. As shown in the above equation, the curve fitting equation uses the fixed value of 100 as the numerator. 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%.

For each test substance concentration and replicate the estimated $\log_{10}IC_{50}$ ($\mu$), the within replicate standard error of $\mu$, the $IC_{50}$, the slope ($\beta$), the within replicate standard error of $\beta$, and the "Status" of each response curve was displayed in a Table 12 (also see Appendix G for full statistical analysis).

### 3.8 Retention of Records

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

## 4.0 RESULTS

### 4.1 Radiochemical Purity

The radiochemical purity for the substrate androstenedione was 97% as reported by RTI International (Appendix C).

4.2 Stock Formulation Analysis

The 0.01 M solution of 4-hydroxyandrostenedione in 95% ethanol was prepared by the Sponsor's Chemical Repository. The actual concentration was within 10 % of the target concentration (Appendix D).

4.3 Protein Analysis

Protein concentration measurements were done according to the procedure provided in Section 3.5 of this report. To measure the protein concentration a 1:10 microsome dilution in assay buffer were processed. The results of measuring the protein concentration are provided in Table 8.

**Table 8. Protein Concentration**

| Replicate Number | Measured Protein Concentration (1:10 dilution) (mg/mL) | Stock Microsomes Protein Concentration (mg/mL) | Working Protein Concentration (mg/mL) | Final Protein Concentration (in the Assay) (mg/mL) |
|---|---|---|---|---|
| 1 | 0.6456 | 6.456 | 0.0105 | 0.0053 |
| 2 | 0.5415 | 5.415 | 0.00885 | 0.0044 |
| 3 | 0.5437 | 5.437 | 0.00888 | 0.0044 |
| 4 | 0.5396 | 5.396 | 0.00881 | 0.0044 |

4.4 Aromatase Activity

Table 9 summarizes the full aromatase activity control values measured at the beginning and at the end of each assay run (in full activity control samples). Four independent measurements of the full aromatase activity (in duplicate, at the beginning and at the end of each assay) were performed. The overall full activity (mean ± sd, n=4) for all measurements was 0.187 ± 0.014 nmol/mg protein/min. Background aromatase activity in control samples (two at the beginning and two at the end of each assay) was very low (<0.2% of full control aromatase activity) suggesting that there was no nonspecific product formation or unintentional contamination with β-NADPH (Appendix E, Individual Replicate Spreadsheets for individual background activity values). The differences between the beginning and the end activity of full aromatase activity values were between 17.1% (for the first replicate) and 4.8% (for the third replicate).

**Table 9. Full Aromatase Activity Controls (FAAC, nmoles/mg protein/min)**

| Replicate | FAAC Beginning | FAAC End | Within | | | | Overall | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | mean | sd | sem | % CV | mean | sd | sem | % CV |
| 1 | 0.1963 | 0.1788 | 0.1864 | 0.0225 | 0.0112 | 12.06 | | | | |
| 1 | 0.2113 | 0.1592 | | | | | | | | |
| 2 | 0.1800 | 0.1511 | 0.1701 | 0.0132 | 0.0066 | 7.77 | 0.1815[a] | 0.0099 | 0.0057 | 5.45 |
| 2 | 0.1781 | 0.1711 | | | | | 0.1872[b] | 0.0141 | 0.0071 | 7.52 |
| 3 | 0.1961 | 0.1861 | 0.1879 | 0.0065 | 0.0032 | 3.45 | 0.1875[c] | 0.0172 | 0.0100 | 9.19 |
| 3 | 0.1890 | 0.1805 | | | | | | | | |
| 4 | 0.2112 | 0.1945 | 0.2046 | 0.0112 | 0.0056 | 5.49 | | | | |
| 4 | 0.2169 | 0.1956 | | | | | | | | |

a. Calculated for first three replicates.
b. Calculated for all four replicates.
c. Calculated for 2 to 4 replicates.

4.5 Percent of Control

Table 10 summarizes aromatase activity (expressed as a percent of full activity) detected in assays with various inhibitor (4-OH ASDN) concentrations. Increasing the 4-OH ASDN concentration affected the aromatase activity in a concentration-dependent manner. The highest applied concentration of 4-OH ASDN ($1.0 \times 10^{-6}$ M) inhibited aromatase activity to approximately 9.3% of full enzyme activity (ca. 90% inhibition); the lowest concentration of 4-OH ASDN ($1.0 \times 10^{-9}$M) inhibited aromatase activity only approximately 7% (ca. 93% of aromatase activity remains intact, Table 11).

**Table 10. Individual Percent of Control Values by Tube and Replicate**

| Test Substance | Replicate | Log [Test Substance] | Percent of Control | | | mean | sd | sem | % CV |
|---|---|---|---|---|---|---|---|---|---|
| | | | Tube 1 | Tube 2 | Tube 3 | | | | |
| 4-OH ASDN | 1 | -6.00 | 9.59 | 9.42 | 8.88 | 9.30 | 0.37 | 0.21 | 3.99 |
| | | -7.00 | 46.49 | 48.25 | 48.08 | 47.61 | 0.97 | 0.56 | 2.04 |
| | | -7.30 | 65.03 | 64.00 | 63.23 | 64.09 | 0.90 | 0.52 | 1.41 |
| | | -7.60 | 76.72 | 75.94 | 79.58 | 77.41 | 1.92 | 1.11 | 2.48 |
| | | -8.00 | 80.12 | 80.05 | 83.56 | 81.24 | 2.01 | 1.16 | 2.47 |
| | | -9.00 | 87.98 | 95.43 | 90.18 | 91.20 | 3.83 | 2.21 | 4.20 |
| | 2 | -6.00 | 7.86 | 7.49 | 8.13 | 7.83 | 0.32 | 0.19 | 4.11 |
| | | -7.00 | 40.78 | 39.10 | 39.71 | 39.86 | 0.85 | 0.49 | 2.13 |
| | | -7.30 | 55.77 | 55.12 | 57.93 | 56.27 | 1.47 | 0.85 | 2.61 |
| | | -7.60 | 74.92 | 73.52 | 71.73 | 73.39 | 1.60 | 0.92 | 2.18 |
| | | -8.00 | 84.67 | 82.88 | 80.47 | 82.67 | 2.11 | 1.22 | 2.55 |
| | | -9.00 | 90.34 | 90.11 | 95.58 | 92.01 | 3.09 | 1.79 | 3.36 |
| | 3 | -6.00 | 8.82 | 8.50 | 8.83 | 8.72 | 0.19 | 0.11 | 2.15 |
| | | -7.00 | 45.54 | 43.25 | 44.13 | 44.31 | 1.16 | 0.67 | 2.61 |
| | | -7.30 | 66.80 | 64.11 | 64.31 | 65.07 | 1.50 | 0.87 | 2.30 |
| | | -7.60 | 77.46 | 75.98 | 70.71 | 74.72 | 3.55 | 2.05 | 4.75 |
| | | -8.00 | 96.48 | 91.24 | 86.62 | 91.45 | 4.93 | 2.85 | 5.39 |
| | | -9.00 | 98.98 | 95.86 | 103.99 | 99.61 | 4.10 | 2.37 | 4.12 |
| | 4 | -6.00 | 11.34 | 11.22 | 11.38 | 11.31 | 0.08 | 0.05 | 0.74 |
| | | -7.00 | 48.64 | 54.37 | 51.57 | 51.53 | 2.87 | 1.66 | 5.56 |
| | | -7.30 | 63.77 | 67.64 | 66.29 | 65.90 | 1.96 | 1.13 | 2.98 |
| | | -7.60 | 71.97 | 79.29 | 75.76 | 75.67 | 3.66 | 2.12 | 4.84 |
| | | -8.00 | 85.11 | 83.28 | 84.36 | 84.25 | 0.92 | 0.53 | 1.09 |
| | | -9.00 | 89.34 | 87.01 | 86.51 | 87.62 | 1.51 | 0.87 | 1.72 |

**Table 11. Replicate Mean and Overall Mean Percent of Control Values**

| Test Substance | Log [Test Substance] | Mean Percent of Control | | | | Overall | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Repl 1 | Repl 2 | Repl 3 | Repl 4 | mean | sd | sem | % CV |
| 4-OH ASDN | -6.00 | 9.30 | 7.83 | 8.72 | 11.31 | 9.29 | 1.48 | 0.74 | 15.89 |
| | -7.00 | 47.61 | 39.86 | 44.31 | 51.53 | 45.83 | 4.95 | 2.48 | 10.81 |
| | -7.30 | 64.09 | 56.27 | 65.07 | 65.90 | 62.83 | 4.44 | 2.22 | 7.06 |
| | -7.60 | 77.41 | 73.39 | 74.72 | 75.67 | 75.30 | 1.69 | 0.85 | 2.25 |
| | -8.00 | 81.24 | 82.67 | 91.45 | 84.25 | 84.90 | 4.53 | 2.27 | 5.34 |
| | -9.00 | 91.20 | 92.01 | 99.61 | 87.62 | 92.61 | 5.04 | 2.52 | 5.44 |

4.6 $IC_{50}$

Based on the curve-fit of the percent of control aromatase activity across six concentrations of 4-OH ASDN, the calculated $IC_{50}$ values are presented in Table 12. The overall $IC_{50}$ value based on calculations for all four replicates is 83.8 nM (16.1% CV) and overall $IC_{50}$ value based on calculations for three (2-4) replicates is 82.9 nM (19.7% CV).

**Table 12. Calculated $IC_{50}$ Values**

| Test Substance | Replicate | Log [$IC_{50}$] | SE Log [$IC_{50}$] | $IC_{50}$ (nM) | Slope | SE Slope | Status* | Overall $IC_{50}$** (nM) | sd | sem | % CV |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 4-OH ASDN | 1 | -7.062 | 0.02430 | 86.79 | -0.9022 | 0.03667 | C | 79.10[a] | 11.75 | 6.78 | 14.85 |
| | 2 | -7.183 | 0.01653 | 65.58 | -0.9031 | 0.02662 | C | 82.86[b] | 16.34 | 9.44 | 19.72 |
| | 3 | -7.071 | 0.01571 | 84.93 | -0.9678 | 0.02755 | C | | | | |
| | 4 | -7.008 | 0.02920 | 98.07 | -0.8528 | 0.04000 | C | 83.84[c] | 13.49 | 6.74 | 16.09 |

* The Status of each response curve is indicated as "C" Complete (response curve ranging from essentially 0 percent to 100 percent of control).

** Arithmetic calculations.

a. Calculated for 1 to 3 replicates.

b. Calculated for replicate 2 to 4.

c. Calculated for all four replicates.

The following figures (Figure 1 through Figure 3) present concentration response curves. Figure 1 presents average percent of control activity of each replicate (1 to 4), Figure 2 represents overall average concentration response curve across 1 to 4 replicates and average responses across repetitions, and Figure 3 represents overall average concentration response curve across each replicate (2 to 4) and average responses across repetitions.

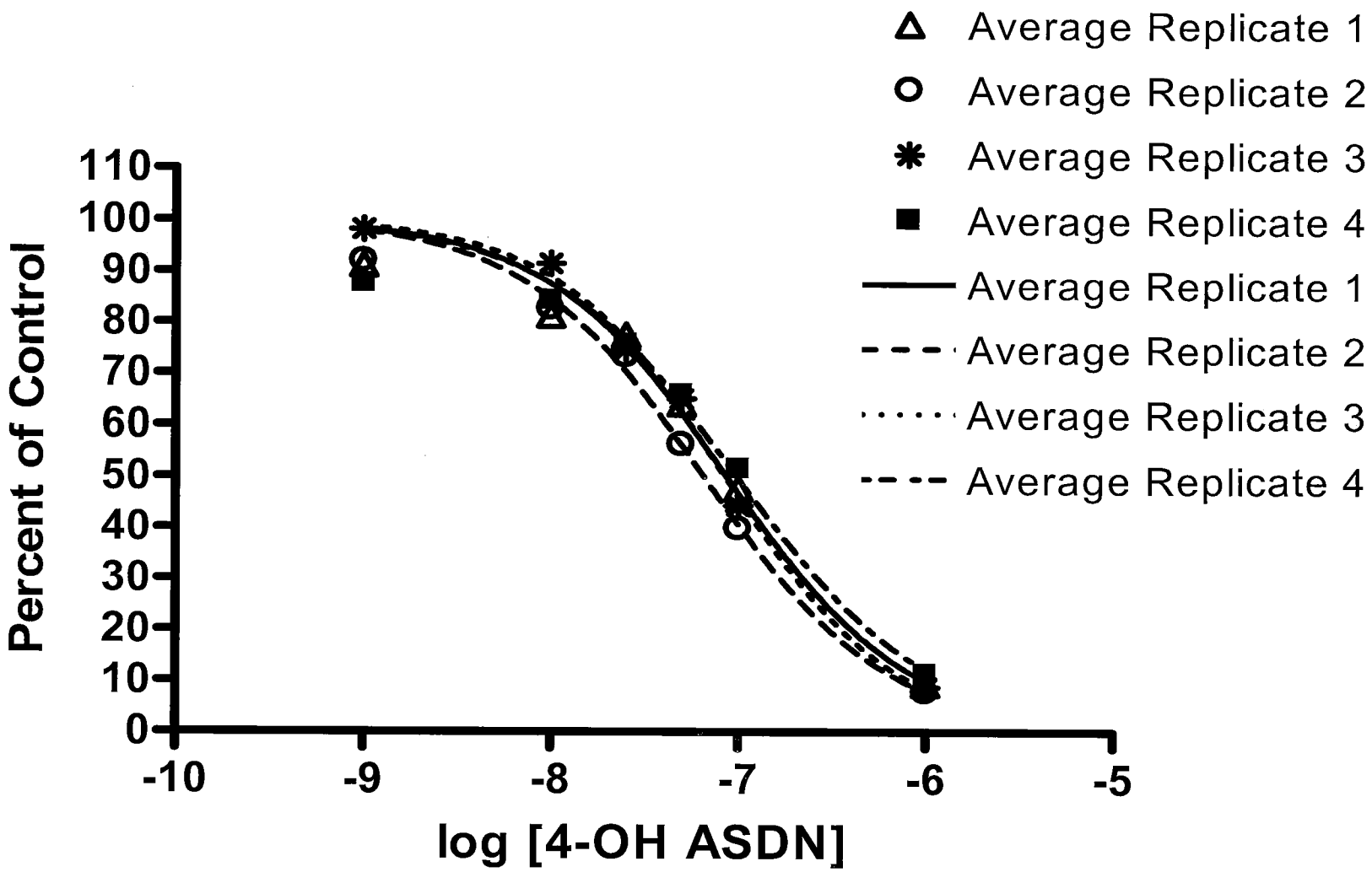


**Figure 1.** **Concentration Response Curves and Averages of Repetitions Within 4-OH ASDN Concentrations. Recombinant Aromatase Assay. By Replicate.**

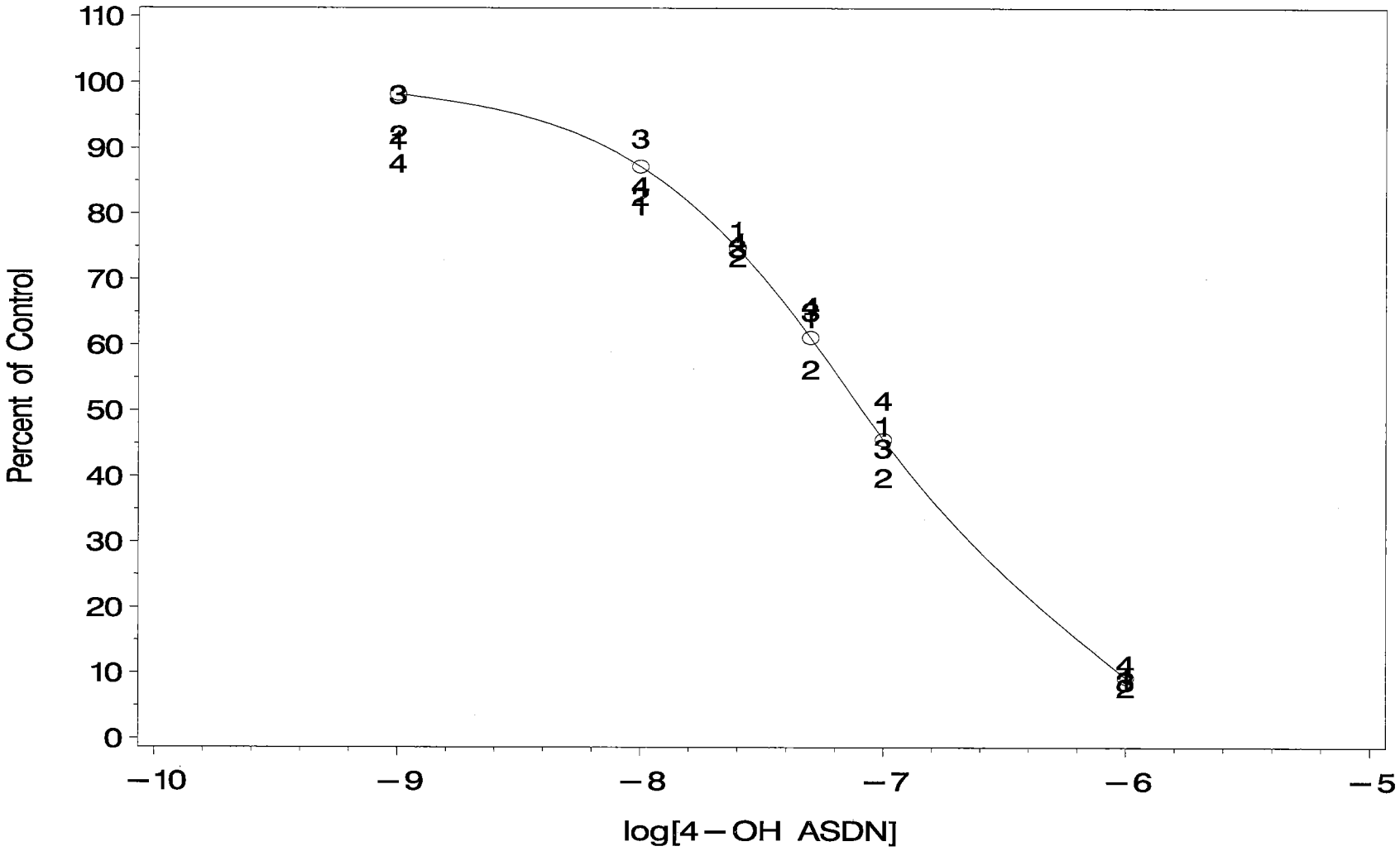


**Figure 2.** **Overall Average Concentration Response Curve Across Replicates 1 to 4 and Average Responses Across Repetitions Within 4-OH ASDN Concentrations. Recombinant Aromatase Assay. Parameters of Average Curve Based on One-Way Analysis of Variance Across Replicate 1 to 4 Parameter Values.**

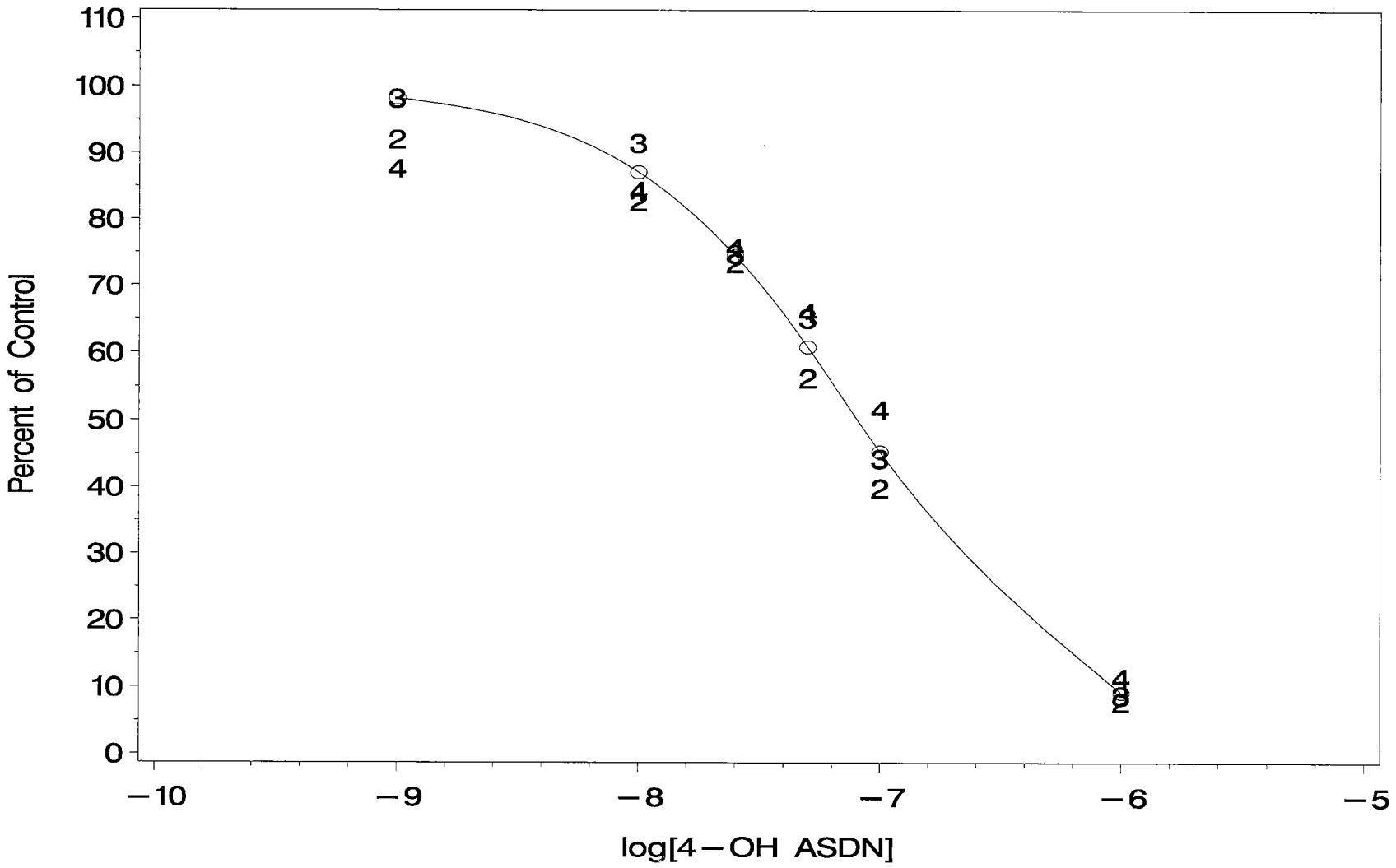


**Figure 3.** **Overall Average Concentration Response Curve Across Replicates 2 to 4 and Average Responses Across Repetitions Within 4-OH ASDN Concentrations. Recombinant Aromatase Assay. Parameters of Average Curve Based on One-Way Analysis of Variance Across Replicate 2 to 4 Parameter Values.**

## 4.7 Statistical Analysis

The full statistical analysis report is presented in Appendix G. There are some small differences in data obtained from the Prism output and data presented in the Statistical report obtained by applying the SAS statistical analysis system. Two-way analysis of variance was carried out, 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), replicate by portion interaction. The error corresponded to repetition within replicate and portion. The results are presented in Table 13. No significant differences between the beginning and the end, averaged across replicates, were observed for background activity controls. Highly significant differences between the beginning and the end portions were observed for the full enzyme activity control.

**Table 13. Variance Components of Full Enzyme Activity Control and Background Activity Control Percent of Control Values. Position Effects and Variation Across Replicates of Portion Effects Within Replicates.**

| Parameter | Difference Between Beginning and End Portions | | Variance Components | | | |
|---|---|---|---|---|---|---|
| | Estimate (%) (Std. Error) | p-Value/ Degree of Freedom | Replicate[a] | Portion* Replicate | Residual (Repetition) | Total Variance |
| **Replicates 1 to 4** | | | | | | |
| **Background Activity** | 0.1001 (0.0724) | 0.1883/df=14 | 0 | <0.00001 | 0.0210 | 0.0210 |
| **Full Enzyme Activity Control** | 10.8440 (2.2042) | 0.0002/df=14 | 0 | 0 | 19.4339 | 19.4339 |
| **Replicates 2 to 4** | | | | | | |
| **Background Activity** | 0.1109 (0.0976) | 0.2821/df=10 | 0 | <0.00001 | 0.0286 | 0.0286 |
| **Full Enzyme Activity Control** | 8.2467 (1.8565) | 0.0013/df=10 | 0 | 0 | 10.3403 | 10.3403 |

a. The replicate component of variation is constrained to be 0, by definition of background activity and full enzyme activity control responses.

The average $\log_{10}IC_{50}$ ($\mu$) and slope ($\beta$) estimates across replicates and associated 95% confidence intervals are presented in Table 14 (for graph see Appendix G). Replicate 2 had a lower $\log_{10}IC_{50}$ than the other three replicates. Replicate 4 had a higher estimated $\log_{10}IC_{50}$ and less negative slope than the other replicates.

The results of analyses of variance for $\log_{10}IC_{50}$ ($\mu$) and slope ($\beta$) are presented in Table 15. For each replicate, the squares of the standard errors associated with each parameter ($\mu$ and $\beta$) are given. The variance for $IC_{50}$ among the replicates was high compared to the variances within replicates; it was at least 8 times higher when only replicates 2 to 4 considered or 5 times higher when all 4 replicates were considered.

**Table 14. Parameter Estimates of the Concentration Response Curves and Associated 95% Confidence Intervals. Percent of Control Activity. Recombinant Aromatase Assay.**

| Parameter | Estimate (95% CI) | | | | | |
|---|---|---|---|---|---|---|
| | Replicate 1[a] | Replicate 2[a] | Replicate 3[a] | Replicate 4[a] | Mean of Replicates 2 to 4 [b] | Mean of Replicates 1 to 4 [b] |
| $Log_{10}IC_{50}$ | -7.062 (-7.114, -7.010) | -7.183 (-7.218, -7.148) | -7.071 (-7.104, -7.038) | -7.009 (-7.071, -6.947) | -7.090 (-7.309, -6.870) | -7.083 (-7.199, -6.967) |
| Slope | -0.902 (-0.980, -0.824) | -0.903 (-0.960, -0.847) | -0.968 (-1.026, -0.909) | -0.853 (-0.938, -0.768) | -0.912 (-1.056, -0.768) | -0.911 (-0.984, -0.839) |

a. Parameter estimates and their associated 95% confidence intervals for each replicate, based on the concentration response curves fitted to the individual repetition data within replicates.

b. Mean and its associated 95% confidence interval, based on a heterogeneous variance one-way analysis of variance model with replicate treated as a random effect.

**Table 15. Variances Associated with Estimated Parameters of Concentration Response Curves. Percent of Control Activity. Recombinant Aromatase Assay.**

| Parameter | Variance/Degree of Freedom[a,b,c] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Replicate 1 | Replicate 2 | Replicate 3 | Replicate 4 | Overall for Replicates 2-4 | | Overall for Replicates 1-4 | |
| | | | | | Random Replicate (p-value)[d] | Variance of Mean | Random Replicate (p-value)[d] | Variance of Mean |
| $Log_{10}IC_{50}$ | 0.000590 /df=16 | 0.000273 /df=16 | 0.000247 /df=16 | 0.000854 /df=16 | 0.007237 /df=2 (p=0.1745) | 0.002562 /df=1.982 | 0.004915 /df=3 (p=0.1310) | 0.001349 /df=3.042 |
| Slope | 0.001345 /df=16 | 0.000709 /df=16 | 0.000759 /df=16 | 0.001602 /df=16 | 0.002104 /df=2 (p=0.2548) | 0.001026 /df=1.912 | 0.001157 /df=3 (p=0.2585) | 0.000549 /df=3.154 |

a. The variance estimates for each replicate were based on the concentration response curves fitted to the individual repetition data within each concentration level.

b. Variance estimates for the random replicate were estimated based on a one-way random effects analysis of variance. The variances for each replicate were fixed at their reported values.

c. Degrees of freedom for the variance of mean were estimated by $2*((1/K)*(S_r^2 + S_i^2))^2/(var(S_r^2)+(2/K^2)*(S_i^4/d\ f_i))$, where $S_r^2$ is random replicate variance, $S_i^2$ and $df_i$ are estimated variance and degree of freedom for a given replicate, $var(S_r^2)$ is the variance associated with the estimation of $S_r^2$ and K is the number of replicates (Hartung and Makambi, 2001).

d. p-value is based on the Wald Z-test result.

## 5.0 DISCUSSION

4-Hydroxyandrostenedione (4-OH ASDN) is the known aromatase inhibitor. Six different concentrations of 4-OH ASDN ranging from 1 x $10^{-9}$ to 1 x $10^{-6}$ M were applied to create the dose response curve. Four independent replicates for aromatase assays were performed (one replicate was added because $IC_{50}$ calculated for the second replicate was slightly out of range observed for replicate 1 and 3). At the inhibitor concentration at 1 x $10^{-9}$ M, almost no inhibition was observed (92.6% of control activity) and at 1 x $10^{-6}$ M almost full inhibition (9.29% of control activity) was observed. The concentration response curves were similar across four replicates.

The overall $IC_{50}$ value for 1 to 4 replicates was 83.8 nM and 82.9 nM for 2 to 4 replicates. The $IC_{50}$ for second replicate was significantly lower than the average across the other three replicates. Replicate 3 had a more negative slope than the other three replicates. Replicate 4 had higher estimated $IC_{50}$ and a less negative slope than the other replicates.

No background enzyme activity was detected through all four replicates. There was some difference between full enzyme activity control at the beginning and at the end of each assay within each replicate. The highest difference was observed in first replicate (ca. 17%), for the remaining three replicates difference was between 4.8 and 10%. No significant difference in full enzyme activity was observed between replicates.

## 6.0 CONCLUSION

The responsiveness (in concentration dependent manner) of the recombinant microsomes aromatase assay to 4-hydroxyandrostenedione was confirmed.

## APPENDIX A

Protocol (with Amendments) ..... A-1

# BATTELLE STUDY PROTOCOL

# AROMATASE ASSAY VALIDATION POSITIVE CONTROL STUDY: RECOMBINANT MICROSOMES

*Prepared By:*

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*Prepared For:*

EPA Contract: 68-W-01-023
EPA Work Assignment: WA 4-17, Task 3

Battelle
*The Business of Innovation*

Experiment Start Date: December 7, 2004
Experiment End Date: January 15, 2005

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# AROMATASE ASSAY VALIDATION
# POSITIVE CONTROL STUDY: RECOMBINANT MICROSOMES

## APPROVED, BATTELLE:

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# AROMATASE ASSAY VALIDATION
# POSITIVE CONTROL STUDY: RECOMBINANT MICROSOMES

## 1.0 OBJECTIVES

The objective of this protocol is to describe procedures for conduct of the aromatase assay using recombinant 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 recombinant 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 recombinant microsomes enhances its predictive potential.

Route of administration and reason for its choice: The route of administration is not applicable since the test system uses recombinant microsomes. The microsomes, reagents, and test substance will be incubated in a common reaction vessel so that the effect of test substance on aromatase enzymatic activity can be evaluated.

## 2.0 MATERIALS RECEIPT AND/OR PREPARATION

A sufficient supply of chemical reagents, radiolabeled and non-radiolabeled androstenedione, and human recombinant 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 Repository (CR). The CR will forward all applicable information regarding supplier, lot numbers and reported/measured purity for the substrate to the laboratories and this information will be included in study reports. The radiochemical purity of the [$^{3}$H]ASDN (of each lot that is used) will be assessed by the lead laboratory (RTI). The radiochemical purity will be greater then approximately 95% and, if less then 95%, then the Sponsor will be notified.

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2.1.2 Preparation of Substrate Solution for use in Aromatase Assay

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

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

2.2 Test Substance

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: 063K4069
Purity: 99% (as per Sigma, assessed by TLC)
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 Chemical Repository (CR) for the EDSP and distributed to the laboratories. 4-OH ASDN will be formulated in 95% 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 activity. 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.

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2.3 Microsomes

Recombinant microsomes will be supplied to each laboratory by the lead laboratory. The microsomes must be stored at approximately -70°C. Bulk microsomes (not diluted) could be thawed/freeze several times. 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. used in the preparation or usage of microsomes is free of detergent residue.

On the day of use, microsomes will be thawed quickly in a 37 ± 1°C water bath and then 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 will be 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 will 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, that have been thawed and diluted for use, be refrozen and used again.

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 and sodium phosphate dibasic will be used in the preparation of the buffer. Solutions of each reagent at 0.1 M will be prepared in deionized water and then the solutions will be combined to a final pH of 7.4. The assay buffer may be stored for up to one month in the refrigerator (ca. 2-8 °C).

2.4.2 Propylene Glycol

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

2.4.3 NADPH

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

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## 3.0 PROTEIN ASSAY

The protein concentration in the microsomes will be determined each day of microsome use in the aromatase assay by using a DC Protein Assay kit purchased from Bio-Rad (Hercules, CA). 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). To a 25 µL aliquot of microsomes solution (1:50 dilution of microsomes may be required) or standard, 125 µL of BioRad DC Protein Kit Reagent A will be added and mixed. Next, 1 mL of BioRad DC Protein Kit Reagent B will be added to each standard or microsomes solution and the samples will be gently mixed. The samples will be allowed to sit at room temperature for at least 15 min to allow color development. (The absorbances are stable for about 1 hour.) Each sample (unknown and standards) will be transferred to disposable polystyrene cuvettes and the absorbance (750 nm) will be measured using a spectrophotometer. The protein concentration of the microsomal sample will be determined by interpolation, reading the concentration of protein on the standard curve that corresponds to its absorbance.

## 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 1.0 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 approximately five minutes prior to initiation of the assay by the addition of 1 mL of the diluted microsomal suspension. The total assay volume will be 2.0 mL, and the tubes will be incubated for 15 min. The incubations will be stopped by the addition of methylene chloride (2.0 mL); the tubes will be vortex-mixed for ca. 5 s and placed on ice. The tubes will be then vortex-mixed an additional 20-25 s. The tubes will then be centrifuged using a Beckman GS-6 centrifuge with GH-3.8 rotor for 10 minutes at a setting of 1000 rpm. The methylene chloride layer will be removed and discarded; the aqueous layers are extracted again with methylene chloride (2.0 mL). This extraction procedure will be performed one additional time, each time discarding the methylene chloride layer. The aqueous layers will be transferred to vials and duplicate aliquots (0.5 mL) will be transferred to 20-mL liquid scintillation counting vials. Liquid scintillation cocktail (Ultima Gold, Packard, 10 mL) will be added to each counting vial and shaken to mix the solution.

**Table 1. Optimized Aromatase Assay Conditions**

| | Assay Type |
|---|---|
| Assay factor (units) | Human Recombinant |
| Microsomal Protein (mg/mL)[a] | 0.004 |
| NADPH (mM)[a] | 0.3 |
| [$^3$H]ASDN (nM)[a] | 100 |
| Incubation Time (min) | 15 |

[a] Final concentrations

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

Results will be presented as the activity (velocity) of the enzyme (aromatase). The amount of estrogen product formed will 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). The activity of the enzyme will be expressed in nmol (mg protein)$^{-1}$min$^{-1}$ and will be calculated by dividing the amount of estrogen formed by the amount of microsomal protein used (in mg) 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 aromatase activity inhibition in the presence of 4-OH ASDN. Six different concentrations of 4-OH ASDN will be used. Each concentration of 4-OH ASDN will be run in triplicate. Three replicates of aromatase assay will be run independently. See Table 2 for the study design. Full and background activity control samples will be included in each assay run. Full activity controls will contain substrate, NADPH, propylene glycol, buffer, vehicle (used for preparation of 4-OH ASDN solutions), and microsomes. Background activity controls will contain all full activity control assay components except NADPH and will serve as assay blanks. Four full activity and four background activity controls will be included with each assay run and will be processed in the same manner as the other samples. The controls sets will be split so that two tubes (of each full and background activity control) will be 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.

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**Table 2. Positive Control Study Design**

| Sample type | Repetitions (test tubes) | Description | Final 4-OH ASDN concentration (M) |
|---|---|---|---|
| Full Activity Control | 4 | Complete assay[a] with inhibitor vehicle control | N/A |
| Background Activity Control | 4 | Complete assay with inhibitor vehicle control omitting NADPH | N/A |
| 4-OH ASDN Concentration 1 | 3 | Complete assay with 4-OH ASDN added | $1 \times 10^{-6}$ |
| 4-OH ASDN Concentration 2 | 3 | Complete assay with 4-OH ASDN added | $1 \times 10^{-7}$ |
| 4-OH ASDN Concentration 3 | 3 | Complete assay with 4-OH ASDN added | $5 \times 10^{-8}$ |
| 4-OH ASDN Concentration 4 | 3 | Complete assay with 4-OH ASDN added | $2.5 \times 10^{-8}$ |
| 4-OH ASDN Concentration 5 | 3 | Complete assay with 4-OH ASDN added | $1 \times 10^{-8}$ |
| 4-OH ASDN Concentration 6 | 3 | Complete assay with 4-OH ASDN added | $1 \times 10^{-9}$ |

[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, inhibitor, total dpm - background dpm, and % activity. The average background dpm values should be subtracted from the assayed samples dpm values to provide dpm 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 dpm 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 control 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 is expressed in nmol (mg protein)$^{-1}$min$^{-1}$ and is calculated by dividing the amount of estrogen formed (nmol) by the amount of microsomal

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protein used (in mg) times the incubation time (in min). Average activity in the full activity control samples for a given Study is calculated. Percent of activity remaining in the presence of various inhibitor concentrations is calculated by dividing the aromatase activity at a given inhibitor concentration by the average full activity control and multiplying by 100.

$IC_{50}$ will be calculated using Prism (Version 4.0) 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 dpm 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.
- Graphs of activity versus log chemical concentration.
- Table of $IC_{50}$s by date, run, technician, assay method.

## 6.0 STATISTICAL ANALYSES

Statistical analysis of the data will be conducted by a statistical analysis plan developed by Battelle's EDSP Data Coordination Center. The salient aspects of the plan are described below.

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 $\varepsilon$ 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 $IC_{50}$ ($10^{\mu}$) and slope ($\beta$). 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.

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The average value and standard error of $\log_{10}IC_{50}$ or $\beta$ can be calculated based on a one-way random effects analysis of variance model fit.

6.1 Graphical and Analysis of Variance Comparison among Concentration Response Curve Fits

For each replicate treat ($\beta$, $\mu$) as a random variable with mean ($\beta_{avg}$, $\mu_{avg}$) 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 ($\beta$) and $\log_{10}IC_{50}$ ($\mu$) will also be compared across replicates based on random effects analysis of variance, treating the replicates as random effects. $\beta$ and $\mu$ are estimated, separately within each replicate, and plotted along with the average and associated 95% confidence interval across replicates.

6.2 Negative and Positive Control Values Across Replicates

Within each replicate, quadruplicate repetitions will be made of the background activity tubes and the full 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) control activity. The average of the four background activity samples within a replicate must necessarily be 0 and the average of the four full postive 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 full 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

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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.

6.3 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 full and background activity groups. The factors in the mixed effects analysis of variance will be concentration group (including full 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 dpm 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.

6.4 Statistical Software

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). The inter-laboratory statistical analysis will be carried out by Battelle's EDSP Data Coordination Unit.

6.5 Inter-laboratory Statistical Analysis

The inter-laboratory statistical analysis will be carried out by Battelle's EDSP Data Coordination Unit.

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

## 9.0 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

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**PROTOCOL AMENDMENT NUMBER 1**

STUDY NUMBER: **G608317**

STUDY TITLE: **Aromatase Assay Validation: Positive Control Study: Recombinant Microsomes (WA 4-17, Task 3)**

PART TO BE CHANGED: The second sentence of the Section 3, page 7 is changed from: A 6-point standard curve will be prepared, ranging from 0.13-1.5 mg protein/mL.

CHANGED TO: A 6-point standard curve will be prepared, ranging from 0.14-1.0 mg protein/mL.

REASON FOR CHANGE: The protein standard (bovine serum albumin) stock solution from BioRad Laboratories is 1.4 mg/mL and not 2.5 mg/mL as was originally believed based on information obtained from the Lead Laboratory, Research Triangle Institute, International.

EFFECTIVE DATE: December 16, 2004

APPROVED BY:

Bozena D. Lusiak
Bozena D. Lusiak, Study Director

12-29-04
Date

Jerry D. Johnson
Sponsor Representative

12-29-04
Date

**PROTOCOL AMENDMENT NUMBER 2**

STUDY NUMBER: **G608317**

STUDY TITLE: **Aromatase Assay Validation: Positive Control Study: Recombinanat Microsomes (WA 4-17, Task 3)**

PART TO BE CHANGED: Paragraph 5.2 (Data Analysis and Presentation) and Section 6.0 (Statistical Analyses); See Attachment 1.

CHANGE TO: See Attachment 2.

REASON FOR CHANGE: Revision done on the request by Sponsor.

EFFECTIVE DATE: January 14, 2005

APPROVED BY:

Bozena D. Lusiak, Study Director — 1-24-05 Date

Sponsor Representative — 1-24-05 Date

**ATTACHMENT 1**

5.2 Data Analysis and Presentation

The data to be reported will include the following information: assay date and run number, technician, inhibitor, total dpm - background dpm, and % activity. The average background dpm values should be subtracted from the assayed samples dpm values to provide dpm 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 dpm 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 control 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 is expressed in nmol (mg protein)$^{-1}$min$^{-1}$ and is calculated by dividing the amount of estrogen formed (nmol) by the amount of microsomal protein used (in mg) times the incubation time (in min). Average activity in the full activity control samples for a given Study is calculated. Percent of activity remaining in the presence of various inhibitor concentrations is calculated by dividing the aromatase activity at a given inhibitor concentration by the average full activity control and multiplying by 100.

$IC_{50}$ will be calculated using Prism (Version 4.0) software to fit the percent of control activity and log concentration data to a curve using the following equation:

$$Y=\text{Bottom} + (\text{Top}-\text{Bottom})/(1+10^{((\text{Log}IC_{50}-X)*\text{HillSlope})})$$

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 dpm 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,

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(2) the day to day (study-to-study) variation.

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

## 6.0 STATISTICAL ANALYSES

Statistical analysis of the data will be conducted by a statistical analysis plan developed by Battelle's EDSP Data Coordination Center. The salient aspects of the plan are described below.

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 $\varepsilon$ 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 $IC_{50}$ ($10^{\mu}$) and slope ($\beta$). 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 $\beta$ can be calculated based on a one-way random effects analysis of variance model fit.

### 6.1 Graphical and Analysis of Variance Comparison among Concentration Response Curve Fits

For each replicate treat ($\beta$, $\mu$) as a random variable with mean ($\beta_{avg}$, $\mu_{avg}$) and covariance $\Sigma_{(\beta, \mu)}$ 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 $\Sigma_{(\beta, \mu)}$ 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

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

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

6.2 Negative and Positive Control Values Across Replicates

Within each replicate, quadruplicate repetitions will be made of the background activity tubes and the full 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) control activity. The average of the four background activity samples within a replicate must necessarily be 0 and the average of the four full postive 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 full 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 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.

6.3 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 full and background activity groups. The factors in the mixed effects analysis of variance will be concentration group (including full 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 dpm across all the replicates and repetitions within

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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 [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 [Average dpm]$^{1/2}$ for their concentration group to adjust for differing variability across concentration groups.

6.4 Statistical Software

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). The inter-laboratory statistical analysis will be carried out by Battelle's EDSP Data Coordination Unit.

6.5 Inter-laboratory Statistical Analysis

The inter-laboratory statistical analysis will be carried out by Battelle's EDSP Data Coordination Unit.

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**ATTACHMENT 2**

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 average of the dpm for the background tubes should be subtracted from the tubes with Total dpm to provide dpm 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 dpm 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 $(\text{mg protein})^{-1}\text{min}^{-1}$ 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:

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

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

The data will be formatted as follows:

- One spreadsheet or table will display the dpm for all assay tubes, calculations of activity $(\text{nmol (mg protein)})^{-1}\text{min}^{-1}$ 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.

## 6.0 STATISTICAL ANALYSES

### 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. 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 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
DAVG / average dpms across the repeat tubes with the same inhibitor concentration
$\beta$ / slope of the concentration response curve ($\beta$ 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 logarithm of concentration within each replicate:

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

where $\varepsilon$ 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 $IC_{50}$ ($10^{\mu}$) and slope ($\beta$). 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 $\log_{10}IC_{50}$ or $\beta$ can be calculated based on a one-way random effects analysis of variance model fit.

For each test substance and replicate the estimated $\log_{10}IC_{50}$ ($\Phi$), the within replicate standard error of $\mu$, the $IC_{50}$, the slope ($\beta$), the within replicate standard error of $\beta$, 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".

[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%.

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 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.

For each replicate treat ($\beta$, $\mu$) as a random variable with mean ($\beta_{avg}$, $\mu_{avg}$). 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 ($\beta$) and $\log_{10}IC_{50}$ ($\mu$) will also be compared across replicates based on random effects analysis of variance, treating the replicates as random effects. $\beta$ and $\mu$ 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).

6.3 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.

6.4 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).

6.5 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.

## APPENDIX B

Quality Assurance Project Plan (QAPP) ..... B-1

## 1.0 TITLE AND APPROVAL

**Quality Assurance Project Plan (QAPP)**
**For Work Assignment 4-17**
**Recombinant Aromatase Validation Study**

**Task 3 - Conduct Positive Control Studies**
**in Participating Laboratories**

for

**EPA CONTRACT NUMBER 68-W-01-023**

**December 7, 2004**

## SIGNATURE PAGE

**Quality Assurance Project Plan for WA 4-17**
**Recombinant Aromatase Validation Study**
**EPA CONTRACT NUMBER 68-W-01-023**

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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 recombinant microsomes, demonstrate the utility of the microsomal assay to detect known aromatase inhibitors, and compare the performance of the 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, and (2) to test up to 10 reference chemicals with different modes of action in order to test 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 3 and 4 of the work assignment.

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

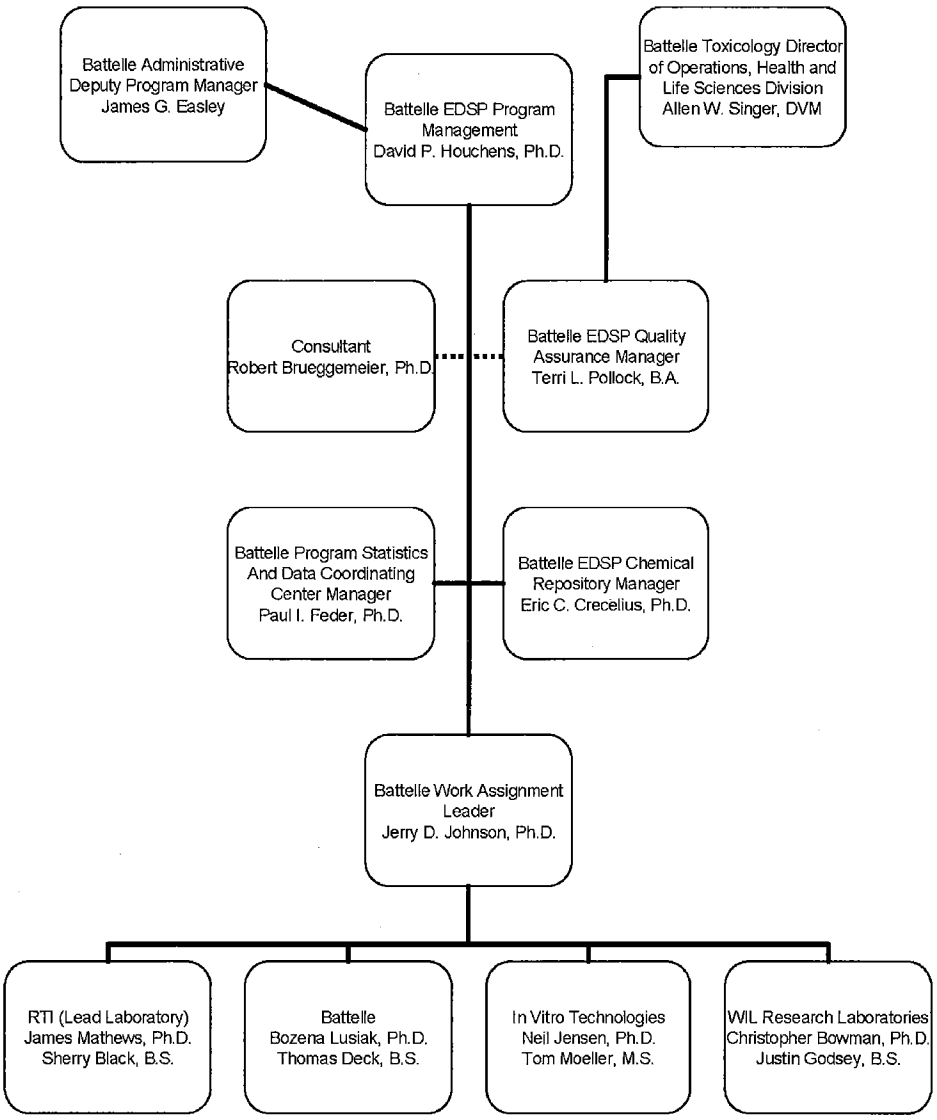


**Figure 4-1. WA 4-17 Organizational Chart**

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 WAL/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 recombinant aromatase assay (WA 2-24) were conducted to optimize the microsomal aromatase assay protocol for recombinant microsomes, demonstrate the utility of the microsomal assay to detect known aromatase inhibitors, and compare the performance of the recombinant assay system and the human placental microsomal assay system.

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 recombinant aromatase assay. A companion work assignment (WA 4-16) has been issued for the conduct of the human placental aromatase assay validation.

The work assignment is comprised of 6 tasks of which two tasks involve experimentation. Only the work in Task 3 is subject to this QAPP. Table 1 summarizes the validation tasks and the laboratory(ies) involved for each experimental task.

**Table 1. WA 4-17 Tasks**

| 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 | Conduct Positive Control Studies in the Participating Laboratories | 3 Participating Laboratories |
| 4 | Conduct Multiple Chemical Studies with Microsomes Supplied by RTI (RTI/Participating Laboratories) | Lead Laboratory + 3 Participating Laboratories |
| 5 | Prepare Study Reports (RTI/Participating Laboratories) | Not an experimental task |
| 6 | Prepare Presentation for EDMVAC | Not an experimental task |

EDMVAC = Endocrine Disruptor Method Validation Committee

## 5.2 Background

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

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

Aromatase is a cytochrome P450 enzyme complex responsible for estrogen biosynthesis and converts androgens, such as testosterone and androstenedione, into the estrogens estradiol and estrone. Aromatase is present in the ovary, placenta, uterus, testis, brain, and extraglandular adipose tissues. Two proteins, cytochrome $P450_{arom}$ 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 recombinant 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, demonstrate the utility of the microsomal assay to detect known aromatase inhibitors, and compare the performance of a recombinant assay system and the human 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 recombinant microsome aromatase assay.

## 6.0 PROJECT/TASK DESCRIPTION

Only Task 3 is under the control by this QAPP. However, this QAPP also addresses the other task in this work assignment and will be reissued prior to the start of the other task together with a finalized task-specific protocol included as an attachment. The Task 3 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 3: Conduct of the 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 recombinant 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) is tested in the aromatase assay at 6 concentrations to construct a dose/response curve from which an $IC_{50}$ may be calculated. Control runs are also 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.

**Task 4: Conduct Studies on Reference Chemicals**

Each participating laboratory will conduct the studies in this task with recombinant microsomes supplied by RTI.

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 must 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 4-17 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 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.

Staff from the participating laboratories will be trained on the performance of the aromatase assay at RTI International as part of Task 3 of WA 4-16. 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 tasks. 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 each task subject to this QAPP will be contained in GLP compliant protocols. A template protocol for Task 3 is attached as an Appendix.

## 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 WA Leader/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 be also 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 is 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 Mamager (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 Recombinant Aromatase Validation Study, EPA Contract Number 68-W-01-023, Work Assignment 4-17. 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 3

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|---|---|---|

EPA Contract No.:
EPA Work Assignment No.:

TITLE: Template Protocol for WA 4-17 Task 3:
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 D. 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 3: 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 recombinant 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 recombinant 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 a human recombinant microsome 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 recombinant microsomes will be obtained prior to initiation of the first set of experiments to ensure that sufficient quantities are available to conduct the studies.

### 2.1 Substrate

#### 2.1.1 Substrate Name/Supplier

The substrate for the aromatase assay is androstenedione (ASDN). Non-radiolabeled and radiolabeled ASDN will be used. The non-radiolabeled ASDN and the radiolabeled androstenedione ([1β-$^3$H]-androstenedione, [$^3$H]ASDN) will be provided to the laboratories by Battelle's Chemical 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.

#### 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

Recombinant 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.

Human recombinant microsomes will be obtained from Gentest™ (Woburn, MA; www.gentest.com). The product name is Human CYP19 (Aromatase) Supersomes™ and the catalog number is 456260. The Supersomes™ package size is 0.5 nmoles cytochrome P450 in 0.5 mL. Supplier-provided values for protein concentration, cytochrome c reductase activity, and aromatase activity will be found on the data sheet accompanying each shipment and will be included in the report.

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 controls 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 | $1 \times 10^{-6}$ |
| 4-OH ASDN Concentration 2 | 3 | Complete assay with 4-OH ASDN added | $1 \times 10^{-7}$ |
| 4-OH ASDN Concentration 3 | 3 | Complete assay with 4-OH ASDN added | $5 \times 10^{-8}$ |
| 4-OH ASDN Concentration 4 | 3 | Complete assay with 4-OH ASDN added | $2.5 \times 10^{-8}$ |
| 4-OH ASDN Concentration 5 | 3 | Complete assay with 4-OH ASDN added | $1 \times 10^{-8}$ |
| 4-OH ASDN Concentration 6 | 3 | Complete assay with 4-OH ASDN added | $1 \times 10^{-9}$ |

[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:

$$Y=\text{Bottom} + (\text{Top-Bottom})/(1+10^{((\text{Log}IC_{50}-X)*\text{HillSlope})})$$

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
$\beta$ ≡ slope of the concentration response curve ($\beta$ 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 $\epsilon$ 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 $IC_{50}$ ($10^{\mu}$) and slope ($\beta$). 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 $\beta$ 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 ($\beta$, $\mu$) as a random variable with mean ($\beta_{avg}$, $\mu_{avg}$) 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 ($\beta$) and $\log_{10}IC_{50}$ ($\mu$) will also be compared across replicates based on random effects analysis of variance, treating the replicates as random effects. $\beta$ and $\mu$ 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 [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.

### 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 C

[$^{3}$H]ASDN Purity Assessment Report (RTI International) ..... C-1

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

Author:

Sherry J Black 9/28/05

Sherry Black — Date
Research Chemist

Approved:

J Mathews 9-28-05

James Mathews, Ph. D, DABT — Date
Study Director

RTI INTERNATIONAL

## Quality Assurance Statement

| | |
|---|---|
| **Study Title:** | **[$^3$H] ASDN Radiochemical Purity Determination WA 4-16 and WA 4-17** |
| **Sponsor:** | **Battelle Memorial Institute** |
| **Study Code:** | **An05-928** |
| **Protocol Number:** | **RTI-928-AN** |

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

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

K. Collier
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).

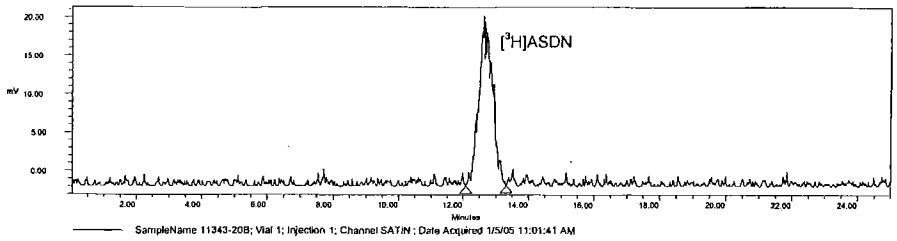
The radiochemical purity of the [$^3$H]ASDN (1:100 dilution in ethanol) was determined using high performance liquid chromatography (HPLC) and liquid scintillation counting. The HPLC system consists of a Waters 2690 Separations Module, a Waters 2487 Dual $\lambda$ Absorbance Detector and a $\beta$-RAM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

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

## Results

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

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




## Conclusion

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

## APPENDIX D

Chemistry Reports (from Battelle) ..... D-1

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

Battelle Study No. WA 4-16/17

## 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** | $C_{19}H_{28}O_3$ |
| **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.

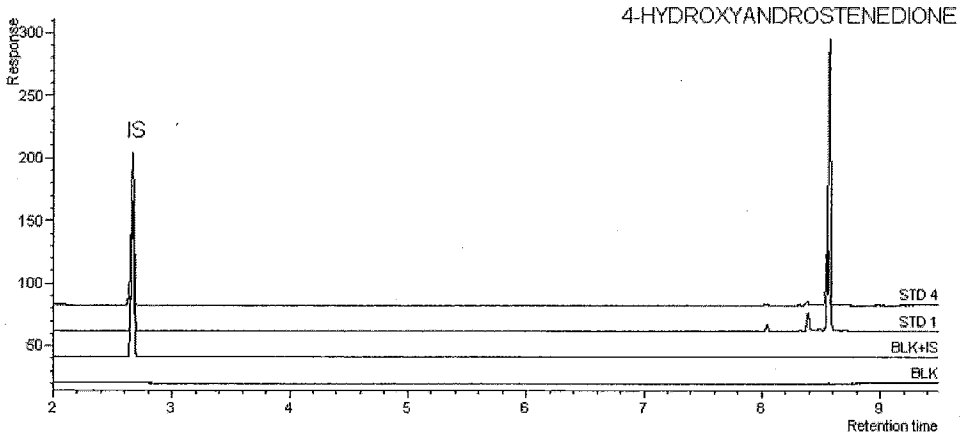



**Figure 2 – Representative Overlaid Chromatograms from a High and Low Vehicle/Calibration Standard, Blank with Internal Standard, and Blank from the Validation (Shown Top to Bottom)**

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

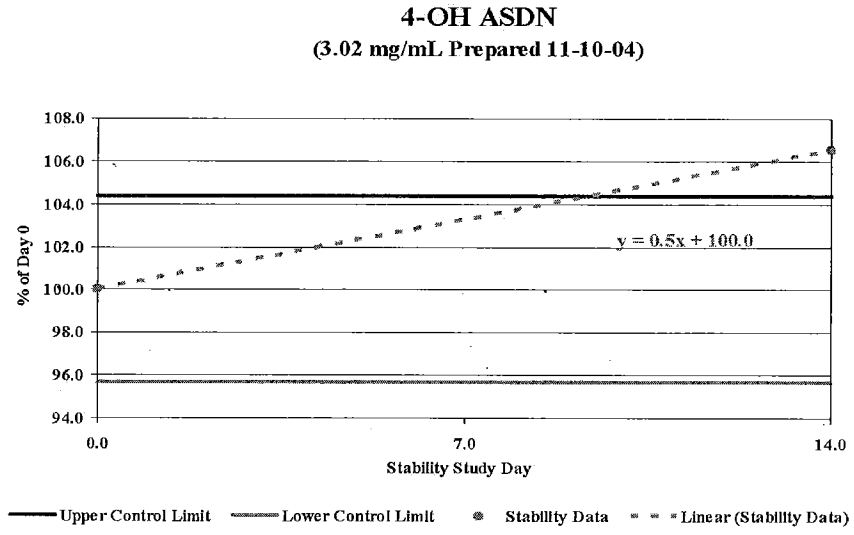
The results from the storage stability study are shown in Table 5 and presented in control chart format in Figure 3.

**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.



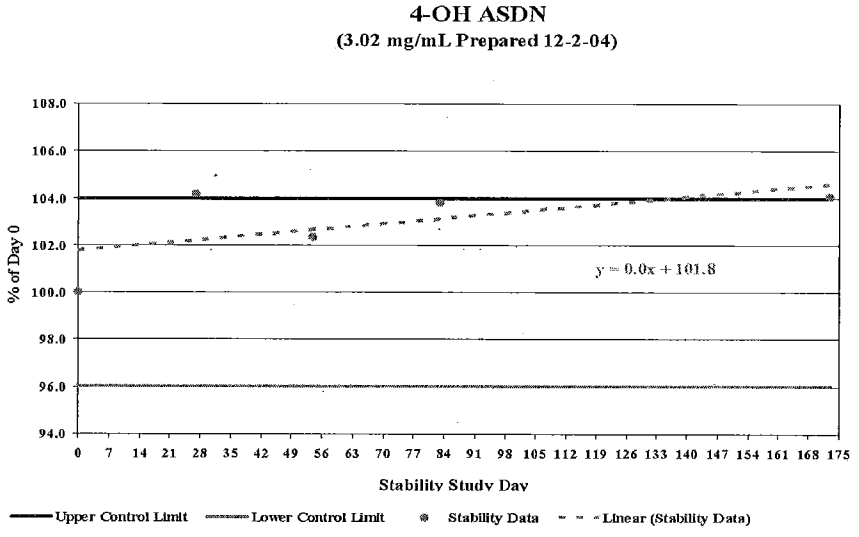




**Figure 3 – Control Charts for the Storage Stability Studies**

### 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.

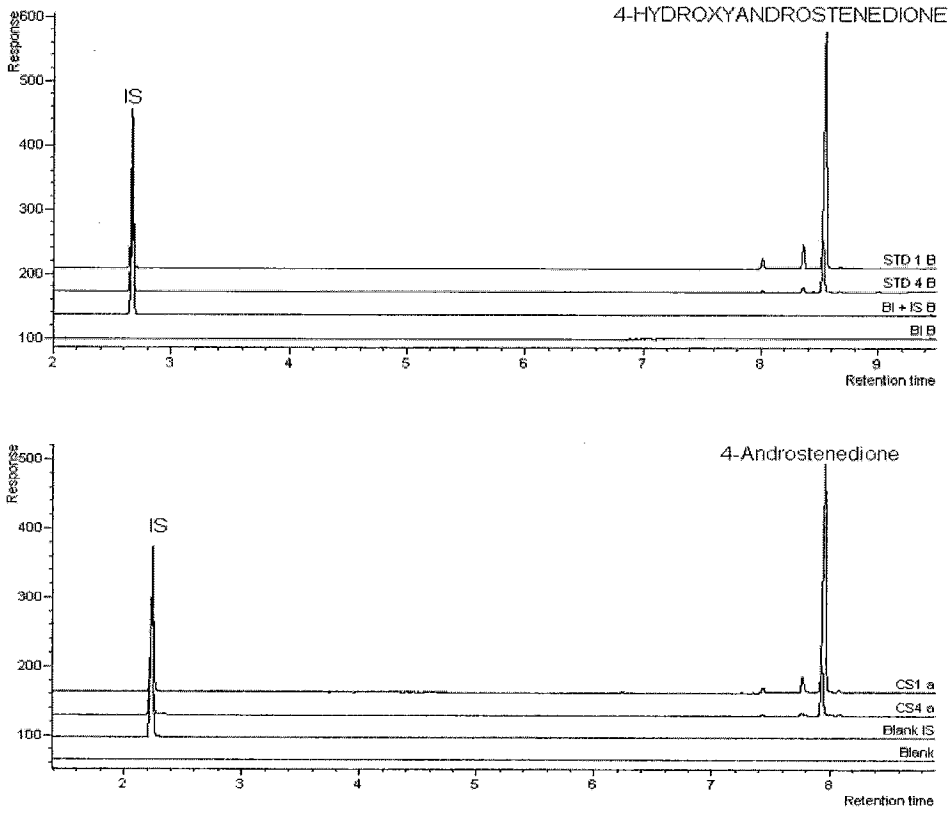



**Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from Formulation Analysis Batch 1-ASDN and Batch 2-ASDN (Shown Top to Bottom)**

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 E

Spreadsheets – The Aromatase Activity Calculation Page of the Spreadsheet for Each Replicate ..... E-1

| Aliquot # | Weight of aliquot (g) | DPM/Aliq. | DPM/g soln. | |
|---|---|---|---|---|
| 1 | 0.0195 | 23147 | 1187026 | |
| 2 | 0.0192 | 25358 | 1320729 | |
| 3 | 0.0195 | 26069 | 1336872 | |
| 4 | 0.0192 | 26418 | 1375938 | |
| 5 | 0.0191 | 26414 | 1382932 | |
| | | | Average DPM/g soln | 1320699 |
| | | | SD | 79143 |
| | | | CV | 5.99 |
| | | | µCi/g soln | 0.595 |

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 | 8.0517 g |
| Mass of dilution B used in substrate prep | 4.5294 g |
| Concentration of nonradiolabeled ASDN in substrate soln. | 0.57379 µg/g |

Calculation of Substrate Solution Specific Activity

1) Calculate µg [$^3$H]ASDN/g soln. = 0.00673 µg/g soln.
µg/g soln.

a. µCi/g soln 0.595
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.

= 0.573790 + 0.00673
= 0.580525 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.025 µCi/µg ASDN

651563 dpm/nmol

Assay Date 12/20/2004 Test Chemical ID 4OH ASDN # Concentrations tested 6

Technician ID BDL/TD Replicate # 1 Microsome type Recombinant Microsome ID BD Gentest, lot#5

| | | | | | | | | Protein stock (mg BSA) | Total volume of stock (mL) | Protein stock ID |
|---|---|---|---|---|---|---|---|---|---|---|
| Standards: | 1 | 0.8 | 0.6 | 0.4 | 0.2 | 0.14 | Blank | 28 | 20 | 210000238 |
| | 0.266 | 0.184 | 0.200 | 0.124 | 0.065 | 0.056 | 0.000 | | | |
| | 0.273 | 0.232 | 0.190 | 0.131 | 0.073 | 0.042 | 0.000 | | | |
| | 0.287 | 0.231 | 0.184 | 0.108 | 0.063 | 0.046 | 0.000 | | | |
| Samples: | | | | | | | | | | |
| | 0.171 | | | | | | | | | |
| | 0.195 | | | | | | | | | |
| | 0.190 | | | | | | | | | |

| Standard concentration (mg/mL) | Volume of stock used | Final volume of Std | mg Protein per µL | µL Standard Used | mg Protein Measured | $A_{raw}$ | $A_{adj}$ | Curve Output |
|---|---|---|---|---|---|---|---|---|
| 1 | 17.9 | 25 | 0.00100 | 25 | 0.0251 | 0.275 | 0.275 | 0.0247 |
| 0.8 | 14.3 | 25 | 0.00080 | 25 | 0.0200 | 0.216 | 0.216 | 0.0190 |
| 0.6 | 10.7 | 25 | 0.00060 | 25 | 0.0150 | 0.191 | 0.191 | 0.0167 |
| 0.4 | 7.1 | 25 | 0.00040 | 25 | 0.0099 | 0.121 | 0.121 | 0.0100 |
| 0.2 | 3.6 | 25 | 0.00020 | 25 | 0.0050 | 0.067 | 0.067 | 0.0049 |
| 0.14 | 2.5 | 25 | 0.00014 | 25 | 0.0035 | 0.048 | 0.048 | 0.0032 |

Blank 0.000

$r^2$= 0.988
m= 0.095
b= -0.001

| Variables | Regression results | |
|---|---|---|
| m, b | 0.095 | -0.001 |
| $se_m$, $se_b$ | 0.005 | 0.001 |
| $r^2$, $se_y$ | 0.988 | 0.001 |
| F, df | 334 | 4 |
| $ss_{reg}$, $ss_{resid}$ | 0.000 | 0.000 |

Regression results are calculated using the function LINEST

| $A_{raw}$ | $A_{adj}$ | mg protein measured | µL diluted µSOMES | Vol usome prep. (µL) | Final vol. Diluted usomes (µL) | mg protein/µL Prep. | average mg/µL | mg/mL |
|---|---|---|---|---|---|---|---|---|
| 0.171 | 0.171 | 0.015 | 25 | 100 | 1000 | 0.006 | 0.006 | 6.456 |
| 0.195 | 0.195 | 0.017 | 25 | 100 | 1000 | 0.007 | | |
| 0.190 | 0.190 | 0.017 | 25 | 100 | 1000 | 0.007 | | |

① AC, recorded manually by BDL on 04-21-05

G608317

| Assay Date | Test Chemical ID | # Concentrations tested | Microsome type | Recombinant Microsome ID | BD Gentest Technician ID | BDL/TD # | Replicate # |
|---|---|---|---|---|---|---|---|
| 12/20/04 ① ② ~~########~~ | 4OH ASDN | 6 | | | | | 1 |

| Microsome Dilution Details | | |
|---|---|---|
| Dilution A | 0.1 | mL microsome Stock used |
| | 1 | mL total volume |
| | 10 | dilution factor |
| Dilution B | 0.49 | mL microsome Dilution A used |
| | 30 | mL total volume |
| | 61.22449 | dilution factor |
| Dilution C (if applicable) | | mL microsome Dilution B used |
| | | mL total volume |
| | NA | dilution factor |
| | 612.2449 | 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): | 6.456 |
| Protein Concentration (dilution added to assay, mg/mL): | 0.010545 |

② AC, computer truncated date and date was recorded manually. 06-16-05 BDL

| Assay Date | 12/20/2004 | Test Chemical ID | 4OH ASDN | # Concentrations tested | | S9 Microsome type | Recombinan Microsome ID | BD Gentest, lot#5 | Technician ID | BDL/TD | 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 | 5081 | 10162 | | | 0.1 | 132070 | | | | | | | |
| | | | 0.5 | 2 | 5081 | 10162 | | | | | | | | | | | |
| | 2 | 2 | 0.5 | 1 | 5537 | 11074 | 10937 | 21874 | 0.1 | 132070 | 16.58 | 21776 | 0.0334 | 1 | 0.005 | 15 | 0.2113 |
| | | | 0.5 | 2 | 5400 | 10800 | | | | | | | | | | | |
| | 3 | 2 | 0.5 | 1 | 4623 | 9246 | 9264 | 18528 | 0.1 | 132070 | 14.03 | 18430 | 0.0283 | 1 | 0.005 | 15 | 0.1788 |
| | | | 0.5 | 2 | 4641 | 9282 | | | | | | | | | | | |
| | 4 | 2 | 0.5 | 1 | 4099 | 8198 | 8255 | 16510 | 0.1 | 132070 | 12.50 | 16412 | 0.0252 | 1 | 0.005 | 15 | 0.1592 |
| | | | 0.5 | 2 | 4156 | 8312 | | | | | | | | | | | |
| Background control | 1 | 2 | 0.5 | 1 | 28 | 56 | 57 | 114 | 0.1 | 132070 | 0.09 | 16 | 0.0000 | 1 | 0.005 | 15 | 0.0002 |
| | | | 0.5 | 2 | 29 | 58 | | | | | | | | | | | |
| | 2 | 2 | 0.5 | 1 | 19 | 38 | 48 | 96 | 0.1 | 132070 | 0.07 | -5 | 0.0000 | 1 | 0.005 | 15 | 0.0000 |
| | | | 0.5 | 2 | 29 | 58 | | | | | | | | | | | |
| | 3 | 2 | 0.5 | 1 | 20 | 40 | 49 | 98 | 0.1 | 132070 | 0.07 | -1 | 0.0000 | 1 | 0.005 | 15 | 0.0000 |
| | | | 0.5 | 2 | 26 | 52 | | | | | | | | | | | |
| | 4 | 2 | 0.5 | 1 | 18 | 35 | 43 | 86 | 0.1 | 132070 | 0.07 | -13 | 0.0000 | 1 | 0.005 | 15 | -0.0001 |
| | | | 0.5 | 2 | 25 | 50 | | | | | | | | | | | |
| Positive control | 1 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 2 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 3 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 4 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| Negative Control | 1 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 2 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 3 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 4 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| 4OH ASDN | 1-1 | 2 | 0.5 | 1 | 480 | 960 | 970 | 1940 | | 132070 | 1.47 | 1642 | 0.0028 | 1 | 0.005 | 15 | 0.0179 |
| | | | 0.5 | 2 | 490 | 980 | | | | | | | | | | | |
| | 1-2 | 2 | 0.5 | 1 | 479 | 958 | 954 | 1908 | 0.1 | 132070 | 1.44 | 1610 | 0.0028 | 1 | 0.005 | 15 | 0.0176 |
| | | | 0.5 | 2 | 475 | 950 | | | | | | | | | | | |
| | 1-3 | 2 | 0.5 | 1 | 454 | 908 | 902 | 1804 | 0.1 | 132070 | 1.37 | 1706 | 0.0026 | 1 | 0.005 | 15 | 0.0165 |
| | | | 0.5 | 2 | 448 | 896 | | | | | | | | | | | |
| | 2-1 | 2 | 0.5 | 1 | 3267 | 4534 | 4515 | 9030 | 0.1 | 132070 | 6.84 | 8532 | 0.0137 | 1 | 0.005 | 15 | 0.0867 |
| | | | 0.5 | 2 | 3248 | 4496 | | | | | | | | | | | |
| | 2-2 | 2 | 0.5 | 1 | 3326 | 4652 | 4684 | 9368 | 0.1 | 132070 | 7.09 | 8870 | 0.0142 | 1 | 0.005 | 15 | 0.0899 |
| | | | 0.5 | 2 | 3358 | 4716 | | | | | | | | | | | |
| | 2-3 | 2 | 0.5 | 1 | 3354 | 4708 | 4667 | 9334 | 0.1 | 132070 | 7.07 | 8238 | 0.0142 | 1 | 0.005 | 15 | 0.0896 |
| | | | 0.5 | 2 | 3313 | 4626 | | | | | | | | | | | |
| | 3-1 | 2 | 0.5 | 1 | 3140 | 5296 | 5296 | 12592 | 0.1 | 132070 | 9.53 | 12494 | 0.0192 | 1 | 0.005 | 15 | 0.1212 |
| | | | 0.5 | 2 | 3156 | 8312 | | | | | | | | | | | |
| | 3-2 | 2 | 0.5 | 1 | 3018 | 6036 | 6157 | 12294 | 0.1 | 132070 | 9.38 | 12296 | 0.0189 | 1 | 0.005 | 15 | 0.1193 |
| | | | 0.5 | 2 | 3179 | 6358 | | | | | | | | | | | |
| | 3-3 | 2 | 0.5 | 1 | 3022 | 6044 | 6123 | 12246 | 0.1 | 132070 | 9.27 | 12148 | 0.0186 | 1 | 0.005 | 15 | 0.1179 |
| | | | 0.5 | 2 | 3101 | 6202 | | | | | | | | | | | |
| | 4-1 | 2 | 0.5 | 1 | 3753 | 7505 | 7418 | 14835 | 0.1 | 132070 | 11.23 | 14738 | 0.0226 | 1 | 0.005 | 15 | 0.1430 |
| | | | 0.5 | 2 | 3666 | 7330 | | | | | | | | | | | |
| | 4-2 | 2 | 0.5 | 1 | 3654 | 7309 | 7343 | 14688 | 0.1 | 132070 | 11.12 | 14589 | 0.0224 | 1 | 0.005 | 15 | 0.1415 |
| | | | 0.5 | 2 | 3689 | 7378 | | | | | | | | | | | |
| | 4-3 | 2 | 0.5 | 1 | 3789 | 7578 | 7693 | 15386 | 0.1 | 132070 | 11.65 | 15288 | 0.0235 | 1 | 0.005 | 15 | 0.1483 |
| | | | 0.5 | 2 | 3904 | 7808 | | | | | | | | | | | |
| | 5-1 | 2 | 0.5 | 1 | 3915 | 7830 | 7745 | 15490 | 0.1 | 132070 | 11.73 | 15392 | 0.0236 | 1 | 0.005 | 15 | 0.1493 |
| | | | 0.5 | 2 | 3830 | 7660 | | | | | | | | | | | |
| | 5-2 | 2 | 0.5 | 1 | 3830 | 7660 | 7738 | 15476 | 0.1 | 132070 | 11.72 | 15378 | 0.0236 | 1 | 0.005 | 15 | 0.1492 |
| | | | 0.5 | 2 | 3908 | 7816 | | | | | | | | | | | |
| | 5-3 | 2 | 0.5 | 1 | 3962 | 7924 | 8075 | 16150 | 0.1 | 132070 | 12.23 | 16052 | 0.0246 | 1 | 0.005 | 15 | 0.1558 |
| | | | 0.5 | 2 | 4113 | 8226 | | | | | | | | | | | |
| | 6-1 | 2 | 0.5 | 1 | 4303 | 8606 | 8500 | 17000 | 0.1 | 132070 | 12.87 | 16902 | 0.0259 | 1 | 0.005 | 15 | 0.1640 |
| | | | 0.5 | 2 | 4197 | 8394 | | | | | | | | | | | |
| | 6-2 | 2 | 0.5 | 1 | 4264 | 9168 | 9216 | 18432 | 0.1 | 132070 | 13.96 | 18334 | 0.0281 | 1 | 0.005 | 15 | 0.1779 |
| | | | 0.5 | 2 | 4632 | 9264 | | | | | | | | | | | |
| | 6-3 | 2 | 0.5 | 1 | 4413 | 8826 | 8711 | 17422 | 0.1 | 132070 | 13.19 | 17324 | 0.0266 | 1 | 0.005 | 15 | 0.1681 |
| | | | 0.5 | 2 | 4298 | 8596 | | | | | | | | | | | |

| Assay Date | 12/20/2004 | Test Chemical ID | 4OH ASDN | # Concentrations tested | 6 | Microsome type | Recombinant | Microsome ID | BD Gentest, lot | Technician ID | BDL/TD | Replicate # | 1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Control Type | Portion | Average | SD |
|---|---|---|---|
| Full activity | Beginning | 0.2038 | 0.0106 |
| Full activity | End | 0.1690 | 0.0138 |
| Full activity | Overall | 0.1864 | 0.0224 |
| Background | Beginning | 0.0001 | 0.000123501 |
| Background | End | -0.0001 | 8.23342E-05 |
| Background | Overall | 0.0000 | 0.000112462 |
| 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 |
|---|---|---|---|---|---|
| 4OH ASDN | 1 | 1 | 1.00E-06 | -6.00 | 0.0179 |
| 4OH ASDN | 1 | 2 | 1.00E-06 | -6.00 | 0.0176 |
| 4OH ASDN | 1 | 3 | 1.00E-06 | -6.00 | 0.0165 |
| 4OH ASDN | 2 | 1 | 1.00E-07 | -7.00 | 0.0867 |
| 4OH ASDN | 2 | 2 | 1.00E-07 | -7.00 | 0.0899 |
| 4OH ASDN | 2 | 3 | 1.00E-07 | -7.00 | 0.0896 |
| 4OH ASDN | 3 | 1 | 5.00E-08 | -7.30 | 0.1212 |
| 4OH ASDN | 3 | 2 | 5.00E-08 | -7.30 | 0.1193 |
| 4OH ASDN | 3 | 3 | 5.00E-08 | -7.30 | 0.1179 |
| 4OH ASDN | 4 | 1 | 2.50E-08 | -7.60 | 0.1430 |
| 4OH ASDN | 4 | 2 | 2.50E-08 | -7.60 | 0.1415 |
| 4OH ASDN | 4 | 3 | 2.50E-08 | -7.60 | 0.1483 |
| 4OH ASDN | 5 | 1 | 1.00E-08 | -8.00 | 0.1493 |
| 4OH ASDN | 5 | 2 | 1.00E-08 | -8.00 | 0.1492 |
| 4OH ASDN | 5 | 3 | 1.00E-08 | -8.00 | 0.1558 |
| 4OH ASDN | 6 | 1 | 1.00E-09 | -9.00 | 0.1640 |
| 4OH ASDN | 6 | 2 | 1.00E-09 | -9.00 | 0.1779 |
| 4OH ASDN | 6 | 3 | 1.00E-09 | -9.00 | 0.1681 |

| | Percent of control values | | | |
|---|---|---|---|---|
| | | Replicate | | |
| Level | Log[test substance] | 1 | 2 | 3 |
| 1 | -6.00 | 9.59 | 9.42 | 8.88 |
| 2 | -7.00 | 46.49 | 48.25 | 48.08 |
| 3 | -7.30 | 65.03 | 64.00 | 63.23 |
| 4 | -7.60 | 76.72 | 75.94 | 79.58 |
| 5 | -8.00 | 80.12 | 80.05 | 83.56 |
| 6 | -9.00 | 87.98 | 95.43 | 90.18 |

| Aliquot # | Weight of aliquot (g) | DPM/Aliq. | DPM/g soln. | |
|---|---|---|---|---|
| 1 | 0.0196 | 28440 | 1451020 | |
| 2 | 0.0196 | 29417 | 1500867 | |
| 3 | 0.0194 | 30069 | 1549948 | |
| 4 | 0.0195 | 29872 | 1531897 | |
| 5 | 0.0195 | 29435 | 1509487 | |
| | | | Average DPM/g soln | 1508644 |
| | | | SD | 37511 |
| | | | CV | 2.49 |
| | | | µCi/g soln | 0.680 |

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

| ASDN solution | mg ASDN added | total volume (mL) | dilution factor | [ASDN] in solution (µg/mL) |
|---|---|---|---|---|
| Stock | 10.2 | 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 | 8.0344 g |
| Mass of dilution B used in substrate prep | 4.5198 g |
| Concentration of nonradiolabeled ASDN in substrate soln. | 0.573807 µg/g |

Calculation of Substrate Solution Specific Activity

1) Calculate µg [$^3$H]ASDN/g soln. = 0.00769 µg/g soln.
µg/g soln.

a. µCi/g soln 0.680
b. Specific activity of [$^3$H]ASDN (µCi/mmol) 25300000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [$^3$H]ASDN/g soln.

= 0.573807 + 0.00769
= 0.581500 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.169 µCi/µg ASDN

743036 dpm/nmol

| Assay Date | 12/21/2004 | Test Chemical ID | 4OH ASDN | # Concentrations tested | 6 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Technician ID | BDL/TD | Replicate # | 2 | Microsome type | Recombinant | Microsome ID | BD Gentest, lot#5 | | |

| | 1 | 0.8 | 0.6 | 0.4 | 0.2 | 0.14 | Blank | Protein stock (mg BSA) | Total volume of stock (mL) | Protein stock ID |
|---|---|---|---|---|---|---|---|---|---|---|
| Standards: | 0.274 | 0.248 | 0.211 | 0.124 | 0.080 | 0.043 | 0.000 | 28 | 20 | 210000238 |
| | 0.286 | 0.243 | 0.185 | 0.131 | 0.076 | 0.029 | 0.000 | | | |
| | 0.266 | 0.231 | 0.176 | 0.136 | 0.055 | 0.035 | 0.000 | | | |
| Samples: | 0.173 | | | | | | | | | |
| | 0.156 | | | | | | | | | |
| | 0.158 | | | | | | | | | |

| Standard concentration (mg/mL) | Volume of stock used | Final volume of Std | mg Protein per µL | µL Standard Used | mg Protein Measured | $A_{raw}$ | $A_{adj}$ | Curve Output |
|---|---|---|---|---|---|---|---|---|
| 1 | 17.9 | 25 | 0.00100 | 25 | 0.0251 | 0.275 | 0.275 | 0.0236 |
| 0.8 | 14.3 | 25 | 0.00080 | 25 | 0.0200 | 0.241 | 0.241 | 0.0205 |
| 0.6 | 10.7 | 25 | 0.00060 | 25 | 0.0150 | 0.191 | 0.191 | 0.0161 |
| 0.4 | 7.1 | 25 | 0.00040 | 25 | 0.0099 | 0.130 | 0.130 | 0.0107 |
| 0.2 | 3.6 | 25 | 0.00020 | 25 | 0.0050 | 0.070 | 0.070 | 0.0054 |
| 0.14 | 2.5 | 25 | 0.00014 | 25 | 0.0035 | 0.036 | 0.036 | 0.0023 |

| Variables | Regression results | |
|---|---|---|
| m, b | 0.089 | -0.001 |
| $se_m$, $se_b$ | 0.006 | 0.001 |
| $r^2$, $se_y$ | 0.984 | 0.001 |
| F, df | 247 | 4 |
| $ss_{reg}$, $ss_{resid}$ | 0.000 | 0.000 |

Regression results are calculated using the function LINEST

| | | | |
|---|---|---|---|
| Blank | 0.000 | $r^2$= | 0.984 |
| | | m= | 0.089 |
| | | 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 |
|---|---|---|---|---|---|---|---|---|
| 0.173 | 0.173 | 0.014 | 25 | 100 | 1000 | 0.006 | 0.005 | 5.415 |
| 0.156 | 0.156 | 0.013 | 25 | 100 | 1000 | 0.005 | | |
| 0.158 | 0.158 | 0.013 | 25 | 100 | 1000 | 0.005 | | |

| Assay Date | 12/21/04 | Test Chemical ID | 4OH ASDN | # Concentrations tested | 6 | Microsome type | Recombina | Microsome ID | BD Gentes | Technician ID | BDL/TD | Replicate # | 2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Microsome Dilution Details | | |
|---|---|---|
| Dilution A | 0.1 | mL microsome Stock used |
| | 1 | mL total volume |
| | 10 | dilution factor |
| Dilution B | 0.49 | mL microsome Dilution A used |
| | 30 | mL total volume |
| | 61.22449 | dilution factor |
| Dilution C (if applicable) | | mL microsome Dilution B used |
| | | mL total volume |
| | NA | dilution factor |
| | 612.2449 | total dilution factor |

| | |
|---|---|
| Protein Concentration (stock microsomes, mg/mL): | 5.415 |
| Protein Concentration (dilution added to assay, mg/mL): | 0.008845 |

| 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 | 12/21/2004 | Test Chemical ID | 4OH ASDN | # Concentrations tested | | S Microsome type | Recombinen | Microsome ID | BD Gentest, lot#5 | Technician ID | BDL/TD | Replicate # | 2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Sample ID | | Calculate DPM in aqueous portion after extraction | | | | | | | Calculate % turnover | | | Calculate nmol $^3H_2O$ formed | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample type | Replicate/Level | Nominal total volume (mL) | Aliq Volume (mL) | Aliq. # | DPM/aliq | DPM/mL | Ave DPM/mL | Total DPM | Volume of substrate solution used/assay tube (mL) | total DPM in assay tube (initial) | % conversion to product | Total DPM corrected for background (Background Tubes) | nmol $^3H_2O$ formed | Volume diluted microsomes used in assay tube (mL) | Final [protein] in assay (mg/mL) | Incubation time (min) | Aromatase activity (nmol estrogen formed/mg protein/min |
| Full activity control | 1 | 2 | 0.5 | 1 | 4406 | 8812 | 8938 | 17876 | 0.1 | 150864 | 11.86 | 17744 | 0.0239 | 1 | 0.004 | 15 | 0.1800 |
| | | | 0.5 | 2 | 4532 | 9064 | | | | | | | | | | | |
| | 2 | 2 | 0.5 | 1 | 4354 | 8708 | 8844 | 17688 | 0.1 | 150864 | 11.72 | 17556 | 0.0236 | 1 | 0.004 | 15 | 0.1781 |
| | | | 0.5 | 2 | 4490 | 8980 | | | | | | | | | | | |
| | 3 | 2 | 0.5 | 1 | 3726 | 7452 | 7512 | 15024 | 0.1 | 150864 | 9.86 | 14892 | 0.0200 | 1 | 0.004 | 15 | 0.1511 |
| | | | 0.5 | 2 | 3786 | 7572 | | | | | | | | | | | |
| | 4 | 2 | 0.5 | 1 | 4222 | 8444 | 8501 | 17002 | 0.1 | 150864 | 11.27 | 16870 | 0.0227 | 1 | 0.004 | 15 | 0.1711 |
| | | | 0.5 | 2 | 4278 | 8556 | | | | | | | | | | | |
| Background control | 1 | 2 | 0.5 | 1 | 55 | 110 | 106 | 212 | 0.1 | 150864 | 0.14 | 80 | 0.0001 | 1 | 0.004 | 15 | 0.0008 |
| | | | 0.5 | 2 | 51 | 102 | | | | | | | | | | | |
| | 2 | 2 | 0.5 | 1 | 23 | 46 | 55 | 110 | 0.1 | 150864 | 0.07 | -23 | 0.0000 | 1 | 0.004 | 15 | -0.0002 |
| | | | 0.5 | 2 | 32 | 64 | | | | | | | | | | | |
| | 3 | 2 | 0.5 | 1 | 25 | 50 | 55 | 110 | 0.1 | 150864 | 0.07 | -23 | 0.0000 | 1 | 0.004 | 15 | -0.0002 |
| | | | 0.5 | 2 | 30 | 60 | | | | | | | | | | | |
| | 4 | 2 | 0.5 | 1 | 22 | 44 | 49 | 98 | 0.1 | 150864 | 0.06 | -35 | 0.0000 | 1 | 0.004 | 15 | -0.0003 |
| | | | 0.5 | 2 | 27 | 54 | | | | | | | | | | | |
| Positive control | 1 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 2 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 3 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 4 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| Negative Control | 1 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 2 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 3 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 4 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| 4OH ASDN | 1-1 | 2 | 0.5 | 1 | 359 | 718 | 725 | 1450 | 0.1 | 150864 | 0.98 | 1318 | 0.0018 | | 0.004 | 15 | 0.0134 |
| | | | 0.5 | 2 | 366 | 732 | | | | | | | | | | | |
| | 1-2 | 2 | 0.5 | 1 | 347 | 694 | 694 | 1388 | 0.1 | 150864 | 0.92 | 1256 | 0.0017 | | 0.004 | 15 | 0.0127 |
| | | | 0.5 | 2 | 347 | 694 | | | | | | | | | | | |
| | 1-3 | 2 | 0.5 | 1 | 379 | 758 | 748 | 1496 | 0.1 | 150864 | 0.99 | 1364 | 0.0018 | | 0.004 | 15 | 0.0136 |
| | | | 0.5 | 2 | 369 | 738 | | | | | | | | | | | |
| | 2-1 | 2 | 0.5 | 1 | 1763 | 3526 | 3485 | 6970 | 0.1 | 150864 | 4.62 | 6838 | 0.0092 | 1 | 0.004 | 15 | 0.0694 |
| | | | 0.5 | 2 | 1722 | 3444 | | | | | | | | | | | |
| | 2-2 | 2 | 0.5 | 1 | 1686 | 3372 | 3344 | 6688 | 0.1 | 150864 | 4.43 | 6556 | 0.0088 | 1 | 0.004 | 15 | 0.0665 |
| | | | 0.5 | 2 | 1658 | 3316 | | | | | | | | | | | |
| | 2-3 | 2 | 0.5 | 1 | 1689 | 3378 | 3395 | 6790 | 0.1 | 150864 | 4.50 | 6658 | 0.0090 | 1 | 0.004 | 15 | 0.0675 |
| | | | 0.5 | 2 | 1706 | 3412 | | | | | | | | | | | |
| | 3-1 | 2 | 0.5 | 1 | 2297 | 4594 | 4741 | 9482 | 0.1 | 150864 | 6.29 | 9350 | 0.0126 | 1 | 0.004 | 15 | 0.0948 |
| | | | 0.5 | 2 | 2444 | 4888 | | | | | | | | | | | |
| | 3-2 | 2 | 0.5 | 1 | 2343 | 4686 | 4687 | 9374 | 0.1 | 150864 | 6.21 | 9242 | 0.0124 | 1 | 0.004 | 15 | 0.0937 |
| | | | 0.5 | 2 | 2344 | 4688 | | | | | | | | | | | |
| | 3-3 | 2 | 0.5 | 1 | 2437 | 4874 | 4822 | 9644 | 0.1 | 150864 | 6.53 | 9712 | 0.0131 | 1 | 0.004 | 15 | 0.0985 |
| | | | 0.5 | 2 | 2485 | 4970 | | | | | | | | | | | |
| | 4-1 | 2 | 0.5 | 1 | 3190 | 6380 | 6346 | 12692 | 0.1 | 150864 | 8.41 | 12580 | 0.0169 | 1 | 0.004 | 15 | 0.1274 |
| | | | 0.5 | 2 | 3156 | 6312 | | | | | | | | | | | |
| | 4-2 | 2 | 0.5 | 1 | 3105 | 6210 | 6229 | 12458 | 0.1 | 150864 | 8.26 | 12326 | 0.0166 | 1 | 0.004 | 15 | 0.1250 |
| | | | 0.5 | 2 | 3124 | 6248 | | | | | | | | | | | |
| | 4-3 | 2 | 0.5 | 1 | 3058 | 6116 | 6079 | 12158 | 0.1 | 150864 | 8.06 | 12026 | 0.0162 | 1 | 0.004 | 15 | 0.1220 |
| | | | 0.5 | 2 | 3020 | 6040 | | | | | | | | | | | |
| | 5-1 | 2 | 0.5 | 1 | 3551 | 7102 | 7164 | 14328 | 0.1 | 150864 | 9.50 | 14196 | 0.0191 | 1 | 0.004 | 15 | 0.1440 |
| | | | 0.5 | 2 | 3613 | 7226 | | | | | | | | | | | |
| | 5-2 | 2 | 0.5 | 1 | 3538 | 7076 | 7014 | 14028 | 0.1 | 150864 | 9.30 | 13896 | 0.0187 | 1 | 0.004 | 15 | 0.1410 |
| | | | 0.5 | 2 | 3476 | 6952 | | | | | | | | | | | |
| | 5-3 | 2 | 0.5 | 1 | 3419 | 6838 | 6812 | 13624 | 0.1 | 150864 | 9.03 | 13492 | 0.0182 | 1 | 0.004 | 15 | 0.1369 |
| | | | 0.5 | 2 | 3383 | 6766 | | | | | | | | | | | |
| | 6-1 | 2 | 0.5 | 1 | 3867 | 7734 | 7639 | 15278 | 0.1 | 150864 | 10.13 | 15146 | 0.0204 | 1 | 0.004 | 15 | 0.1536 |
| | | | 0.5 | 2 | 3772 | 7544 | | | | | | | | | | | |
| | 6-2 | 2 | 0.5 | 1 | 3796 | 7592 | 7620 | 15240 | 0.1 | 150864 | 10.10 | 15108 | 0.0203 | 1 | 0.004 | 15 | 0.1533 |
| | | | 0.5 | 2 | 3824 | 7648 | | | | | | | | | | | |
| | 6-3 | 2 | 0.5 | 1 | 4123 | 8246 | 8078 | 16156 | 0.1 | 150864 | 10.71 | 16024 | 0.0216 | 1 | 0.004 | 15 | 0.1625 |
| | | | 0.5 | 2 | 3955 | 7910 | | | | | | | | | | | |

| Assay Date | 12/21/2004 | Test Chemical ID | 4OH ASDN | # Concentrations tested | | Microsome 6 type | Recombinant | Microsome ID | BD Gentest, lot | Technician ID | BDL/TD | Replicate # | 2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Control Type | Portion | Average | SD |
|---|---|---|---|
| Full activity | Beginning | 0.1790 | 0.0013 |
| Full activity | End | 0.1611 | 0.0142 |
| Full activity | Overall | 0.1701 | 0.0132 |
| Background | Beginning | 0.0003 | 0.000731662 |
| Background | End | -0.0003 | 8.60779E-05 |
| Background | Overall | 0.0000 | 0.000540706 |
| 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 |
|---|---|---|---|---|---|
| 4OH ASDN | 1 | 1 | 1.00E-06 | -6.00 | 0.0134 |
| 4OH ASDN | 1 | 2 | 1.00E-06 | -6.00 | 0.0127 |
| 4OH ASDN | 1 | 3 | 1.00E-06 | -6.00 | 0.0138 |
| 4OH ASDN | 2 | 1 | 1.00E-07 | -7.00 | 0.0694 |
| 4OH ASDN | 2 | 2 | 1.00E-07 | -7.00 | 0.0665 |
| 4OH ASDN | 2 | 3 | 1.00E-07 | -7.00 | 0.0675 |
| 4OH ASDN | 3 | 1 | 5.00E-08 | -7.30 | 0.0948 |
| 4OH ASDN | 3 | 2 | 5.00E-08 | -7.30 | 0.0937 |
| 4OH ASDN | 3 | 3 | 5.00E-08 | -7.30 | 0.0985 |
| 4OH ASDN | 4 | 1 | 2.50E-08 | -7.60 | 0.1274 |
| 4OH ASDN | 4 | 2 | 2.50E-08 | -7.60 | 0.1250 |
| 4OH ASDN | 4 | 3 | 2.50E-08 | -7.60 | 0.1220 |
| 4OH ASDN | 5 | 1 | 1.00E-08 | -8.00 | 0.1440 |
| 4OH ASDN | 5 | 2 | 1.00E-08 | -8.00 | 0.1410 |
| 4OH ASDN | 5 | 3 | 1.00E-08 | -8.00 | 0.1369 |
| 4OH ASDN | 6 | 1 | 1.00E-09 | -9.00 | 0.1536 |
| 4OH ASDN | 6 | 2 | 1.00E-09 | -9.00 | 0.1533 |
| 4OH ASDN | 6 | 3 | 1.00E-09 | -9.00 | 0.1625 |

| Percent of control values | | | | |
|---|---|---|---|---|
| | Log[test | Replicate | | |
| Level | substance] | 1 | 2 | 3 |
| 1 | -6.00 | 7.86 | 7.49 | 8.13 |
| 2 | -7.00 | 40.78 | 39.10 | 39.71 |
| 3 | -7.30 | 55.77 | 55.12 | 57.93 |
| 4 | -7.60 | 74.92 | 73.52 | 71.73 |
| 5 | -8.00 | 84.67 | 82.88 | 80.47 |
| 6 | -9.00 | 90.34 | 90.11 | 95.58 |

| Aliquot # | Weight of aliquot (g) | DPM/Aliq. | DPM/g soln. | |
|---|---|---|---|---|
| 1 | 0.0194 | 25693 | 1324381 | |
| 2 | 0.0194 | 26379 | 1359742 | |
| 3 | 0.0196 | 27013 | 1378214 | |
| 4 | 0.0194 | 26254 | 1353299 | |
| 5 | 0.0195 | 27051 | 1387231 | |
| | | | Average DPM/g soln | 1360574 |
| | | | SD | 24420 |
| | | | CV | 1.79 |
| | | | µCi/g soln | 0.613 |

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 | 10.6 | 10 | | 1060.00 |
| Dilution A | | | 100 | 10.60 |
| Dilution B | | | 10 | 1.06 |

Calculation of concentration nonradiolabeled ASDN in substrate solution

| | |
|---|---|
| Total g substrate solution | 8.0368 g |
| Mass of dilution B used in substrate prep | 4.5239 g |
| Concentration of nonradiolabeled ASDN in substrate soln. | 0.596672 µg/g |

Calculation of Substrate Solution Specific Activity

1) Calculate µg [$^3$H]ASDN/g soln. = 0.00694 µg/g soln.
µg/g soln.

| | |
|---|---|
| a. µCi/g soln | 0.613 |
| 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.

= 0.596672 + 0.00694
= 0.603610 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.015 µCi/µg ASDN

645563 dpm/nmol

| Assay Date | 12/22/2004 | Test Chemical ID | 4OH ASDN | # Concentrations tested | 6 |
|---|---|---|---|---|---|
| Technician ID | BDL/TD | Replicate # | 3 | Microsome type | Recombinant |

| Microsome ID | BD Gentest, lot#5 | Protein stock (mg BSA) | Total volume of stock (mL) | Protein stock ID |
|---|---|---|---|---|
| | | 28 | 20 | 210000238 |

| Standards: | 1 | 0.8 | 0.6 | 0.4 | 0.2 | 0.14 | Blank |
|---|---|---|---|---|---|---|---|
| | 0.268 | 0.212 | 0.175 | 0.132 | 0.062 | 0.052 | 0.000 |
| | 0.308 | 0.213 | 0.181 | 0.135 | 0.074 | 0.053 | 0.000 |
| | 0.286 | 0.232 | 0.189 | 0.120 | 0.051 | 0.061 | 0.000 |

Samples:

0.160
0.164
0.162

| Standard concentration (mg/mL) | Volume of stock used | Final volume of Std | mg Protein per µL | µL Standard Used | mg Protein Measured | $A_{raw}$ | $A_{adj}$ | Curve Output |
|---|---|---|---|---|---|---|---|---|
| 1 | 17.9 | 25 | 0.00100 | 25 | 0.0251 | 0.287 | 0.287 | 0.0252 |
| 0.8 | 14.3 | 25 | 0.00080 | 25 | 0.0200 | 0.219 | 0.219 | 0.0189 |
| 0.6 | 10.7 | 25 | 0.00060 | 25 | 0.0150 | 0.185 | 0.185 | 0.0158 |
| 0.4 | 7.1 | 25 | 0.00040 | 25 | 0.0099 | 0.129 | 0.129 | 0.0106 |
| 0.2 | 3.6 | 25 | 0.00020 | 25 | 0.0050 | 0.062 | 0.062 | 0.0044 |
| 0.14 | 2.5 | 25 | 0.00014 | 25 | 0.0035 | 0.055 | 0.055 | 0.0037 |

| Blank | 0.000 |
|---|---|

$r^2$= 0.992
m= 0.093
b= -0.001

| Variables | Regression results | |
|---|---|---|
| m, b | 0.093 | -0.001 |
| $se_m$, $se_b$ | 0.004 | 0.001 |
| $r^2$, $se_y$ | 0.992 | 0.001 |
| F, df | 506 | 4 |
| $ss_{reg}$, $ss_{resid}$ | 0.000 | 0.000 |

Regression results are calculated using the function LINEST

| $A_{raw}$ | $A_{adj}$ | mg protein measured | µL diluted µSOMES | Vol usome prep. (µL) | Final vol. Diluted usomes (µL) | mg protein/µL Prep. | average mg/µL | mg/mL |
|---|---|---|---|---|---|---|---|---|
| 0.160 | 0.160 | 0.013 | 25 | 100 | 1000 | 0.005 | 0.005 | 5.437 |
| 0.164 | 0.164 | 0.014 | 25 | 100 | 1000 | 0.006 | | |
| 0.162 | 0.162 | 0.014 | 25 | 100 | 1000 | 0.005 | | |

| Assay Date | 12/22/04 | Test Chemical ID | 4OH ASDN | # Concentrations tested | 6 | Microsome type | Recombina | Microsome ID | BD Gentes | Technician ID | BDL/TD | Replicate # | 3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Microsome Dilution Details | | |
|---|---|---|
| Dilution A | 0.1 | mL microsome Stock used |
| | 1 | mL total volume |
| | 10 | dilution factor |
| Dilution B | 0.49 | mL microsome Dilution A used |
| | 30 | mL total volume |
| | 61.22449 | dilution factor |
| Dilution C (if applicable) | | mL microsome Dilution B used |
| | | mL total volume |
| | NA | dilution factor |
| | 612.2449 | total dilution factor |

| | |
|---|---|
| Protein Concentration (stock microsomes, mg/mL): | 5.437 |
| Protein Concentration (dilution added to assay, mg/mL): | 0.00888 |

| 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 | 12/22/2004 | Test Chemical ID | 4OH ASDN | # Concentrations tested | | S Microsome type | Recombinan | Microsome ID | | BD Gentest, lot# S | | Technician ID | | BDL/TD | | Replicate # | 3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Sample ID | | Calculate DPM in aqueous portion after extraction | | | | | | | Calculate % turnover | | | Calculate nmol $^3H_2O$ formed | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample type | Replicate/Level | Nominal total volume (mL) | Aliq Volume (mL) | Aliq. # | DPM/aliq | DPM/mL | Ave DPM/mL | Total DPM | Volume of substrate solution used/assay tube (mL) | total DPM in assay tube (initial) | % conversion to product | Total DPM corrected for background (Background Tubes) | nmol $^3H_2O$ formed | Volume diluted microsomes used in assay tube (mL) | Final [protein] in assay (mg/mL) | Incubation time (min) | Aromatase activity (nmol estrogen formed/mg protein/min |
| Full activity control | 1 | 2 | 0.5 | 1 | 4132 | 8264 | 8478 | 16956 | 0.1 | 136057 | 12.46 | 16862 | 0.0281 | 1 | 0.004 | 15 | 0.1961 |
| | | | 0.5 | 2 | 4346 | 8692 | | | | | | | | | | | |
| | 2 | 2 | 0.5 | 1 | 4070 | 8140 | 8174 | 16348 | 0.1 | 136057 | 12.02 | 16254 | 0.0252 | 1 | 0.004 | 15 | 0.1690 |
| | | | 0.5 | 2 | 4104 | 8208 | | | | | | | | | | | |
| | 3 | 2 | 0.5 | 1 | 4039 | 8078 | 8051 | 16102 | 0.1 | 136057 | 11.83 | 16006 | 0.0248 | 1 | 0.004 | 15 | 0.1661 |
| | | | 0.5 | 2 | 4012 | 8024 | | | | | | | | | | | |
| | 4 | 2 | 0.5 | 1 | 3916 | 7832 | 7810 | 15620 | 0.1 | 136057 | 11.49 | 15526 | 0.0240 | 1 | 0.004 | 15 | 0.1605 |
| | | | 0.5 | 2 | 3894 | 7788 | | | | | | | | | | | |
| Background control | 1 | 2 | 0.5 | 1 | 21 | 42 | 47 | 94 | 0.1 | 136057 | 0.07 | -1 | 0.0000 | 1 | 0.004 | 15 | 0.0000 |
| | | | 0.5 | 2 | 26 | 52 | | | | | | | | | | | |
| | 2 | 2 | 0.5 | 1 | 27 | 54 | 48 | 96 | 0.1 | 136057 | 0.07 | 2 | 0.0000 | 1 | 0.004 | 15 | 0.0000 |
| | | | 0.5 | 2 | 21 | 42 | | | | | | | | | | | |
| | 3 | 2 | 0.5 | 1 | 25 | 50 | 45 | 90 | 0.1 | 136057 | 0.07 | -5 | 0.0000 | 1 | 0.004 | 15 | -0.0001 |
| | | | 0.5 | 2 | 20 | 40 | | | | | | | | | | | |
| | 4 | 2 | 0.5 | 1 | 24 | 48 | 49 | 98 | 0.1 | 136057 | 0.07 | 4 | 0.0000 | 1 | 0.004 | 15 | 0.0000 |
| | | | 0.5 | 2 | 25 | 50 | | | | | | | | | | | |
| Positive control | 1 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 2 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 3 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 4 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| Negative Control | 1 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 2 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 3 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 4 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| 4OH ASDN | 1-1 | 2 | 0.5 | 1 | 381 | 762 | 760 | 1520 | 0.1 | 136057 | 1.12 | 1426 | 0.0022 | 1 | 0.004 | 15 | 0.0166 |
| | | | 0.5 | 2 | 379 | 758 | | | | | | | | | | | |
| | 1-2 | 2 | 0.5 | 1 | 372 | 744 | 734 | 1468 | 0.1 | 136057 | 1.08 | 1374 | 0.0021 | 1 | 0.004 | 15 | 0.0160 |
| | | | 0.5 | 2 | 362 | 724 | | | | | | | | | | | |
| | 1-3 | 2 | 0.5 | 1 | 387 | 774 | 761 | 1522 | 0.1 | 136057 | 1.12 | 1426 | 0.0022 | 1 | 0.004 | 15 | 0.0166 |
| | | | 0.5 | 2 | 374 | 748 | | | | | | | | | | | |
| | 2-1 | 2 | 0.5 | 1 | 1873 | 3746 | 3727 | 7454 | 0.1 | 136057 | 5.48 | 7360 | 0.0114 | 1 | 0.004 | 15 | 0.0856 |
| | | | 0.5 | 2 | 1854 | 3708 | | | | | | | | | | | |
| | 2-2 | 2 | 0.5 | 1 | 1781 | 3562 | 3542 | 7084 | 0.1 | 136057 | 5.21 | 6990 | 0.0108 | 1 | 0.004 | 15 | 0.0813 |
| | | | 0.5 | 2 | 1761 | 3522 | | | | | | | | | | | |
| | 2-3 | 2 | 0.5 | 1 | 1811 | 3622 | 3613 | 7226 | 0.1 | 136057 | 5.31 | 7132 | 0.0110 | 1 | 0.004 | 15 | 0.0829 |
| | | | 0.5 | 2 | 1802 | 3604 | | | | | | | | | | | |
| | 3-1 | 2 | 0.5 | 1 | 2734 | 5468 | 5445 | 10890 | 0.1 | 136057 | 8.00 | 10798 | 0.0157 | 1 | 0.004 | 15 | 0.1255 |
| | | | 0.5 | 2 | 2711 | 5422 | | | | | | | | | | | |
| | 3-2 | 2 | 0.5 | 1 | 2600 | 5200 | 5228 | 10456 | 0.1 | 136057 | 7.68 | 10082 | 0.0151 | 1 | 0.004 | 15 | 0.1205 |
| | | | 0.5 | 2 | 2628 | 5256 | | | | | | | | | | | |
| | 3-3 | 2 | 0.5 | 1 | 2628 | 5256 | 5244 | 10488 | 0.1 | 136057 | 7.71 | 10394 | 0.0161 | 1 | 0.004 | 15 | 0.1209 |
| | | | 0.5 | 2 | 2616 | 5232 | | | | | | | | | | | |
| | 4-1 | 2 | 0.5 | 1 | 3170 | 6340 | 6307 | 12614 | 0.1 | 136057 | 9.27 | 12520 | 0.0194 | 1 | 0.004 | 15 | 0.1456 |
| | | | 0.5 | 2 | 3137 | 6274 | | | | | | | | | | | |
| | 4-2 | 2 | 0.5 | 1 | 3072 | 6144 | 6187 | 12374 | 0.1 | 136057 | 9.09 | 12280 | 0.0190 | 1 | 0.004 | 15 | 0.1428 |
| | | | 0.5 | 2 | 3115 | 6230 | | | | | | | | | | | |
| | 4-3 | 2 | 0.5 | 1 | 2903 | 5806 | 5781 | 11522 | 0.1 | 136057 | 8.47 | 11428 | 0.0177 | 1 | 0.004 | 15 | 0.1329 |
| | | | 0.5 | 2 | 2858 | 5716 | | | | | | | | | | | |
| | 5-1 | 2 | 0.5 | 1 | 3946 | 7892 | 7844 | 15688 | 0.1 | 136057 | 11.53 | 15594 | 0.0242 | 1 | 0.004 | 15 | 0.1813 |
| | | | 0.5 | 2 | 3896 | 7792 | | | | | | | | | | | |
| | 5-2 | 2 | 0.5 | 1 | 3726 | 7452 | 7420 | 14840 | 0.1 | 136057 | 10.91 | 14748 | 0.0228 | 1 | 0.004 | 15 | 0.1715 |
| | | | 0.5 | 2 | 3694 | 7388 | | | | | | | | | | | |
| | 5-3 | 2 | 0.5 | 1 | 3569 | 7138 | 7047 | 14094 | 0.1 | 136057 | 10.36 | 14000 | 0.0217 | 1 | 0.004 | 15 | 0.1628 |
| | | | 0.5 | 2 | 3478 | 6956 | | | | | | | | | | | |
| | 6-1 | 2 | 0.5 | 1 | 4073 | 8146 | 8048 | 16092 | 0.1 | 136057 | 11.83 | 15998 | 0.0248 | 1 | 0.004 | 15 | 0.1860 |
| | | | 0.5 | 2 | 3973 | 7946 | | | | | | | | | | | |
| | 6-2 | 2 | 0.5 | 1 | 3874 | 7748 | 7794 | 15588 | 0.1 | 136057 | 11.46 | 15494 | 0.0240 | 1 | 0.004 | 15 | 0.1802 |
| | | | 0.5 | 2 | 3920 | 7840 | | | | | | | | | | | |
| | 6-3 | 2 | 0.5 | 1 | 4238 | 8476 | 8451 | 16902 | 0.1 | 136057 | 12.42 | 16808 | 0.0260 | 1 | 0.004 | 15 | 0.1956 |
| | | | 0.5 | 2 | 4213 | 8426 | | | | | | | | | | | |

| Assay Date | 12/22/2004 | Test Chemical ID | 4OH ASDN | # Concentrations tested | | Microsome 6 type | Recombinant | Microsome ID | BD Gentest, lot | Technician ID | BDL/TD | Replicate # | 3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Control Type | Portion | Average | SD |
|---|---|---|---|
| Full activity | Beginning | 0.1925 | 0.0050 |
| Full activity | End | 0.1833 | 0.0040 |
| Full activity | Overall | 0.1879 | 0.0065 |
| Background | Beginning | 0.0000 | 1.64456E-05 |
| Background | End | 0.0000 | 6.57826E-05 |
| Background | Overall | 0.0000 | 3.972E-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 |
|---|---|---|---|---|---|
| 4OH ASDN | 1 | 1 | 1.00E-06 | -6.00 | 0.0166 |
| 4OH ASDN | 1 | 2 | 1.00E-06 | -6.00 | 0.0160 |
| 4OH ASDN | 1 | 3 | 1.00E-06 | -6.00 | 0.0166 |
| 4OH ASDN | 2 | 1 | 1.00E-07 | -7.00 | 0.0856 |
| 4OH ASDN | 2 | 2 | 1.00E-07 | -7.00 | 0.0813 |
| 4OH ASDN | 2 | 3 | 1.00E-07 | -7.00 | 0.0829 |
| 4OH ASDN | 3 | 1 | 5.00E-08 | -7.30 | 0.1255 |
| 4OH ASDN | 3 | 2 | 5.00E-08 | -7.30 | 0.1205 |
| 4OH ASDN | 3 | 3 | 5.00E-08 | -7.30 | 0.1209 |
| 4OH ASDN | 4 | 1 | 2.50E-08 | -7.60 | 0.1456 |
| 4OH ASDN | 4 | 2 | 2.50E-08 | -7.60 | 0.1428 |
| 4OH ASDN | 4 | 3 | 2.50E-08 | -7.60 | 0.1329 |
| 4OH ASDN | 5 | 1 | 1.00E-08 | -8.00 | 0.1813 |
| 4OH ASDN | 5 | 2 | 1.00E-08 | -8.00 | 0.1715 |
| 4OH ASDN | 5 | 3 | 1.00E-08 | -8.00 | 0.1628 |
| 4OH ASDN | 6 | 1 | 1.00E-09 | -9.00 | 0.1860 |
| 4OH ASDN | 6 | 2 | 1.00E-09 | -9.00 | 0.1802 |
| 4OH ASDN | 6 | 3 | 1.00E-09 | -9.00 | 0.1955 |

| Percent of control values | | | | |
|---|---|---|---|---|
| | Log[test | Replicate | | |
| Level | substance] | 1 | 2 | 3 |
| 1 | -6.00 | 8.82 | 8.50 | 8.83 |
| 2 | -7.00 | 45.54 | 43.25 | 44.13 |
| 3 | -7.30 | 66.80 | 64.11 | 64.31 |
| 4 | -7.60 | 77.46 | 75.98 | 70.71 |
| 5 | -8.00 | 96.48 | 91.24 | 86.62 |
| 6 | -9.00 | 98.98 | 95.86 | 103.99 |

| Aliquot # | Weight of aliquot (g) | DPM/Aliq. | DPM/g soln. | |
|---|---|---|---|---|
| 1 | 0.0194 | 22763 | 1173351 | |
| 2 | 0.0198 | 24452 | 1234949 | |
| 3 | 0.0197 | 24719 | 1254772 | |
| 4 | 0.0197 | 24232 | 1230051 | |
| 5 | 0.0196 | 25685 | 1310459 | |
| | | | Average DPM/g soln | 1240716 |
| | | | SD | 49363 |
| | | | CV | 3.98 |
| | | | µCi/g soln | 0.559 |

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

| ASDN solution | mg ASDN added | total volume (mL) | dilution factor | [ASDN] in solution (µg/mL) |
|---|---|---|---|---|
| Stock | 11.3 | 10 | | 1130.00 |
| Dilution A | | | 100 | 11.30 |
| Dilution B | | | 10 | 1.13 |

Calculation of concentration nonradiolabeled ASDN in substrate solution

| | |
|---|---|
| Total g substrate solution | 8.0484 g |
| Mass of dilution B used in substrate prep | 4.5245 g |
| Concentration of nonradiolabeled ASDN in substrate soln. | 0.635242 µg/g |

Calculation of Substrate Solution Specific Activity

1) Calculate µg [$^3$H]ASDN/g soln. = 0.00633 µg/g soln.
µg/g soln.

| | |
|---|---|
| a. µCi/g soln | 0.559 |
| 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.

= 0.635242 + 0.00633
= 0.641569 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 0.871 µCi/µg ASDN

553863 dpm/nmol

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Assay Date | 3/11/2005 | Test Chemical ID | 4OH ASDN | | # Concentrations tested | 6 | | | | |
| Technician ID | BDL/TD | Replicate # | 4 | Microsome type | Recombinant | Microsome ID | BD Gentest, lot#5 | | | |

| | | | | | | | | Protein stock (mg BSA) | Total volume of stock (mL) | Protein stock ID |
|---|---|---|---|---|---|---|---|---|---|---|
| Standards: | 1 | 0.8 | 0.6 | 0.4 | 0.2 | 0.11 | Blank | 28 | 20 | 210000238 |
| | 0.301 | 0.235 | 0.196 | 0.133 | 0.079 | 0.045 | 0.000 | | | |
| | 0.297 | 0.250 | 0.197 | 0.120 | 0.079 | 0.053 | 0.000 | | | |
| | 0.303 | 0.255 | 0.195 | 0.149 | 0.067 | 0.053 | 0.000 | | | |
| Samples: | | | | | | | | | | |
| | 0.173 | 0.038 | 0.152 | 0.276 | | | | | | |
| | 0.171 | 0.039 | 0.157 | 0.298 | | | | | | |
| | 0.175 | | | | | | | | | |

| Standard concentration (mg/mL) | Volume of stock used | Final volume of Std | mg Protein per µL | µL Standard Used | mg Protein Measured | $A_{raw}$ | $A_{adj}$ | Curve Output | Variables | Regression results | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 17.9 | 25 | 0.00100 | 25 | 0.0251 | 0.300 | 0.300 | 0.0247 | m, b | 0.088 | -0.002 |
| 0.8 | 14.3 | 25 | 0.00080 | 25 | 0.0200 | 0.247 | 0.247 | 0.0200 | $se_m$, $se_b$ | 0.002 | 0.000 |
| 0.6 | 10.7 | 25 | 0.00060 | 25 | 0.0150 | 0.196 | 0.196 | 0.0155 | $r^2$, $se_y$ | 0.999 | 0.000 |
| 0.4 | 7.1 | 25 | 0.00040 | 25 | 0.0099 | 0.134 | 0.134 | 0.0101 | F, df | 3038 | 4 |
| 0.2 | 3.6 | 25 | 0.00020 | 25 | 0.0050 | 0.075 | 0.075 | 0.0048 | $ss_{reg}$, $ss_{resid}$ | 0.000 | 0.000 |
| 0.11 | 2 | 25 | 0.00011 | 25 | 0.0028 | 0.050 | 0.050 | 0.0027 | | | |

Regression results are calculated using the function LINEST

Blank 0.000

$r^2$= 0.999
m= 0.088
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 |
|---|---|---|---|---|---|---|---|---|
| 0.173 | 0.173 | 0.014 | 25 | 100 | 1000 | 0.005 | 0.005 | 5.396 |
| 0.171 | 0.171 | 0.013 | 25 | 100 | 1000 | 0.005 | | |
| 0.175 | 0.175 | 0.014 | 25 | 100 | 1000 | 0.005 | | |
| 0.038 | 0.038 | 0.002 | 25 | 1 | 1 | 0.000 | 0.000 | 0.064 |
| 0.039 | 0.039 | 0.002 | 25 | 1 | 1 | 0.000 | | |
| 0.152 | 0.152 | 0.012 | 25 | 1 | 1 | 0.000 | 0.000 | 0.475 |
| 0.157 | 0.157 | 0.012 | 25 | 1 | 1 | 0.000 | | |
| 0.276 | 0.276 | 0.023 | 25 | 1 | 1 | 0.001 | 0.001 | 0.943 |
| 0.298 | 0.298 | 0.025 | 25 | 1 | 1 | 0.001 | | |

| Assay Date | 3/11/2005 | Test Chemical ID | 4OH ASDN | # Concentrations tested | 6 | Microsome type | Recombina Microsome ID | BD Gentes | Technician ID | BDL/TD | Replicate # |
|---|---|---|---|---|---|---|---|---|---|---|---|

Microsome Dilution Details

| | | |
|---|---|---|
| Dilution A | 0.1 | mL microsome Stock used |
| | 1 | mL total volume |
| | 10 | dilution factor |
| Dilution B | 0.49 | mL microsome Dilution A used |
| | 30 | mL total volume |
| | 61.22449 | dilution factor |
| Dilution C (if applicable) | | mL microsome Dilution B used |
| | | mL total volume |
| | NA | dilution factor |
| | 612.2449 | total dilution factor |

| | |
|---|---|
| Protein Concentration (stock microsomes, mg/mL): | 5.396 |
| Protein Concentration (dilution added to assay, mg/mL): | 0.008813 |

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 | 3/11/2005 | Test Chemical ID | 4OH ASDN | # Concentrations tested | 6 | Microsome type | Recombinan Microsome ID | BD Gentest, lot#5 | Technician ID | BDL/TD | Replicate # | 4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|

| 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 microsom as used in assay tube (mL) | Final (protein) in assay (mg/mL) | Incubation time (min) | Aromatase activity (nmole estrogen formed/mg protein/min |
| Full activity control | 1 | 2 | 0.5 | 1 | 3912 | 7824 | 7779 | 15558 | 0.1 | 124072 | 12.54 | 15465 | 0.0279 | 1 | 0.004 | 15 | 0.2112 |
| | | | 0.5 | 2 | 3867 | 7734 | | | | | | | | | | | |
| | 2 | 2 | 0.5 | 1 | 3986 | 7972 | 7987 | 15974 | 0.1 | 124072 | 12.87 | 15881 | 0.0287 | 1 | 0.004 | 15 | 0.2169 |
| | | | 0.5 | 2 | 4001 | 8002 | | | | | | | | | | | |
| | 3 | 2 | 0.5 | 1 | 3583 | 7166 | 7167 | 14334 | 0.1 | 124072 | 11.55 | 14241 | 0.0257 | 1 | 0.004 | 15 | 0.1945 |
| | | | 0.5 | 2 | 3584 | 7168 | | | | | | | | | | | |
| | 4 | 2 | 0.5 | 1 | 3597 | 7194 | 7207 | 14414 | 0.1 | 124072 | 11.62 | 14321 | 0.0259 | 1 | 0.004 | 15 | 0.1958 |
| | | | 0.5 | 2 | 3610 | 7220 | | | | | | | | | | | |
| Background control | 1 | 2 | 0.5 | 1 | 26 | 52 | 45 | 90 | 0.1 | 124072 | 0.07 | -3 | 0.0000 | 1 | 0.004 | 15 | 0.0000 |
| | | | 0.5 | 2 | 19 | 38 | | | | | | | | | | | |
| | 2 | 2 | 0.5 | 1 | 20 | 40 | 47 | 94 | 0.1 | 124072 | 0.08 | 1 | 0.0000 | 1 | 0.004 | 15 | 0.0000 |
| | | | 0.5 | 2 | 27 | 54 | | | | | | | | | | | |
| | 3 | 2 | 0.5 | 1 | 27 | 54 | 54 | 108 | 0.1 | 124072 | 0.09 | 15 | 0.0000 | 1 | 0.004 | 15 | 0.0002 |
| | | | 0.5 | 2 | 27 | 54 | | | | | | | | | | | |
| | 4 | 2 | 0.5 | 1 | 18 | 36 | 40 | 80 | 0.1 | 124072 | 0.06 | -13 | 0.0000 | 1 | 0.004 | 15 | -0.0002 |
| | | | 0.5 | 2 | 22 | 44 | | | | | | | | | | | |
| Positive control | 1 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 2 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 3 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 4 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| Negative Control | 1 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 2 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 3 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 4 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| 4OH ASDN | 1-1 | 2 | 0.5 | 1 | 451 | 902 | 896 | 1792 | 0.1 | 124072 | 1.44 | 1689 | 0.0031 | 1 | 0.004 | 15 | 0.0232 |
| | | | 0.5 | 2 | 445 | 890 | | | | | | | | | | | |
| | 1-2 | 2 | 0.5 | 1 | 434 | 868 | 887 | 1774 | 0.1 | 124072 | 1.43 | 1661 | 0.0030 | 1 | 0.004 | 15 | 0.0230 |
| | | | 0.5 | 2 | 453 | 906 | | | | | | | | | | | |
| | 1-3 | 2 | 0.5 | 1 | 444 | 888 | 899 | 1798 | 0.1 | 124072 | 1.45 | 1705 | 0.0031 | 1 | 0.004 | 15 | 0.0233 |
| | | | 0.5 | 2 | 455 | 910 | | | | | | | | | | | |
| | 2-1 | 2 | 0.5 | 1 | 1863 | 3726 | 3699 | 7398 | 0.1 | 124072 | 5.95 | 7295 | 0.0132 | 1 | 0.004 | 15 | 0.0965 |
| | | | 0.5 | 2 | 1826 | 3652 | | | | | | | | | | | |
| | 2-2 | 2 | 0.5 | 1 | 2044 | 4088 | 4118 | 8236 | 0.1 | 124072 | 6.64 | 8143 | 0.0147 | 1 | 0.004 | 15 | 0.1112 |
| | | | 0.5 | 2 | 2074 | 4148 | | | | | | | | | | | |
| | 2-3 | 2 | 0.5 | 1 | 1970 | 3940 | 3958 | 7916 | 0.1 | 124072 | 6.30 | 7723 | 0.0139 | 1 | 0.004 | 15 | 0.1055 |
| | | | 0.5 | 2 | 1938 | 3876 | | | | | | | | | | | |
| | 3-1 | 2 | 0.5 | 1 | 2381 | 4762 | 4822 | 9644 | 0.1 | 124072 | 7.77 | 9551 | 0.0172 | 1 | 0.004 | 15 | 0.1304 |
| | | | 0.5 | 2 | 2441 | 4882 | | | | | | | | | | | |
| | 3-2 | 2 | 0.5 | 1 | 2542 | 5084 | 5112 | 10224 | 0.1 | 124072 | 9.24 | 10131 | 0.0183 | 1 | 0.004 | 15 | 0.1384 |
| | | | 0.5 | 2 | 2570 | 5140 | | | | | | | | | | | |
| | 3-3 | 2 | 0.5 | 1 | 2471 | 4942 | 5011 | 10022 | 0.1 | 124072 | 9.09 | 9929 | 0.0179 | 1 | 0.004 | 15 | 0.1366 |
| | | | 0.5 | 2 | 2540 | 5080 | | | | | | | | | | | |
| | 4-1 | 2 | 0.5 | 1 | 2727 | 5454 | 5436 | 10872 | 0.1 | 124072 | 8.78 | 10779 | 0.0195 | 1 | 0.004 | 15 | 0.1472 |
| | | | 0.5 | 2 | 2709 | 5418 | | | | | | | | | | | |
| | 4-2 | 2 | 0.5 | 1 | 2952 | 5904 | 5984 | 11968 | 0.1 | 124072 | 9.65 | 11875 | 0.0214 | 1 | 0.004 | 15 | 0.1622 |
| | | | 0.5 | 2 | 3032 | 6064 | | | | | | | | | | | |
| | 4-3 | 2 | 0.5 | 1 | 2642 | 5284 | 5720 | 11440 | 0.1 | 124072 | 9.22 | 11347 | 0.0205 | 1 | 0.004 | 15 | 0.1550 |
| | | | 0.5 | 2 | 2878 | 5756 | | | | | | | | | | | |
| | 5-1 | 2 | 0.5 | 1 | 3183 | 6366 | 6420 | 12840 | 0.1 | 124072 | 10.35 | 12747 | 0.0230 | 1 | 0.004 | 15 | 0.1741 |
| | | | 0.5 | 2 | 3227 | 6454 | | | | | | | | | | | |
| | 5-2 | 2 | 0.5 | 1 | 3099 | 6198 | 6283 | 12568 | 0.1 | 124072 | 10.13 | 12473 | 0.0225 | 1 | 0.004 | 15 | 0.1703 |
| | | | 0.5 | 2 | 3184 | 6368 | | | | | | | | | | | |
| | 5-3 | 2 | 0.5 | 1 | 3123 | 6246 | 6364 | 12728 | 0.1 | 124072 | 10.26 | 12635 | 0.0228 | 1 | 0.004 | 15 | 0.1726 |
| | | | 0.5 | 2 | 3241 | 6482 | | | | | | | | | | | |
| | 6-1 | 2 | 0.5 | 1 | 3373 | 6746 | 6737 | 13474 | 0.1 | 124072 | 10.88 | 13381 | 0.0242 | 1 | 0.004 | 15 | 0.1827 |
| | | | 0.5 | 2 | 3364 | 6728 | | | | | | | | | | | |
| | 6-2 | 2 | 0.5 | 1 | 3261 | 6522 | 6562 | 13124 | 0.1 | 124072 | 10.58 | 13031 | 0.0235 | 1 | 0.004 | 15 | 0.1780 |
| | | | 0.5 | 2 | 3301 | 6602 | | | | | | | | | | | |
| | 6-3 | 2 | 0.5 | 1 | 3269 | 6538 | 6525 | 13050 | 0.1 | 124072 | 10.52 | 12957 | 0.0234 | 1 | 0.004 | 15 | 0.1770 |
| | | | 0.5 | 2 | 3242 | 6484 | | | | | | | | | | | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Assay Date | 3/11/2005 | Test Chemical ID | 4OH ASDN | # Concentrations tested | 6 | Microsome type | Recombinant | Microsome ID | BD Gentest, lot | Technician ID | BDL/TD | Replicate # | 4 |

| Control Type | Portion | Average | SD |
|---|---|---|---|
| Full activity | Beginning | 0.2140 | 0.0040 |
| Full activity | End | 0.1950 | 0.0008 |
| Full activity | Overall | 0.2045 | 0.0112 |
| Background | Beginning | 0.0000 | 3.86282E-05 |
| Background | End | 0.0000 | 0.000270398 |
| Background | Overall | 0.0000 | 0.000156486 |
| 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 |
|---|---|---|---|---|---|
| 4OH ASDN | 1 | 1 | 1.00E-06 | -6.00 | 0.0232 |
| 4OH ASDN | 1 | 2 | 1.00E-06 | -6.00 | 0.0230 |
| 4OH ASDN | 1 | 3 | 1.00E-06 | -6.00 | 0.0233 |
| 4OH ASDN | 2 | 1 | 1.00E-07 | -7.00 | 0.0995 |
| 4OH ASDN | 2 | 2 | 1.00E-07 | -7.00 | 0.1112 |
| 4OH ASDN | 2 | 3 | 1.00E-07 | -7.00 | 0.1055 |
| 4OH ASDN | 3 | 1 | 5.00E-08 | -7.30 | 0.1304 |
| 4OH ASDN | 3 | 2 | 5.00E-08 | -7.30 | 0.1384 |
| 4OH ASDN | 3 | 3 | 5.00E-08 | -7.30 | 0.1356 |
| 4OH ASDN | 4 | 1 | 2.50E-08 | -7.60 | 0.1472 |
| 4OH ASDN | 4 | 2 | 2.50E-08 | -7.60 | 0.1622 |
| 4OH ASDN | 4 | 3 | 2.50E-08 | -7.60 | 0.1550 |
| 4OH ASDN | 5 | 1 | 1.00E-08 | -8.00 | 0.1741 |
| 4OH ASDN | 5 | 2 | 1.00E-08 | -8.00 | 0.1703 |
| 4OH ASDN | 5 | 3 | 1.00E-08 | -8.00 | 0.1726 |
| 4OH ASDN | 6 | 1 | 1.00E-09 | -9.00 | 0.1827 |
| 4OH ASDN | 6 | 2 | 1.00E-09 | -9.00 | 0.1780 |
| 4OH ASDN | 6 | 3 | 1.00E-09 | -9.00 | 0.1770 |

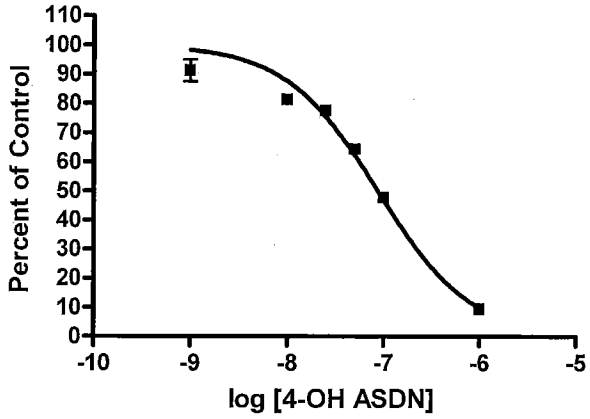
| Percent of control values | | | | |
|---|---|---|---|---|
| | | Replicate | | |
| Level | Log[test substance] | 1 | 2 | 3 |
| 1 | -6.00 | 11.34 | 11.22 | 11.38 |
| 2 | -7.00 | 48.64 | 54.37 | 51.57 |
| 3 | -7.30 | 63.77 | 67.64 | 66.29 |
| 4 | -7.60 | 71.97 | 79.29 | 75.76 |
| 5 | -8.00 | 85.11 | 83.28 | 84.36 |
| 6 | -9.00 | 89.34 | 87.01 | 86.51 |

## APPENDIX F

Prism Output ..... F-1

# Recombinant Assay 12-20-2004

## Recombinant Assay Replicate 1

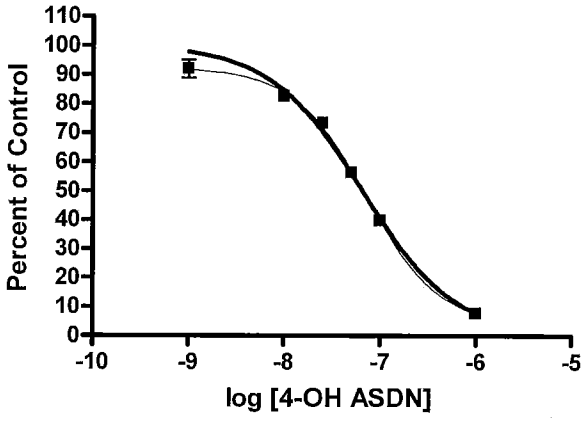



| | | | |
|---|---|---|---|
| -6.00 | 9.59 | 9.42 | 8.88 |
| -7.00 | 46.49 | 48.25 | 48.08 |
| -7.30 | 65.03 | 64.00 | 63.23 |
| -7.60 | 76.72 | 75.94 | 79.58 |
| -8.00 | 80.12 | 80.05 | 83.56 |
| -9.00 | 87.98 | 95.43 | 90.18 |

| | |
|---|---|
| Sigmoidal dose-response (variable slope) | |
| Best-fit values | |
| BOTTOM | 0.0 |
| TOP | 100.0 |
| LOGEC50 | -7.062 |
| HILLSLOPE | -0.9022 |
| EC50 | 8.679e-008 |
| Std. Error | |
| LOGEC50 | 0.02430 |
| HILLSLOPE | 0.03667 |
| 95% Confidence Intervals | |
| LOGEC50 | -7.113 to -7.010 |
| HILLSLOPE | -0.9799 to -0.8245 |
| EC50 | 7.709e-008 to 9.772e-008 |
| Goodness of Fit | |
| Degrees of Freedom | 16 |
| $R^2$ (unweighted) | 0.9744 |
| Weighted Sum of Squares (1/Y) | 4.275 |
| Absolute Sum of Squares | 343.6 |
| Sy.x | 4.634 |
| Constraints | |
| BOTTOM | BOTTOM = 0.0 |
| TOP | TOP = 100.0 |
| Data | |
| Number of X values | 6 |
| Number of Y replicates | 3 |
| Total number of values | 18 |
| Number of missing values | 0 |

# Recombinant Assay 12-21-2004

## Recombinant Assay Replicate 2

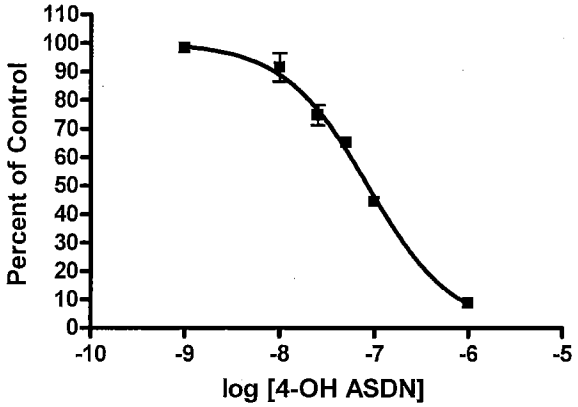



| | | | |
|---|---|---|---|
| -6.00 | 7.86 | 7.49 | 8.13 |
| -7.00 | 40.78 | 39.10 | 39.71 |
| -7.30 | 55.77 | 55.12 | 57.93 |
| -7.60 | 74.92 | 73.52 | 71.73 |
| -8.00 | 84.67 | 82.88 | 80.47 |
| -9.00 | 90.34 | 90.11 | 95.58 |

| | |
|---|---|
| Sigmoidal dose-response (variable slope) | |
| Best-fit values | |
| BOTTOM | 0.0 |
| TOP | 100.0 |
| LOGEC50 | -7.183 |
| HILLSLOPE | -0.9031 |
| EC50 | 6.558e-008 |
| Std. Error | |
| LOGEC50 | 0.01653 |
| HILLSLOPE | 0.02662 |
| 95% Confidence Intervals | |
| LOGEC50 | -7.218 to -7.148 |
| HILLSLOPE | -0.9596 to -0.8467 |
| EC50 | 6.050e-008 to 7.110e-008 |
| Goodness of Fit | |
| Degrees of Freedom | 16 |
| $R^2$ (unweighted) | 0.9878 |
| Weighted Sum of Squares (1/Y) | 2.160 |
| Absolute Sum of Squares | 177.3 |
| Sy.x | 3.329 |
| Constraints | |
| BOTTOM | BOTTOM = 0.0 |
| TOP | TOP = 100.0 |
| Data | |
| Number of X values | 6 |
| Number of Y replicates | 3 |
| Total number of values | 18 |
| Number of missing values | 0 |

# Recombinant Assay 12-22-2004

## Recombinant Assay Replicate 3

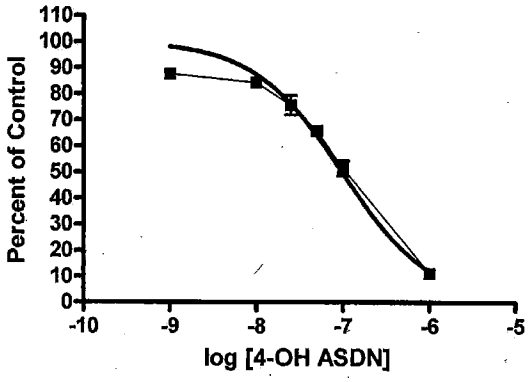



| | | | |
|---|---|---|---|
| -6.00 | 8.82 | 8.50 | 8.83 |
| -7.00 | 45.54 | 43.25 | 44.13 |
| -7.30 | 66.80 | 64.11 | 64.31 |
| -7.60 | 77.46 | 75.98 | 70.71 |
| -8.00 | 96.48 | 91.24 | 86.62 |
| -9.00 | 98.98 | 95.86 | 99.50 |

| | Percent of Control Values |
|---|---|
| Sigmoidal dose-response (variable slope) | |
| Best-fit values | |
| BOTTOM | 0.0 |
| TOP | 100.0 |
| LOGEC50 | -7.071 |
| HILLSLOPE | -0.9678 |
| EC50 | 8.493e-008 |
| Std. Error | |
| LOGEC50 | 0.01571 |
| HILLSLOPE | 0.02755 |
| 95% Confidence Intervals | |
| LOGEC50 | -7.104 to -7.038 |
| HILLSLOPE | -1.026 to -0.9094 |
| EC50 | 7.866e-008 to 9.170e-008 |
| Goodness of Fit | |
| Degrees of Freedom | 16 |
| $R^2$ (unweighted) | 0.9909 |
| Weighted Sum of Squares (1/Y) | 1.988 |
| Absolute Sum of Squares | 149.5 |
| Sy.x | 3.057 |
| Constraints | |
| BOTTOM | BOTTOM = 0.0 |
| TOP | TOP = 100.0 |
| Data | |
| Number of X values | 6 |
| Number of Y replicates | 3 |
| Total number of values | 18 |
| Number of missing values | 0 |

## Recombinant Assay 03-11-05

### Recombinant Assay Replicate 4




| | | | |
|---|---|---|---|
| -6.00 | 11.34 | 11.22 | 11.38 |
| -7.00 | 48.64 | 54.37 | 51.57 |
| -7.30 | 63.77 | 67.64 | 66.29 |
| -7.60 | 71.97 | 79.29 | 75.76 |
| -8.00 | 85.11 | 83.28 | 84.36 |
| -9.00 | 89.34 | 87.01 | 86.51 |

| | |
|---|---|
| Sigmoidal dose-response (variable slope) | |
| Best-fit values | |
| BOTTOM | 0.0 |
| TOP | 100.0 |
| LOGEC50 | -7.008 |
| HILLSLOPE | -0.8528 |
| EC50 | 9.807e-008 |
| Std. Error | |
| LOGEC50 | 0.02920 |
| HILLSLOPE | 0.04000 |
| 95% Confidence Intervals | |
| LOGEC50 | -7.070 to -6.947 |
| HILLSLOPE | -0.9377 to -0.7680 |
| EC50 | 8.504e-008 to 1.131e-007 |
| Goodness of Fit | |
| Degrees of Freedom | 16 |
| $R^2$ (unweighted) | 0.9637 |
| Weighted Sum of Squares (1/Y) | 5.531 |
| Absolute Sum of Squares | 440.3 |
| Sy.x | 5.246 |
| Constraints | |
| BOTTOM | BOTTOM = 0.0 |
| TOP | TOP = 100.0 |
| Data | |
| Number of X values | 6 |
| Number of Y replicates | 3 |
| Total number of values | 18 |
| Number of missing values | 0 |

## APPENDIX G

Statistician's Report ..... G-1

DRAFT REPORT

# RECOMBINANT AROMATASE VALIDATION STUDY
# 4-OH ASDN POSITIVE CONTROL INHIBITOR STUDY

## INTRALABORATORY STATISTICAL ANALYSIS OF BATTELLE DATA

EPA CONTRACT NUMBER 68-W-01-023
WORK ASSIGNMENT 4-17, TASK 3

October 25, 2005

Prepared for

U.S. ENVIRONMENTAL PROTECTION AGENCY
ENDOCRINE DISRUPTOR SCREENING PROGRAM
WASHINGTON, D.C.

Prepared by

BATTELLE
505 King Avenue
Columbus, Ohio 43201

**Recombinant Aromatase Validation Study**
**4-OH ASDN Positive Control Inhibitor Study**
**Intralaboratory Statistical Analysis of Battelle Data**

**EPA CONTRACT NUMBER 68-W-01-023**
**WORK ASSIGNMENT 4-17, TASK 3**

Ying-Liang Chou, Author _____ Date _____

Paul I. Feder, Reviewer _____ Date _____

# Offsite Quality Assurance Statement

**Study Number:**G608317

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

| Phase Inspected | Inspection Date | Date Reported to Study Director/ Management |
|---|---|---|
| Audit study file | 10/12/2005 | 10/12/2005 |
| Audit draft report | 10/12/2005 | 10/12/2005 |

**Quality Assurance Unit** **Date**

Hillary Flory 10-25-05

This report discusses the methods and results of the intralaboratory statistical analysis on the data collected at Battelle with the recombinant aromatase assay in the 4-OH ASDN positive control inhibitor study.

## Summary and Conclusions

Statistical analyses were carried out on the percent of control responses for aromatase activity in four independent replicates. Within each replicate three repeat tubes were run at each of six graded concentrations of the positive control inhibitor 4-OH ASDN. Additionally two full enzyme activity control tubes and two background activity control tubes were run at the beginning of each replicate and two full enzyme activity and two background activity controls were run at the end.

Concentration response curves were fitted within each replicate to describe the relation between 4-OH ASDN concentration and extent of inhibition. The concentration response curves were summarized by the $IC_{50}$ (concentration corresponding to 50 percent inhibition) and slope. Results were compared across replicates. In addition full enzyme activity and background activity control tube responses were compared between beginning and end of each replicate to identify differences within replicates and differences across replicates.

The following results were obtained:

1. Replicate 2 had a lower estimated $IC_{50}$ than the other three replicates. Replicate 3 had a more negative slope than the other three replicates. Replicate 4 had a higher estimated $IC_{50}$ and a less negative slope than the other replicates. The variance for $IC_{50}$ among the replicates was high compared to the variances within replicates; it was at least near 8 times higher when only replicates 2 to 4 considered or 5 times higher when all 4 replicates considered.
2. For the background activity controls, the measurements at the end were similar to those at the beginning for all replicates except replicate 2. Replicate 2 had a higher average at the beginning due to the higher measurement in a repetition at the beginning (close to 0.5% , Table A-2). Without this data, the average at the beginning would be similar to that at the end for replicate 2.
3. For the full enzyme activity controls the average percent of control responses at the end was lower than at the beginning for each replicate. The ends were about 5% to 19% lower than the beginnings. This provides a suggestion of reduction in aromatase enzyme activity between the beginning and end of a replicate.
4. For the background activity controls averaged across replicates there was not a significant difference between the beginning and the end portions whether or not replicate 1 was included in the analysis. For the full enzyme activity controls the average across replicates was highly significantly lower at the end portion than at the beginning, whether or not replicate 1 was included. The variation of portion (end vs. beginning) effects among replicates was zero or near zero for both the background activity and the full

enzyme activity controls.

## Introduction and Background

Task 3 of the Recombinant Aromatase Validation Study involves the individual laboratories independently carrying out the recombinant aromatase assay with positive control inhibitor 4-OH ASDN and centrally prepared microsomes, according to a common protocol. This report discuses the methods and results of the intralaboratory statistical analysis performed on the experimental data collected by Battelle. Aromatase activity levels were determined for the full enzyme activity control, the background activity control, and for six graded concentrations of positive control inhibitor 4-OH ASDN.

Four replicates of the positive control inhibitor study were carried out. Within each replicate three repetitions were run at each of the 4-OH ASDN log (base 10) concentrations -6, -7, -7.3, -7.6, -8, and -9. In addition two repeat tubes of the full enzyme activity and background activity controls were run prior to the 4-OH ASDN runs and two repeat tubes of the full enzyme activity and background activity controls were run following the 4-OH ASDN runs.

Statistical analyses were carried out on the "percent of control" responses. Percent of control is defined as the ratio of the (background adjusted) aromatase activity in the tube under consideration to the (background adjusted) average aromatase activity among the four full enzyme activity control tubes within the replicate, times 100. The average percent of control among the four full enzyme activity control tubes is necessarily 100 percent within each replicate. The average percent of control among the four background activity control tubes is necessarily 0 percent.

Nominally for an inhibitor the percent of control activity values vary between approximately 0% near the high inhibition concentrations and approximately 100% near the low inhibition concentrations, but this may vary with the inhibitor.

## Objectives

The primary objectives of the statistical analysis are:

1. Fit concentration curves within each replicate to describe the trend in the percent of control activity across varying inhibitor concentrations of test substance 4-OH ASDN.
2. Estimate the $IC_{50}$ concentration, slope, and associated standard errors within each replicate.
3. Combine results across replicates to determine the average $IC_{50}$ concentration, average slope, and associated standard errors.
4. Determine whether there are differences between the full enzyme activity and background activity controls obtained at the beginning and those obtained at the end of each replicate.
5. Assess the consistency of conditions within replicates and across replicates based on the

full enzyme activity and background activity control values.

Statistical analyses were carried out based on the results from all four replicates, as well as the results restricted to replicates 2 to 4.

## Statistical Analysis Methods

Concentration Response Trend Curves

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

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

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

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

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

where $\epsilon$ 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 also approximately proportional to the response Y.

The response curve was fitted by weighted least squares nonlinear regression analysis with weights equal to 1/Y. This weighting system gives greater weight to the lower end of the concentration response curve, where greater inhibition occurs.

Model fits were carried out using PRISM software (Version 4). Observed percent of control values above 100% were set to 99.5%. Observed percent of control values below 0% were set to 0.5%. This adjustment tacitly assumes an upper bound of 100% on the concentration response curve and a lower bound of 0%.

For each replicate the estimated $\log_{10}IC_{50}$ ($\mu$) and its associated standard error, the $IC_{50}$

and its associated geometric standard error, the slope ($\beta$) and its associated standard error, and the "Status" of each response curve are reported. The "Status" of each response curve is indicated as "C", complete, if the concentration response curve inhibition ranges from essentially 0 percent to 100 percent of control. Otherwise it is indicated as "II", incomplete but can interpolate to $\log_{10}IC_{50}$ or "IX", incomplete but must extrapolate to $\log_{10}IC_{50}$. These are the definitions of response curve "Status' that were used in this analysis. A modified set of descriptors of response curve status that conform to EPA conventions will be used in subsequent analyses.

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

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

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

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

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

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

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

where $\beta_{avg}$ and $\mu_{avg}$ were estimated across the four replicates, based on the random effects one-way analysis of variance model discussed above. An analogous plot, restricted to replicates 2 to 4, was also prepared.

All concentration response curves were fitted to the data using the non-linear regression analysis features in the PRISM statistical analysis package, Version 4. Supplemental statistical analyses and displays such as summary tables, graphical displays, analysis of variance, and multiple comparisons were carried out using PRISM and the SAS statistical analysis system-Version 9.

Analysis of Variance of Full Enzyme Activity Controls and Background Activity Controls Across Replicates

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

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

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

This analysis was carried out for replicates 1 to 4 and additionally for replicates 2 to 4, using the SAS statistical analysis system, Version 9.

## Statistical Analysis Results

The percent of control responses are displayed in Table A-1 for each replicate and for

each 4-OH ASDN ($log_{10}$) concentration. The full enzyme activity and background activity control percent of control responses are displayed in Table A-2, sorted by replicate and beginning and end within replicate.

Concentration response curves were fitted separately to the repeat tubes data within each replicate and to the averages of the repetitions within each replicate (Table A-1). The parameters of these fitted concentration response curves are displayed in Table 1. The individual repetition data within each replicate are plotted in Figure A-1 through Figure A-4 with the corresponding fitted concentration response curves superimposed in each figure. Figure 1 displays the four concentration response curves fitted to the averages of the three repetitions within each replicate. The concentration response curves for the four replicates are similar.

The parameters of the average concentration response curve, based on random effects analysis of variance model fits with replicate as a random effect are displayed in Table 1. The average parameters based on replicates 1 to 4 and those based on replicates 2 to 4 are displayed. The average concentration response curves, along with the averages of three repetitions within each replicate are plotted together in Figure 2 (replicates 1 to 4) and in Figure 3 (replicates 2 to 4).

The parameter estimates for each replicate and the average parameter estimates across replicates with their associated 95% confidence intervals are displayed in Table 2, and graphed in Figure 4 for $log_{10}IC_{50}$ ($\mu$) and Figure 5 for slope ($\beta$). Regardless whether replicate 1 was included, replicate 2 had a lower estimated $IC_{50}$ than the averages across replicates; replicate 3 had a more negative slope than the averages across replicates; replicate 4 had a higher estimated $IC_{50}$ and a less negative slope than the averages across replicates.

The results of analyses of variance for these estimates are presented in Table 3. For each replicate the squares of the standard errors associated with each parameter are given. These estimates include only within replicate variation. Across replicates, the replicate-to-replicate variation and the square of the standard error of the overall average are displayed. These estimates include both within replicate variation and replicate-to-replicate variation. The variance for $log_{10}IC_{50}$ among the replicates was high compared to the variances within replicates; it was at least 8 times higher when only replicates 2 to 4 considered or 5 times higher when all 4 replicates considered. The variance components across replicates 2 to 4 are seen to be greater than those across replicates 1 to 4.

The background activity control and full enzyme activity control responses for each replicate are displayed in Table A-2. These data are plotted by replicate in Figures 6 and 7, with plotting symbol distinguishing between beginning and end of the replicate. Figures 8 and 9 show the differences between the averages at the beginning and at the end within each replicate (end minus beginning). For the background activity controls, the measurements at the end were similar to those at the beginning for all replicates except replicate 2. Replicate 2 had a higher average at the beginning due to the higher measurement in a repetition at the beginning (close to 0.5% , Table A-2). This value differed considerably from any of the other background activity controls. Without this data value, the average at the beginning would be similar to that at the end for replicate 2. For the full enzyme activity controls the average percent of control responses at

the end were lower than those at the beginning for each replicate. The differences in the full enzyme activity controls between the end and the beginning of each replicate varied between -5% and -19%. This suggests reduction in aromatase enzyme activity between the beginning and end of the replicates.

Mixed effects analysis of variance models were fitted to the background activity control and full enzyme activity control data with portion as a fixed effect and with replicate and replicate by portion interaction as random effects. The component of variation due to replicate is constrained to be 0 by the definitions of the background activity and full enzyme activity control responses. The results are displayed in Table 4. The left panel of the table displays the results of the tests for the differences between the responses collected at the beginning and at the end of a replicate, averaged across replicates (beginning minus end). The right panel displays the estimated variance components. No significant differences were observed between the beginning and the end, averaged across replicates, for the background activity controls, whether replicate 1 was included in the analysis or was excluded. Highly significant differences between the beginning and the end portions were observed for the full enzyme activity controls. The variation in replicate 1 is greater than that in the other replicates. The estimated variance for the portion by replicate interaction is considerably smaller than the residual variation, which is based on the variation between the two repetitions carried out within the same portion of the same replicate. The estimated variance for replicate is zero, which reflects the constraints on the control values.

**Table 1. Estimated Parameters of the Concentration Response Curve Fits by Replicate and Averaged Across Replicates. Percent of Control Activity. Recombinant Aromatase Assay**

| Replicate | $Log_{10}IC_{50}$ (SE) | $IC_{50}$ (GSE)[e] | Slope (SE) | Status |
|---|---|---|---|---|
| **Individual Values[a]** | | | | |
| 1 | -7.062 (0.02430) | $8.679x10^{-8}$ (1.05755) | -0.9022 (0.03667) | C |
| 2 | -7.183 (0.01653) | $6.558x10^{-8}$ (1.03880) | -0.9031 (0.02662) | C |
| 3 | -7.071 (0.01571) | $8.493x10^{-8}$ (1.03684) | -0.9678 (0.02755) | C |
| 4 | -7.009 (0.02922) | $9.805x10^{-8}$ (1.06960) | -0.8527 (0.04002) | C |
| **Mean of Replicates 2-4[c]** | -7.090 (0.05061) | $8.136x10^{-8}$ (1.12360) | -0.9123 (0.03203) | -- |
| **Mean of Replicates 1-4[d]** | -7.083 (0.03673) | $8.252x10^{-8}$ (1.08824) | -0.9114 (0.02344) | -- |
| **Averages Values[b]** | | | | |
| 1 | -7.061 (0.04465) | $8.689x10^{-8}$ (1.10828) | -0.9027 (0.06744) | C |
| 2 | -7.183 (0.02855) | $6.567x10^{-8}$ (1.06795) | -0.9033 (0.04600) | C |
| 3 | -7.070 (0.02124) | $8.512x10^{-8}$ (1.05012) | -0.9693 (0.03734) | C |
| 4 | -7.007 (0.05367) | $9.842x10^{-8}$ (1.13154) | -0.8538 (0.07361) | C |

a. Concentration response curve fitted to the data collected within each replicate, with three repetitions at each 4-OH ASDN concentration level.
b. Concentration response curve fitted to the averages of the three repetitions at each 4-OH ASDN concentration level within each replicate.
c. Weighted averages of the parameter estimates across replicates, estimated based on replicates 2 to 4.
d. Weighted averages of the parameter estimates across replicates, estimated based on replicates 1 to 4.
e. 10 to the power of $log_{10}IC_{50}$ and 10 to the power of its associated standard error.

**Table 2. Parameter Estimates of the Concentration Response Curves and Associated 95% Confidence Intervals. Percent of Control Activity. Recombinant Aromatase Assay**

| Parameter | Estimate (95% CI) | | | | | |
|---|---|---|---|---|---|---|
| | Replicate 1[a] | Replicate 2[a] | Replicate 3[a] | Replicate 4[a] | Mean of Replicates 2-4 [b] | Mean of Replicates 1-4 [b] |
| $Log_{10}IC_{50}$ | -7.062 (-7.114, -7.010) | -7.183 (-7.218, -7.148) | -7.071 (-7.104, -7.038) | -7.009 (-7.071, -6.947) | -7.090 (-7.309, -6.870) | -7.083 (-7.199, -6.967) |
| Slope | -0.902 (-0.980, -0.824) | -0.903 (-0.960, -0.847) | -0.968 (-1.026, -0.909) | -0.853 (-0.938, -0.768) | -0.912 (-1.056, -0.768) | -0.911 (-0.984, -0.839) |

a. Parameter estimates and their associated 95% confidence intervals for each replicate, based on the concentration response curves fitted to the individual repetition data within replicates.

b. Mean and its associated 95% confidence interval, based on a heterogeneous variance one-way analysis of variance model with replicate treated as a random effect.

**Table 3. Variances Associated with Estimated Parameters of Concentration Response Curves. Percent of Control Activity. Recombinant Aromatase Assay**

| Parameter | Variance/Degree of Freedom[a,b,c] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Overall for Replicates 2-4 | | Overall for Replicates 1-4 | |
| | Replicate 1 | Replicate 2 | Replicate 3 | Replicate 4 | Random Replicate (p-value)[d] | Variance of Mean | Random Replicate (p-value)[d] | Variance of Mean |
| $Log_{10}IC_{50}$ | 0.000590 /df=16 | 0.000273 /df=16 | 0.000247 /df=16 | 0.000854 /df=16 | 0.007237 /df=2 (p=0.1745) | 0.002562 /df=1.982 | 0.004915 /df=3 (p=0.1310) | 0.001349 /df=3.042 |
| Slope | 0.001345 /df=16 | 0.000709 /df=16 | 0.000759 /df=16 | 0.001602 /df=16 | 0.002104 /df=2 (p=0.2548) | 0.001026 /df=1.912 | 0.001157 /df=3 (p=0.2585) | 0.000549 /df=3.154 |

a. The variance estimates for each replicate were based on the concentration response curves fitted to the individual repetition data within each concentration level.

b. Variance estimates for the random replicate were estimated based on a one-way random effects analysis of variance. The variances for each replicate were fixed at their reported values.

c. Degrees of freedom for the variance of mean were estimated by $2*((1/K)* \sum(S_r^2 + S_i^2))^2/(var(S_r^2) +(2/K^2)* \sum(S_i^4 /d\ f_i))$, where $S_r^2$ is random replicate variance, $S_i^2$ and $df_i$ are estimated variance and degree of freedom for a given replicate, $var(S_r^2)$ is the variance associated with the estimation of $S_r^2$ and K is the number of replicates (Hartung and Makambi, 2001).

d. p-value is based on the Wald Z-test result.

**Table 4. Variance Components of Full Enzyme Activity Control and Background Activity Control Percent of Control Values. Position Effects and Variation Across Replicates of Portion Effects Within Replicates.**

| Parameter | Difference Between Beginning and End Portions (Beginning Minus End) | | Variance Components | | | |
|---|---|---|---|---|---|---|
| | Estimate (%) (Std. Error) | p-Value/ Degree of Freedom | Replicate[a] | Portion* Replicate | Residual (Repetition) | Total Variance |
| **Replicates 1 to 4** | | | | | | |
| Background Activity | 0.1001 (0.0724) | 0.1883/df=14 | 0 | <0.00001 | 0.0210 | 0.0210 |
| Full Enzyme Activity Control | 10.8440 (2.2042) | 0.0002/df=14 | 0 | 0 | 19.4339 | 19.4339 |
| **Replicates 2 to 4** | | | | | | |
| Background Activity | 0.1109 (0.0976) | 0.2821/df=10 | 0 | <0.00001 | 0.0286 | 0.0286 |
| Full Enzyme Activity Control | 8.2467 (1.8565) | 0.0013/df=10 | 0 | 0 | 10.3403 | 10.3403 |

a. The replicate component of variation is constrained to be 0, by definition of background activity and full enzyme activity control responses.

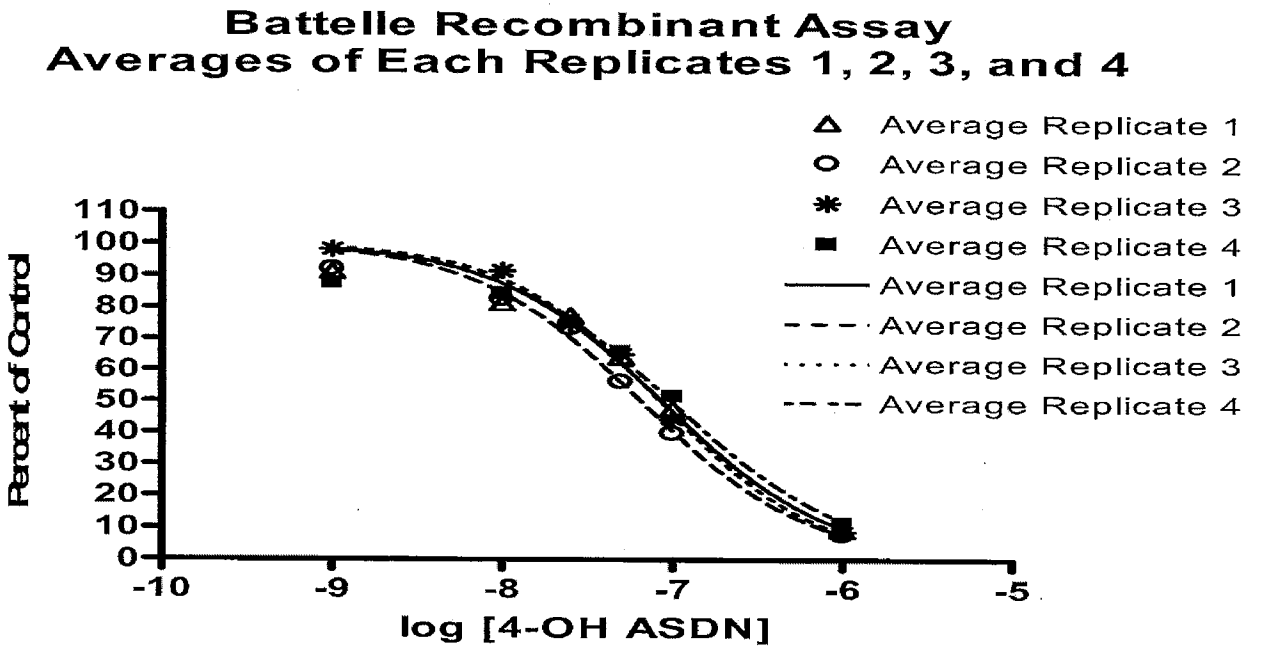



**Figure 1.** **Concentration Response Curves and Averages of Repetitions Within 4-OH ASDN Concentrations. Recombinant Aromatase Assay. By Replicate**

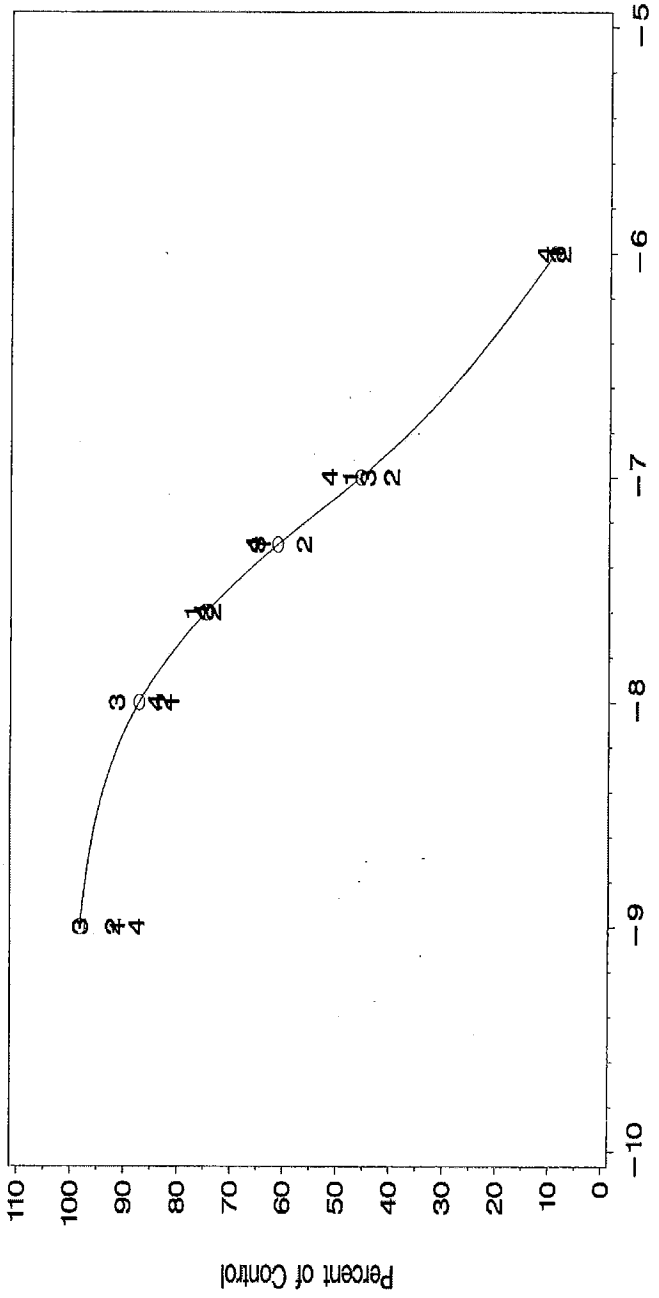



**Figure 2.** **Overall Average Concentration Response Curve Across Replicates 1 to 4 and Average Responses Across Repetitions Within 4-OH ASDN Concentrations. Recombinant Aromatase Assay. Parameters of Average Curve Based on One-Way Analysis of Variance Across Replicate 1 to 4 Parameter Values**

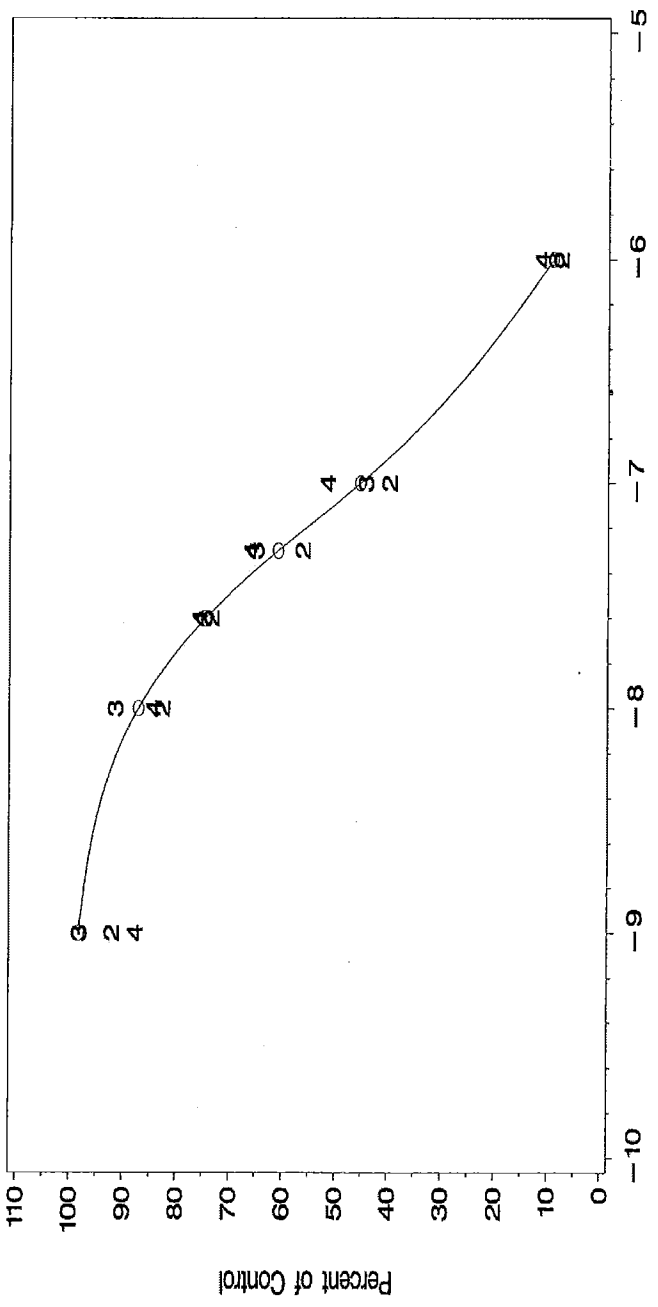



Figure 3. Overall Average Concentration Response Curve Across Replicates 2 to 4 and Average Responses Across Repetitions Within 4-OH ASDN Concentrations. Recombinant Aromatase Assay. Parameters of Average Curve Based on One-Way Analysis of Variance Across Replicate 2 to 4 Parameter Values

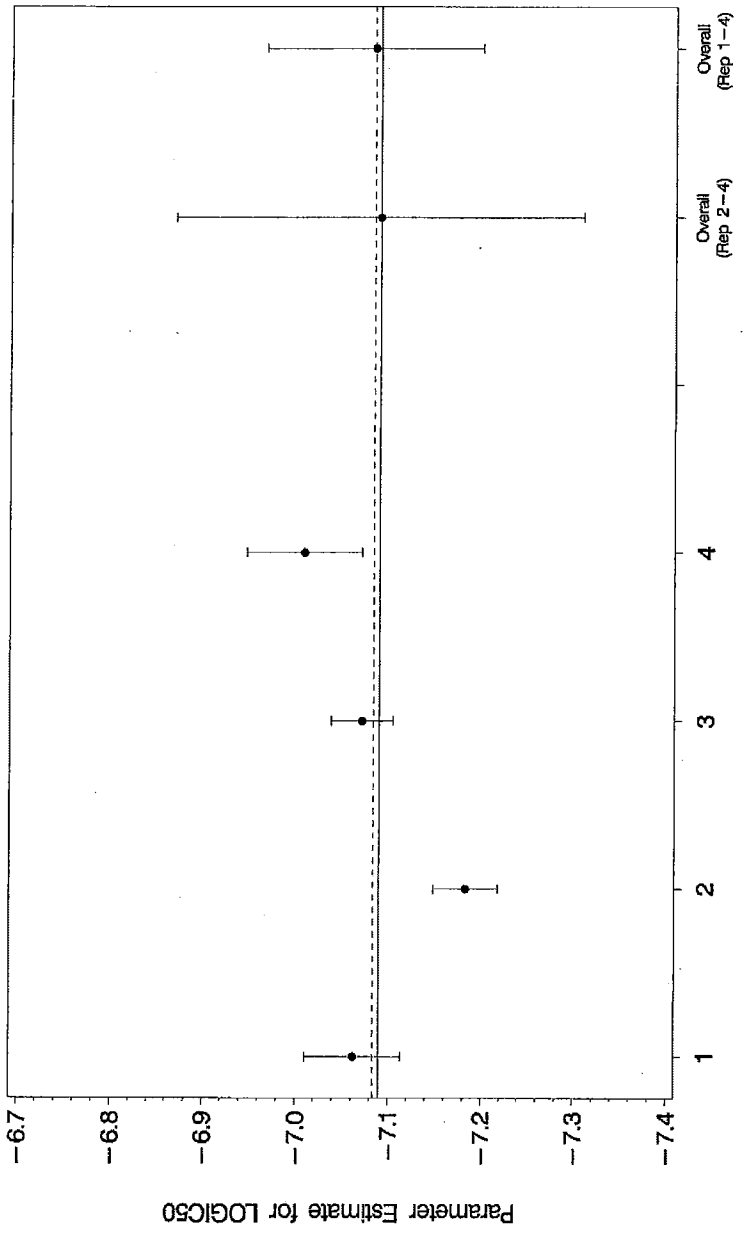



**Figure 4.** **$Log_{10}IC_{50}$ Parameter Estimates and Their Associated 95% Confidence Intervals for Each Replicate and Across Replicates 1 to 4, 2 to 4. Recombinant Aromatase Assay. The Solid Reference Line Corresponds to the Average Across Replicates 2 to 4. The Dotted Reference Line Corresponds to the Average Across Replicates 1 to 4**

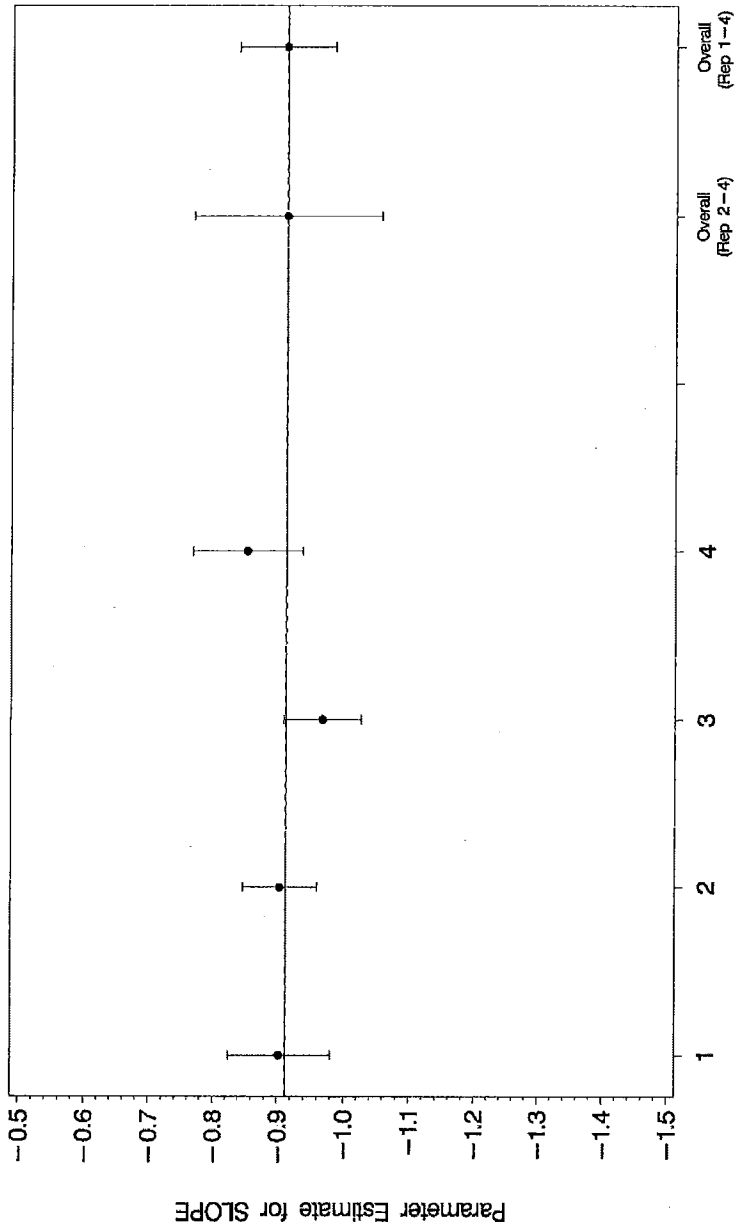



**Figure 5.** Slope (β) Parameter Estimates and Their Associated 95% Confidence Intervals for Each Replicate and Across Replicates 1 to 4, 2 to 4. Recombinant Aromatase Assay. The Solid Reference Line Corresponds to the Average Across Replicates 2 to 4. The Dotted Reference Line Corresponds to the Average Across Replicates 1 to 4

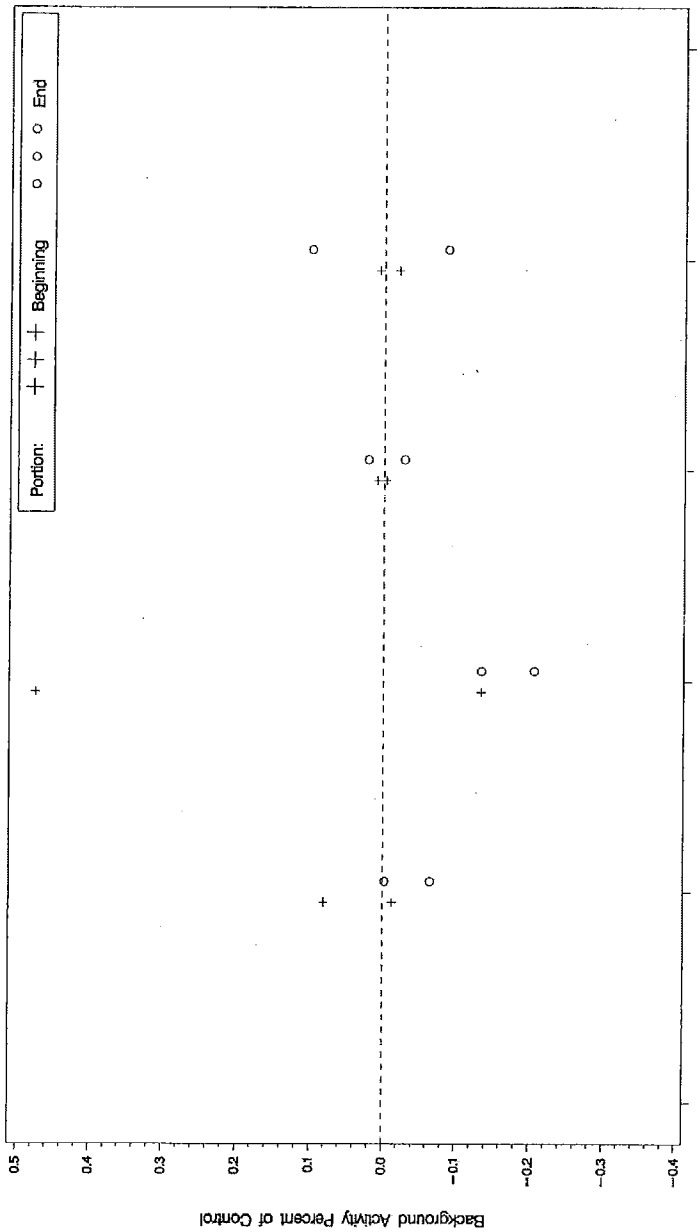



**Figure 6.** **Background Activity Controls. Percent of Control by Replicate and Portion of Replicate (Beginning or End). Recombinant Aromatase Assay. Reference Line at 0%.**

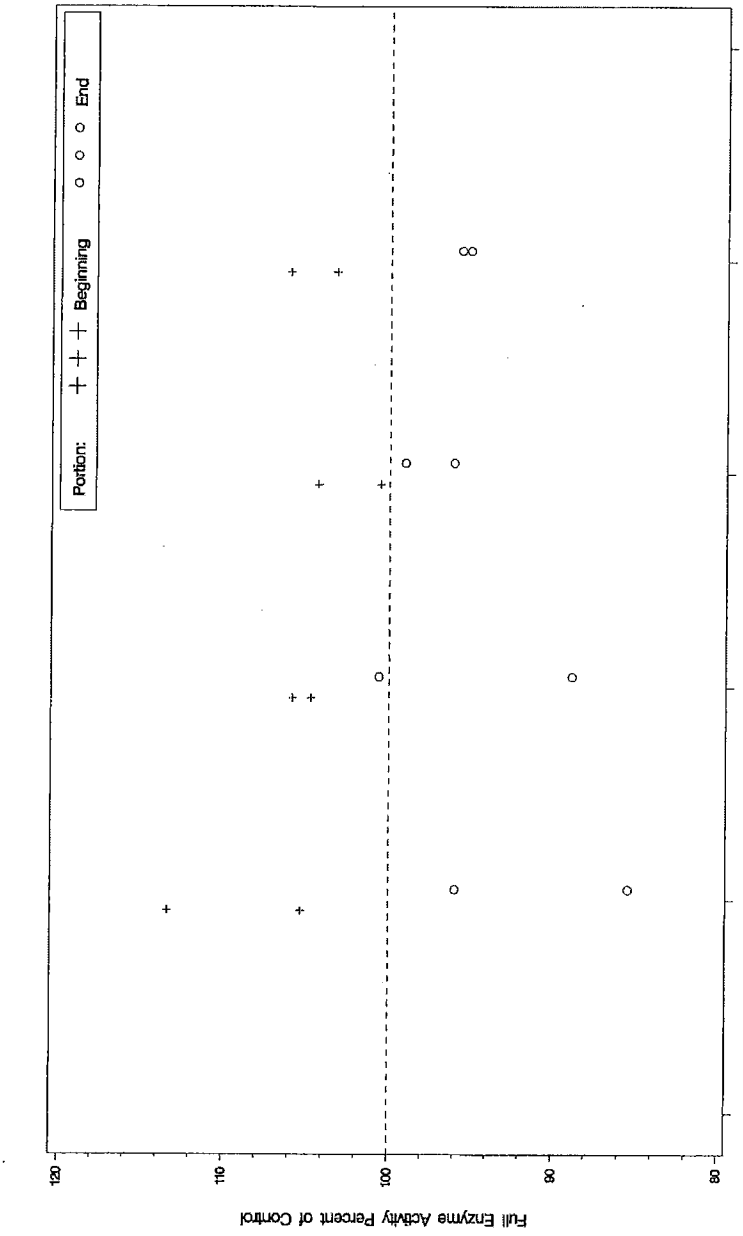



**Figure 7. Full Enzyme Activity Controls. Percent of Control by Replicate and Portion of Replicate (Beginning or End). Recombinant Aromatase Assay. Reference Line at 100%.**

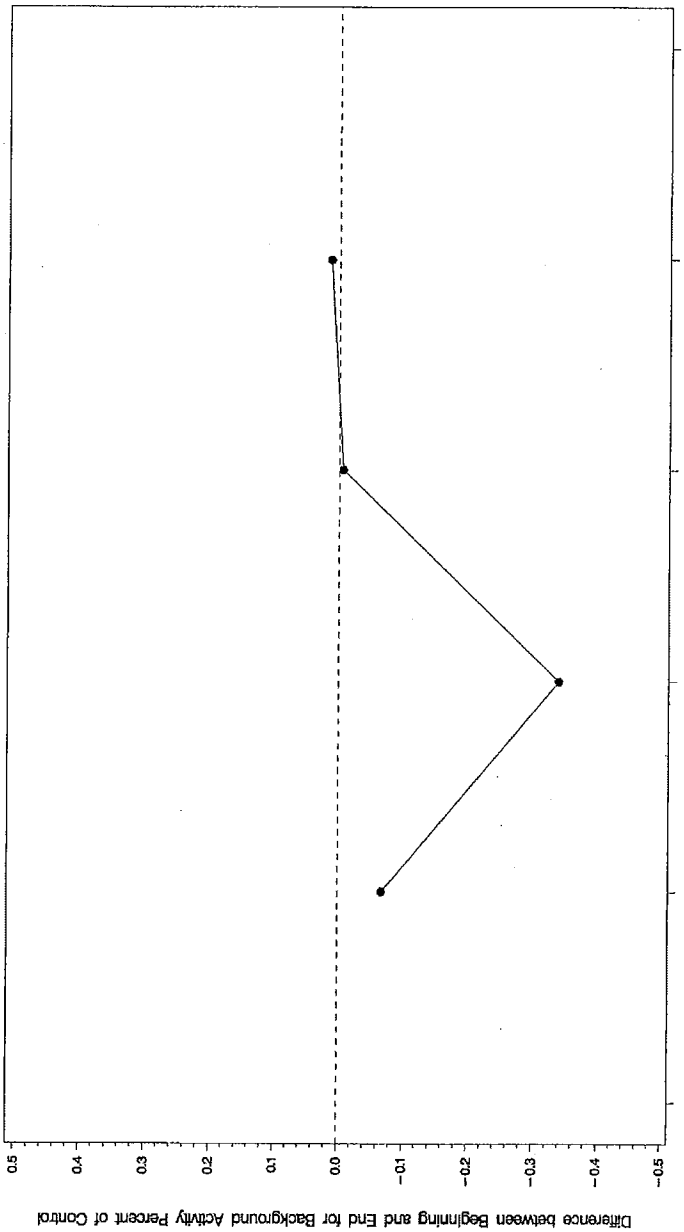



**Figure 8.** **Background Activity Controls. Difference Between the Average of the Two End Percent of Control Responses and the Average of the Two Beginning Responses by Replicate (End Minus Beginning). Recombinant Aromatase Assay. Reference Line at 0%.**

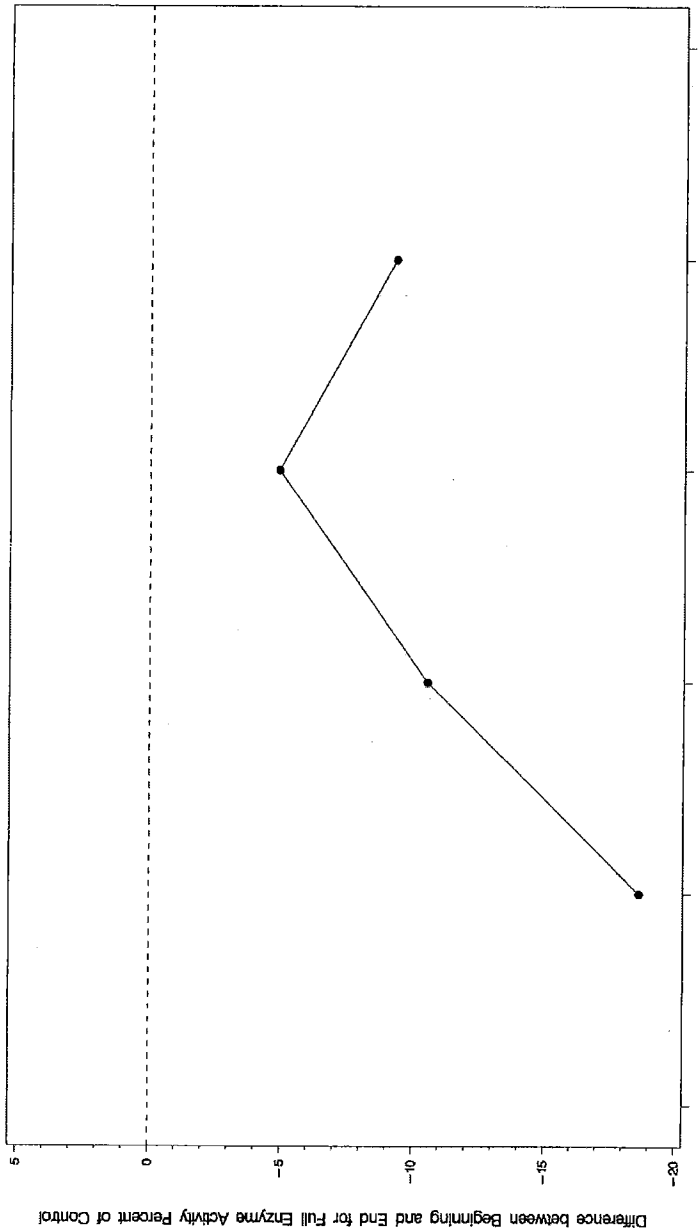



**Figure 9.** **Full Enzyme Activity Controls. Difference Between the Average of the Two End Percent of Control Responses and the Average of the Two Beginning Responses by Replicate (End Minus Beginning). Recombinant Aromatase Assay. Reference Line at 0%.**

**Table A-1. Percent of Control Activity in Recombinant Assay by Replicate, 4-OH ASDN Concentration Within Replicate, and Repetition Within Concentration**

| Replicate | Log [4-OH ASDN] | Percent of Control | | |
|---|---|---|---|---|
| | | Repetition 1 | Repetition 2 | Repetition 3 |
| 1 | -6.00 | 9.59 | 9.42 | 8.88 |
| | -7.00 | 46.49 | 48.25 | 48.08 |
| | -7.30 | 65.03 | 64.00 | 63.23 |
| | -7.60 | 76.72 | 75.94 | 79.58 |
| | -8.00 | 80.12 | 80.05 | 83.56 |
| | -9.00 | 87.98 | 95.43 | 90.18 |
| 2 | -6.00 | 7.86 | 7.49 | 8.13 |
| | -7.00 | 40.78 | 39.10 | 39.71 |
| | -7.30 | 55.77 | 55.12 | 57.93 |
| | -7.60 | 74.92 | 73.52 | 71.73 |
| | -8.00 | 84.67 | 82.88 | 80.47 |
| | -9.00 | 90.34 | 90.11 | 95.58 |
| 3 | -6.00 | 8.82 | 8.50 | 8.83 |
| | -7.00 | 45.54 | 43.25 | 44.13 |
| | -7.30 | 66.80 | 64.11 | 64.31 |
| | -7.60 | 77.46 | 75.98 | 70.71 |
| | -8.00 | 96.48 | 91.24 | 86.62 |
| | -9.00 | 98.98 | 95.86 | 99.50 |
| 4 | -6.00 | 11.34 | 11.22 | 11.38 |
| | -7.00 | 48.64 | 54.37 | 51.57 |
| | -7.30 | 63.77 | 67.64 | 66.28 |
| | -7.60 | 71.92 | 79.29 | 75.76 |
| | -8.00 | 85.11 | 83.28 | 84.36 |
| | -9.00 | 89.33 | 87.01 | 86.51 |

**Table A-2. Background Activity Control and Full Enzyme Activity Control Corrected Aromatase Activity by Replicate and Portion (Beginning or End). Recombinant Aromatase Assay.**

| Aromatase Activity | Replicate | Portion | Corrected Activity | % of Control[a] |
|---|---|---|---|---|
| Background Activity Control | 1 | Beginning | 0.000150 | 0.0807 |
| | | Beginning | -0.000024 | -0.0130 |
| | | End | -0.000005 | -0.0026 |
| | | End | -0.000121 | -0.0651 |
| | 2 | Beginning | 0.000806 | 0.4742 |
| | | Beginning | -0.000228 | -0.1342 |
| | | End | -0.000228 | -0.1342 |
| | | End | -0.000350 | -0.2058 |
| | 3 | Beginning | -0.000006 | -0.0031 |
| | | Beginning | 0.000017 | 0.0093 |
| | | End | -0.000052 | -0.0278 |
| | | End | 0.000041 | 0.0217 |
| | 4 | Beginning | -0.000041 | -0.0200 |
| | | Beginning | 0.000014 | 0.0067 |
| | | End | 0.000205 | 0.1002 |
| | | End | -0.000178 | -0.0868 |
| Full Enzyme Activity Control | 1 | Beginning | 0.196252 | 105.2836 |
| | | Beginning | 0.211292 | 113.3521 |
| | | End | 0.178825 | 95.9345 |
| | | End | 0.159244 | 85.4298 |
| | 2 | Beginning | 0.179997 | 105.8366 |
| | | Beginning | 0.178090 | 104.7152 |
| | | End | 0.151065 | 88.8249 |
| | | End | 0.171131 | 100.6233 |
| | 3 | Beginning | 0.196079 | 104.3281 |
| | | Beginning | 0.189009 | 100.5661 |
| | | End | 0.186148 | 99.0441 |
| | | End | 0.180543 | 96.0617 |
| | 4 | Beginning | 0.211255 | 103.2583 |
| | | Beginning | 0.216937 | 106.0359 |
| | | End | 0.194535 | 95.0858 |
| | | End | 0.195627 | 95.6200 |

a. The corrected aromatase activity values were divided by the average of the four full enzyme activity control activity values within the same replicate and multiplied by 100 percent.

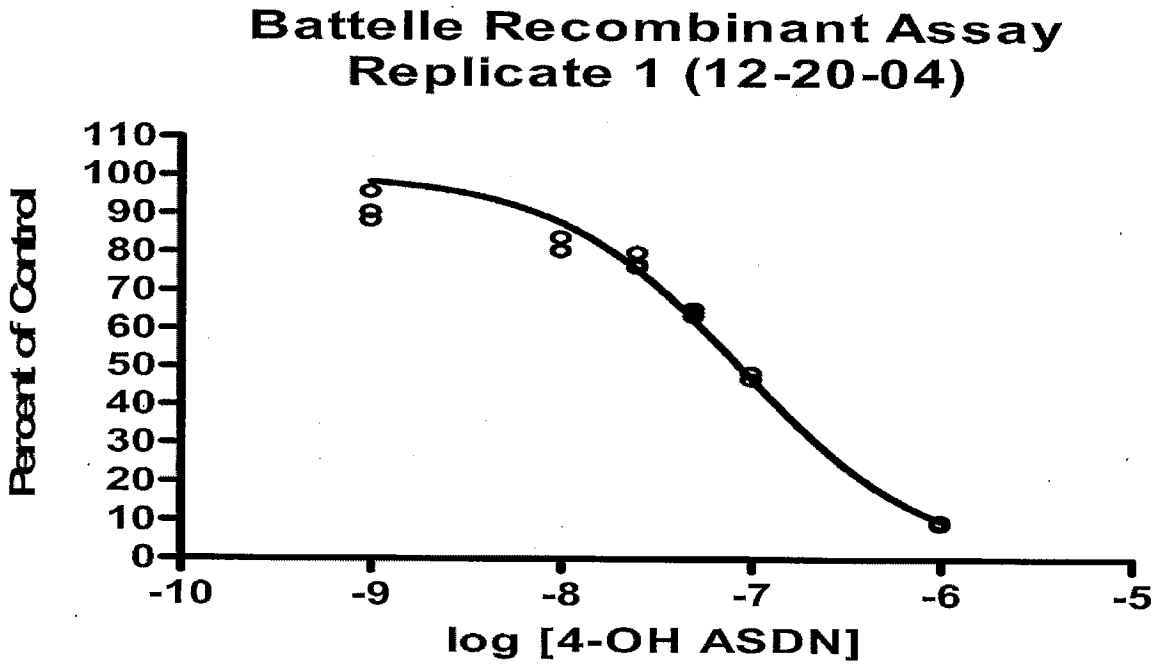



**Figure A-1** **Replicate 1. Individual Percent of Control Values Vs. (Base 10) Logarithm of 4-OH ASDN Inhibitor Concentration. Concentration Response Curve Fitted to Average Responses Within Concentrations. Recombinant Aromatase Assay**

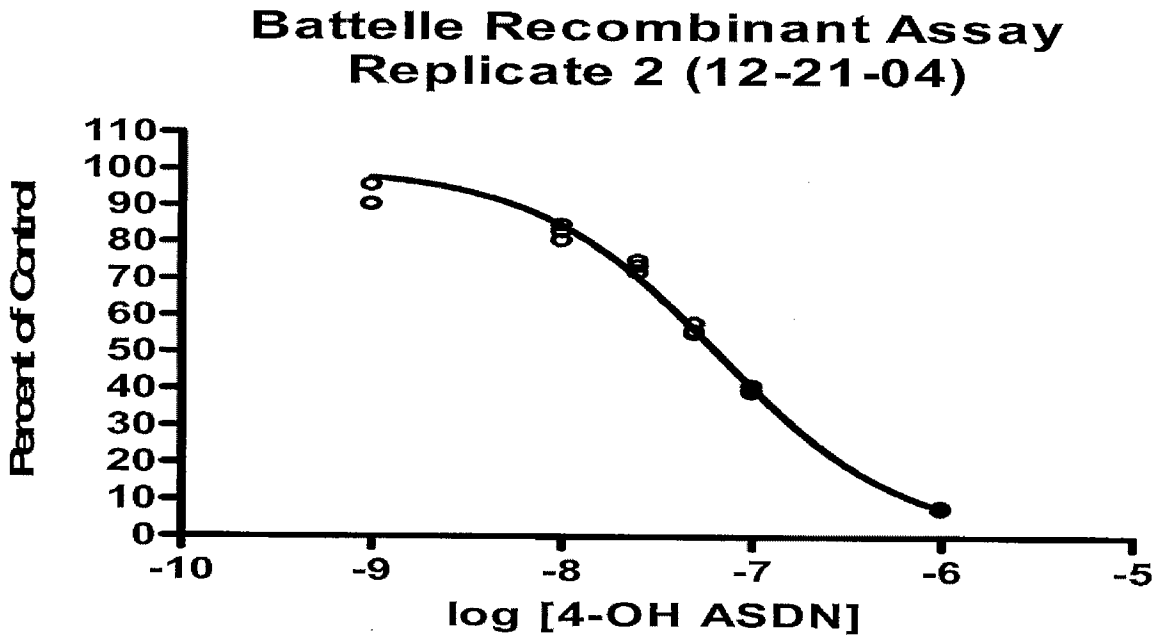



**Figure A-2** **Replicate 2. Individual Percent of Control Values Vs. (Base 10) Logarithm of 4-OH ASDN Inhibitor Concentration. Concentration Response Curve Fitted to Average Responses Within Concentrations. Recombinant Aromatase Assay**

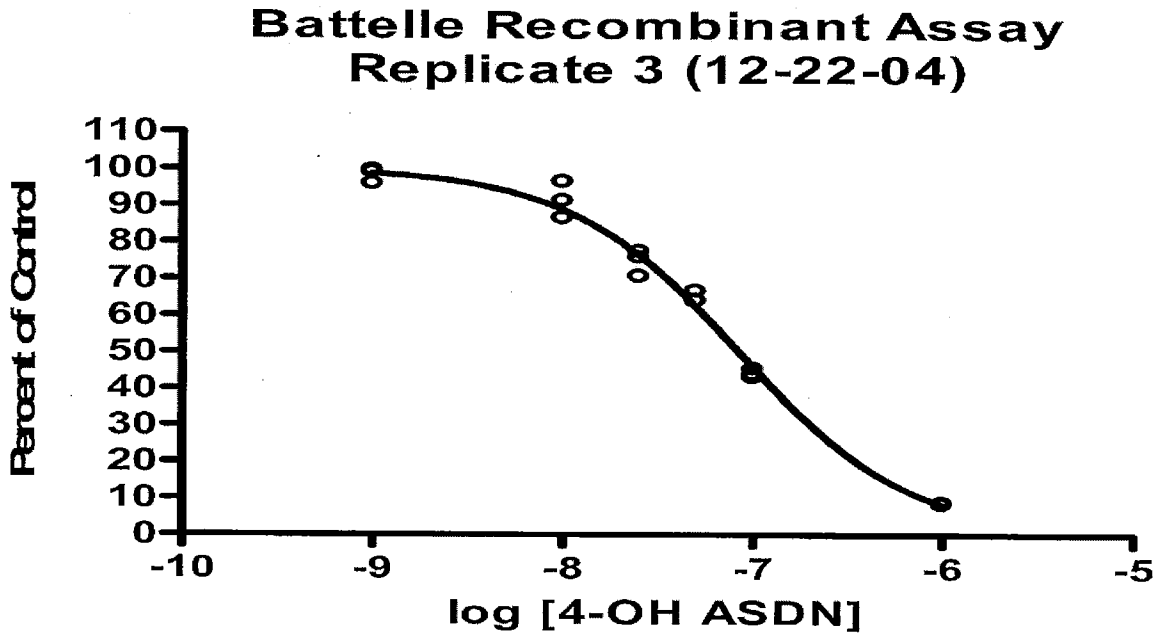



**Figure A-3** **Replicate 3. Individual Percent of Control Values Vs. (Base 10) Logarithm of 4-OH ASDN Inhibitor Concentration. Concentration Response Curve Fitted to Average Responses Within Concentrations. Recombinant Aromatase Assay**

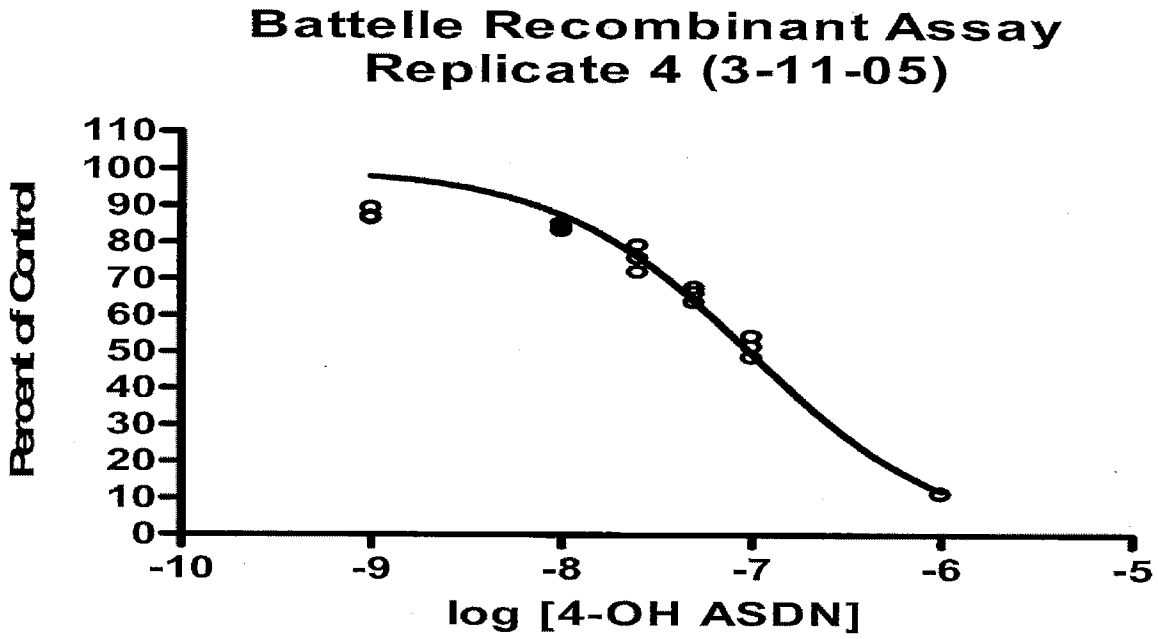



**Figure A-4** **Replicate 4. Individual Percent of Control Values Vs. (Base 10) Logarithm of 4-OH ASDN Inhibitor Concentration. Concentration Response Curve Fitted to Average Responses Within Concentrations. Recombinant Aromatase Assay**

# APPENDIX B

## In Vitro Technologies Draft Final Report

# DRAFT TASK REPORT

# RECOMBINANT AROMATASE VALIDATION STUDY

## WA 4-17 Task 3

**EPA Contract Number 68-W-01-023**
**Work Assignment 4-17**

**Sponsor:**

Battelle Memorial Institute
505 King Avenue
Columbus, OH 43201

**Performing Laboratory:**

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

DRAFT REPORT

| | |
|---|---|
| Title: | RECOMBINANT AROMATASE VALIDATION STUDY<br>WA 4-17 Task 3 |
| Authors: | Neil Jensen, Ph.D. |
| Performing Laboratory: | In Vitro Technologies, Inc.<br>1450 South Rolling Road<br>Baltimore, MD 21227 |
| Sponsor: | Battelle Memorial Institute<br>505 King Avenue<br>Columbus, OH 43201 |
| Sponsor Representatives: | David Houchens, Ph.D.<br>EDSP Program Manager<br>Battelle<br><br>Jerry D. Johnson, Ph.D.<br>Diplomate, A.B.T.<br>Work Assignment Leader<br>Battelle |
| Study Initiation Date: | 25 January 2005 |
| Experimental Dates: | Experimental start date: 25 January 2005<br>Experimental end date: 27 January 2005 |
| Draft Task Report Date | 26 January 2006 |

| | |
|---|---|
| Author: | Approved: |
| _____ | _____ |
| Signature. Date | Signature. Date |

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| 6.0 | References | 16 |


## LIST OF APPENDICES


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| Appendix 2: | Copy of QAPP for Work Assignment W4-17, Task 3 | (39 pages) |
| Appendix 3: | Excel Spreadsheets for Task 3 | (18 pages) |
| Appendix 4: | Prism Output for Task 3 | (3 pages) |
| Appendix 5: | Copy of Battelle Chemistry Report | (18 pages) |
| Appendix 6: | Copy of RTI [$^3$H] ASDN Purity Assessment Report | (4 pages) |
| Appendix 7: | Copy of Statistician's Report | (26 pages) |

# STATEMENT OF COMPLIANCE

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

| Neil Jensen, Ph.D. | | |
|---|---|---|
| Study Director | Signature | Date |

# QUALITY ASSURANCE STATEMENT

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

| Inspection/Audit Dates: | Study Phase Audited: | Date(s) reported to Study Director and Management: |
|---|---|---|
| 24 January 2005 | Protocol Review | 24 January 2005 |
| 26, 27 January 2005 | Sample preparation<br>Analysis<br>Data entry | 28 January 2005 |
| 21, 22 September 2005 | Data and report | 22 September 2005 |

| Quality Assurance | Signature | Date |
|---|---|---|

# PARTICIPATION

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

| | |
|---|---|
| Study Director: | Neil Jensen, Ph.D. |
| Scientists: | Aruna Koganti, Ph.D. |
| | Tim Moeller, M.Sc. |
| Technical Writers: | Barbara Biery, Ph.D. |
| | Blaise Considine, B.A. |

# DATA RETENTION

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

# 1.0 Executive Summary

The objective of this study was to validate the recombinant aromatase assay with a known inhibitor. This study was part of a multi-laboratory effort for the validation of the recombinant aromatase assay. The protocol was specific to the study conducted at In Vitro Technologies, Inc. In Vitro Technologies conducted three separate experiments to evaluate the inhibition of recombinant aromatase by 4-hydroxyandrostenedione (4-OH ASDN).

For replicate 1, the aromatase activity was 0.1774 nmol/mg/min and the $IC_{50}$ was $8.809 \times 10^{-8}$ M.
For replicate 2, the aromatase activity was 0.1795 nmol/mg/min and the $IC_{50}$ was $9.634 \times 10^{-8}$M.
For replicate 3, the aromatase activity was 0.2909 nmol/mg/min and the $IC_{50}$ was $8.340 \times 10^{-8}$ M.

# 2.0 Introduction

## *2.1 Background*

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

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

Aromatase is a cytochrome P450 enzyme complex responsible for estrogen biosynthesis and converts androgens, such as testosterone and androstenedione, into the estrogens estradiol and estrone. Aromatase is present in the ovary, placenta, uterus, testis, brain, and extraglandular adipose tissues. Two proteins, cytochrome P450arom and NADPH-cytochrome P450 reductase, are necessary for enzymatic activity, and the enzyme complex is localized in the smooth endoplasmic reticulum. The aromatase gene, designated CYP19, encodes the cytochrome P450arom and consists of 10 exons, with the exact size of the gene exceeding 70 kilobases. Aromatase is found in breast tissue, and the importance of intratumoral aromatase and local

estrogen production is being unraveled. Effective aromatase inhibitors have been developed as therapeutic agents for estrogen-dependent breast cancer to reduce the growth stimulatory effects of estrogens in breast cancer. Investigations on the development of aromatase inhibitors began in the 1970's and have expanded greatly in the past three decades.

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

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

## 2.2 Task Description and Objectives

The objective of this study was to validate the recombinant aromatase assay with a known inhibitor. This study was part of a multi-laboratory effort for the validation of the recombinant aromatase assay. The protocol was specific to the study to be conducted at In Vitro Technologies, Inc.

# 3.0 Materials and Methods

## 3.1 Substrate

The substrate for the aromatase assay was androstenedione (ASDN). Non-radiolabeled and radiolabeled ASDN were used. The non-radiolabeled ASDN (lot 024K0809) was obtained from Sigma, St. Louis, MO by Battelle's Chemical Repository and was then distributed to the participating laboratories. It had a reported purity of 99%. The radiolabeled androstenedione ([1β-$^3$H]-androstenedione, [$^3$H]ASDN, lot 3538496), was obtained from Perkin Elmer Life Science, Boston, MA and had a reported specific activity of 25.3 Ci/mmol. Radiochemical purity was

reported by the supplier to be > 97%. Radiochemical purity was assessed by high performance liquid chromatography by the lead laboratory. The results of this analysis are presented in the report contained in Appendix 6.

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

The following illustrates the preparation of a substrate solution using a stock of [$^3$H]ASDN with a specific activity of 25.3 Ci/mmol and a concentration of 1 mCi/mL. A 1:100 dilution (10 µCi/mL) of the radiolabeled stock in 0.1 M sodium phosphate was prepared. A 1 mg/mL solution of ASDN in 95% ethanol was prepared. Dilutions were prepared in 0.1 M sodium phosphate to a final concentration of 1 µg/mL. The 1 µg/mL solution of ASDN (4.5 mL), 800 µL of the [$^3$H]ASDN dilution, and 2.7 mL of buffer were combined to make 8 mL of substrate solution (enough for 80 tubes). The weight of each component added to the substrate solution was recorded. After mixing the solution well, aliquots (approximately 20 µL) were weighed and combined with scintillation cocktail for radiochemical content analysis. The addition of 100 µL of the substrate solution to each 2 mL assay volume yielded a final [$^3$H]ASDN concentration of 100 nM with 0.1 µCi/tube.

## 3.2 Test Substances

The test article was identified in this study as follows:

- 4-hydroxyandrostenedione (4-OH ASDN, molecular weight 302.4 g/mol, CAS no.: 566-48-3)

Battelle provided 4-OH ASDN as a stock solution in ethanol. The 4-OH ASDN stock formulation was prepared by the Chemical Repository as a 0.01 M solution in 95% ethanol. In Vitro Technologies prepared fresh dilutions of the stock formulation using 95% ethanol (supplied by the Chemical Repository) according to the procedures described in the following table:

| 4-OH ASDN Stock Formulation Concentrations (mM) | | Volume of Stock (µL) | Volume of Ethanol (µL) | Dilution Number & Concentrations (mM) | | Final Concentration in the Assay (M) |
|---|---|---|---|---|---|---|
| CR Stock[a] | 10 | 20 | 1980 | 1 | 0.1 | $1 \times 10^{-6}$ |
| Working Stock #1 | 0.1 | 100 | 900 | 2 | 0.01 | $1 \times 10^{-7}$ |
| | | 50 | 950 | 3 | 0.005 | $5 \times 10^{-8}$ |
| | | 25 | 975 | 4 | 0.0025 | $2.5 \times 10^{-8}$ |
| Working Stock #2 | 0.01 | 100 | 900 | 5 | 0.001 | $1 \times 10^{-8}$ |
| Working Stock #5 | 0.001 | 100 | 900 | 6 | 0.0001 | $1 \times 10^{-9}$ |

a. Chemical Repository stock formulation.

Battelle'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 Battelle's Chemistry Report, which is appended to this document (Appendix 5).

| Chemical name | Chemical code | Mfr. Purity | CAS No. | Molecular formula | Molecular weight (g/mol) | Stock Solution ID | Target Stock Formulation Concentration | Vehicle | Storage Conditions |
|---|---|---|---|---|---|---|---|---|---|
| ASDN | 270-0010 | 99% | 63-05-8 | $C_{19}H_{26}O_2$ | 286.41 | 1132-1719-5,6,7<br>1132-1721-5,6,7<br>1132-1723-5,6,7 | 1, 0.01, 0.001 mg/mL | 95% ethanol | RT |
| [$^3$H]ASDN | 270-0012 | >97% | 63-05-8 | $C_{19}H_{26}O_2$ | 286.41 | 1132-1719-8,9<br>1132-1721-8,9<br>1132-1723-8,9 | 20 µM, 2 µM | 0.1M sodium phosphate | –20°C |
| 4-OH ADSN | 270-0013 | 99% | 566-48-3 | $C_{19}H_{26}O_3$ | 302.41 | 1132-1719-11<br>1132-1721-11<br>1132-1723-11 | 0.01 M | 95% ethanol | 2–8°C |

RT, room temperature

## 3.3 Microsomes

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

Microsomes (lot no. 5) were obtained from RTI and stored at approximately –70°C until use. The protein concentration was approximately 4.9 mg/mL. Microsomes were thawed rapidly in a 37 ± 1°C water bath, rehomogenized using a Potter Elvejhem homogenizer and then kept on ice until used. For use in the assay, the microsomes were diluted in the assay buffer in two serial dilutions. A 10-fold dilution was made to achieve a concentration of approximately 0.50 mg/mL. Another 60-fold dilution was made to achieve the desired final working stock concentration of approximately 0.008 mg/mL. The final target protein concentration in the incubation mixture was approximately 0.004 mg/mL.

## 3.4 Other assay components

| Chemical | Supplier | Lot Number |
|---|---|---|
| NADPH | Sigma | 103K7046 |
| Propylene glycol | JT Baker | 042343 |
| Sodium phosphate dibasic | JT Baker | A43465 |
| Sodium phosphate monobasic | JT Baker | A28H21 |
| 95% ethanol | Spectrum | SW0045 |

### 3.4.1 NADPH

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

### 3.4.2 Assay Buffer

The assay buffer, 0.1 M sodium phosphate buffer, pH 7.4, was prepared and stored in the refrigerator (2 to 8°C).

## 3.5 Protein Determination

The protein concentration of the microsome preparation was determined on each day of use of the microsomes in the aromatase assay. A six-point standard curve was prepared, ranging from 0.13 to 1.5 mg protein/mL. The protein standards were made from bovine serum albumin (BSA). Protein was determined by using a DC Protein Assay kit purchased from Bio-Rad (Hercules, CA). To a 25 µL aliquot of standard or unknown, 125 µL of Bio-Rad DC Protein Kit Reagent A was added and mixed. Bio-Rad DC Protein Kit Reagent B (1 mL) was added to each standard or unknown and the samples were mixed. The samples were placed at room temperature for at least 15 minutes to allow for color development. The absorbances were stable for approximately 1 hour. Each sample (standards and unknowns) was transferred to disposable polystyrene cuvettes and the absorbance (750 nm) was measured using a spectrophotometer. The protein concentration of the microsomal sample was determined by extrapolation of the absorbance value using the standard curve developed using the protein standards.

## 3.6 Cytochrome P450 Aromatase (CYP19) Activity

The assays were performed in 13 × 100 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) were 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 were placed at 37 ± 1°C in the water bath for 5 minutes prior to initiation of the assay by the addition of 1 mL of the diluted microsomal suspension. The total assay volume was 2.0 mL, and the tubes were incubated for 15 minutes. The incubations were stopped by the addition of 2.0 mL of methylene chloride; the tubes were vortex-mixed for approximately 5 seconds and placed on ice. The tubes were vortex-mixed an additional 20 to 25 seconds. The tubes were spun in a centrifuge for 10 minutes at a setting of 1,000 rpm. The methylene chloride layer was removed and discarded; the aqueous layer was extracted again with 2 mL of methylene chloride. This extraction procedure was repeated once more, each time discarding the methylene chloride layer. The aqueous layers were transferred to vials and duplicate aliquots (0.5 mL) were transferred to 20-mL liquid scintillation counting vials. Liquid scintillation cocktail (Ultima Gold, Packard, 10 mL) was added to each counting vial and shaken to mix the solution. The radiochemical content of each aliquot was determined as described below.

**Table 1. Optimized Aromatase Assay Conditions**

| Assay factor (units) | Assay Type |
|---|---|
| | Human Recombinant |
| Microsomal Protein (mg/mL)[a] | 0.004 |
| NADPH (mM)[a] | 0.3 |
| [$^3$H]ASDN (nM)[a] | 100 |
| Incubation Time (min) | 15 |

[a] Final concentrations

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

Results are presented as the activity (velocity) of the enzyme reaction. The amount of estrogen product formed was determined by dividing the total amount of $^3H_2O$ formed by the specific

activity of the [$^3$H]ASDN substrate (expressed in DPM/nmol). The activity of the enzyme reaction is expressed in nmol (mg protein)$^{-1}$min$^{-1}$ and was calculated by dividing the amount of estrogen formed by the product of mg microsomal protein used times the incubation time (e.g., 15 minutes).

**Full Enzyme Activity Control Study**

Each study tested the response of aromatase activity to the presence of six concentrations of 4-OH ASDN. This study was conducted in three independent replicates. Each concentration of 4-OH ASDN was run in triplicate tubes in each study. See Table 2 below for the study design. Full enzyme activity control and background activity samples were included for each study. Full enzyme activity controls contained substrate, NADPH, propylene glycol, buffer, vehicle (used for preparation of 4-OH ASDN solutions), and microsomes. Background activity samples contained all full enzyme activity control assay components except NADPH, and served as assay blanks. Four full enzyme activity control samples and four background activity samples were included with each study and were treated the same as the other samples. The control sets were split so that two tubes (of each full enzyme activity control and background activity samples) were run at the beginning and two at the end of each study set.

The assay was conducted as described in the Cytochrome P450 Aromatase (CYP19) Activity section above, with the following modification: 4-OH ASDN solution (or vehicle) was 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 was adjusted so the total incubation volume remained at 2 mL.

**Table 2. Full Enzyme Activity Control Study 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 | $1 \times 10^{-4}$ | $1 \times 10^{-6}$ |
| 4-OH ASDN Concentration 2 | 3 | 4-OH ASDN added | $1 \times 10^{-5}$ | $1 \times 10^{-7}$ |
| 4-OH ASDN Concentration 3 | 3 | 4-OH ASDN added | $5 \times 10^{-6}$ | $5 \times 10^{-8}$ |
| 4-OH ASDN Concentration 4 | 3 | 4-OH ASDN added | $2.5 \times 10^{-6}$ | $2.5 \times 10^{-8}$ |
| 4-OH ASDN Concentration 5 | 3 | 4-OH ASDN added | $1 \times 10^{-6}$ | $1 \times 10^{-8}$ |
| 4-OH ASDN Concentration 6 | 3 | 4-OH ASDN added | $1 \times 10^{-7}$ | $1 \times 10^{-9}$ |

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

## *3.7 Data Analysis*

In Vitro Technologies supplied all raw data to Battelle in electronic format using Excel spreadsheets and Prism template developed and provided by Battelle.

## 3.7.1 Data Analysis and Presentation

The data reported 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 DPM for the background tubes was subtracted from the tubes with Total DPM to provide DPM for specific aromatase activity. A spreadsheet was developed by the lead laboratory that was used to process the data into a final form for analysis and evaluation. A working document detailing the conversion of the data from DPM to nmol, as well as the actual methods for calculations of the final aromatase activity, was distributed to the laboratories. This process is briefly summarized below:

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). Multiplication of the volume (mL) of substrate solution added to the incubation by the substrate solution radiochemical content (DPM/mL) yielded 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 yielded the percent of the substrate that was converted to product. The total DPM remaining in the aqueous portion after extraction was 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 was 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 was 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 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 activity and multiplying by 100.

$IC_{50}$ was calculated using GraphPad Prism (Version 4) software to fit the percent of control activity and log concentration data to a curve using the following equation:

$$Y = 100/(1+10^{((LogIC_{50}-X)*HillSlope)})$$

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

The data were formatted as follows:

- One spreadsheet or table displayed the DPM for all assay tubes, calculations of activity (nmol (mg protein)$^{-1}$min$^{-1}$) etc.
- Another table presented the results of the analysis of variability of the assay and included:

  (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 $IC_{50}$ by date, run, technician, assay method

## 3.7.2 Statistical Analysis

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

The statistical analysis described in this section was carried out by Battelle. The resulting data were sent to In Vitro Technologies and are included in the final report.

### 3.7.2.1 Concentration Response Fits for the Test Substance

For the test substance, multiple independent replicates of the concentration response curve fit were carried out. The number of replicates was three. Full enzyme activity and background activity control percent activity values were compared across daily replicate tests for the test substance.

For each replicate, two repeat tubes of the full enzyme activity controls and the background activity controls were 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 samples were prepared after the repetitions of the inhibitor compound were prepared. Three repetitions were prepared for each level of the inhibitor compound (4-OH ASDN).

For each repetition at each level, the Excel database spreadsheet included total DPM per tube (corrected for background DPM) and total aromatase activity per tube. The aromatase activity was 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 was corrected for the background DPM, as measured by the average of the background activity tubes. Percent activity was 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 necessarily equaled 0 within each replicate and the average percent activity across the four full enzyme activity repeat tubes necessarily equaled 100 within each replicate. The total DPM values were 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 were fitted to the percent of control activity values within each of the repeat tubes at each inhibitor concentration. Concentration was expressed on the log scale. In agreement with past convention, logarithms are 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 DPM across the repeat tubes with the same inhibitor concentration
$\beta$ = slope of the concentration response curve ($\beta$ 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 was fitted to relate percent of control activity to logarithm of concentration within each replicate:

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

where ε was the variation among repetitions, distributed with mean 0 and variance proportional to DAVG (based on Poisson distribution theory for radiation counts). The variance was approximated by Y. The response curve was fitted by weighted least squares nonlinear regression analysis with weights equal to 1/Y. Model fits were carried out using Prism software (Version 4). Observed individual percent activity values above 100% were set to 99.5%. Observed individual percent activity values below 0% were set to 0.5%.

The concentration response fits were carried out for each replicate test. Based on the results of the fit within each replicate, the extent of aromatase inhibition is summarized as $IC_{50}$ ($10^{\mu}$) and slope (β). The estimated $IC_{50}$ for an inhibitor compound was a (weighted) geometric mean across the replicates. The estimated overall standard error was based on the standard errors within each replicate and the replicate-to-replicate variability. The average value and standard error of $\log_{10}IC_{50}$ or β was calculated based on a one-way random effects analysis of variance model fit.

For the 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 are 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 could not be fitted (and so an $IC_{50}$ could not be estimated) were referred to as "noninhibitors".

### 3.7.2.2 Graphical and Analysis of Variance Comparisons among Concentration Response Curve Fits

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

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

For each replicate treat (β, μ) as a random variable with mean ($\beta_{avg}$, $\mu_{avg}$). 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) were also compared across replicates based on random effects analysis of variance, treating the replicates as random effects. β and μ were 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).

### Background and Full Enzyme Activity Control Values Across Replicates

Within each replicate, quadruplicate repetitions were made of the background activity tubes and the full enzyme activity control tubes. Half the repetitions were carried out at the beginning of the replicate and half at the end. If the conditions were constant throughout the replicate test, the control tubes at the beginning should be equivalent to those at the end. To assess whether this was the case, the control responses were 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 were 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 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 carried out, 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 corresponded to repetition within replicate and portion. The response was percent of control aromatase activity. If the daily replicates were similar, the portion main effect and portion by replicate interaction would be insignificant. Note that the replicate effects were necessarily zero because of the constrained totals within each replicate. For purposes of evaluation, replicate was treated as a fixed effect. If portion by replicate interaction was significant, the nature of the effect would 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, are presented graphically.

# *4.0* Results

Three replicates of the assay were run without incident. No additional replicates were run. In addition, no samples required reanalysis. Prism and Excel files were populated with results from the three replicates. Tables 4.3 – 4.6 provide a summary of the data.

## *4.1 Radiochemical Purity*

The measured radiochemical purity of the [$^3$H]ASDN was 97%. The RTI [$^3$H]ASDN Purity Assessment Report is Appendix 6 of this study report.

## *4.2 Stock Formulation Analysis*

The Battelle stock formulation and stability analyses are presented in Appendix 5.

## *4.3 Protein Analysis*

| Test chemical ID | Replicate | Assay Date | Protein stock concentration (measured) | Upper/lower [test chem.] | Stock soln ID | Stock soln exp date |
|---|---|---|---|---|---|---|
| Microsomes | 1 | 25 January 2005 | 5.066 mg/mL | 0.13–1.5 mg/mL BSA | 1132-1719-14 | 13 Jan 2006 |
| Microsomes | 2 | 26 January 2005 | 5.533 mg/mL | 0.13–1.5 mg/mL BSA | 1132-1721-14 | 13 Jan 2006 |
| Microsomes | 3 | 27 January 2005 | 3.703 mg/mL | 0.13–1.5 mg/mL BSA | 1132-1723-14 | 13 Jan 2006 |

## 4.4 Aromatase Activity

| Test Chemical | Replicate | Technician | FEAC Beginning (nmol/mg/min) | FEAC End (nmol/mg/min) | Standard Deviation | Overall Mean (±sd) |
|---|---|---|---|---|---|---|
| 4-OH ASDN | 1 | TM | 0.1824 | 0.1725 | 0.0199, 0.0028 | 0.1774 (0.0129) |
| | 2 | TM | 0.1891 | 0.1699 | 0.0129, 0.00028 | 0.1795 (0.0133) |
| | 3 | TM | 0.3081 | 0.2737 | 0.0038, 0.0085 | 0.2909 (0.0206) |

## 4.5 Percent of Control

| Test chemical | Replicate | Log[test chemical] | Percent of Control | | | Mean |
|---|---|---|---|---|---|---|
| | | | Tube 1 | Tube 2 | Tube 3 | |
| 4-OH ASDN | 1 | –6.00 | 10.01 | 9.64 | 10.28 | 9.98 |
| | | –7.00 | 49.15 | 43.37 | 43.00 | 45.17 |
| | | –7.30 | 62.41 | 58.89 | 60.76 | 60.69 |
| | | –7.60 | 72.78 | 73.45 | 71.33 | 72.52 |
| | | –8.00 | 87.54 | 85.00 | 79.86 | 84.13 |
| | | –9.00 | 99.32 | 89.32 | 96.12 | 94.92 |
| | 2 | –6.00 | 9.29 | 9.56 | 7.93 | 8.93 |
| | | –7.00 | 47.83 | 45.80 | 43.98 | 45.87 |
| | | –7.30 | 58.90 | 56.17 | 55.44 | 56.84 |
| | | –7.60 | 74.03 | 71.86 | 71.14 | 72.34 |
| | | –8.00 | 84.36 | 82.04 | 78.26 | 81.55 |
| | | –9.00 | 93.35 | 87.18 | 90.58 | 90.37 |
| | 3 | –6.00 | 10.01 | 9.07 | 8.38 | 9.15 |
| | | –7.00 | 46.65 | 43.55 | 43.76 | 44.65 |
| | | –7.30 | 63.64 | 62.52 | 60.09 | 62.08 |
| | | –7.60 | 76.92 | 72.60 | 74.16 | 74.56 |
| | | –8.00 | 84.67 | 83.95 | 92.36 | 86.99 |
| | | –9.00 | 94.33 | 94.38 | 97.13 | 95.28 |

## 4.6 $IC_{50}$

| Test chemical | Replicate | Log[$IC_{50}$] | SE log[$IC_{50}$] | $IC_{50}$ (M) | Slope | SE slope | Status | Overall $IC_{50}$ (±sd, sem, %CV) |
|---|---|---|---|---|---|---|---|---|
| 4-OH ASDN | 1 | –7.055 | 0.05859 | $8.809 \times 10^{-8}$ | -0.8874 | 0.1088 | C | $8.928 \times 10^{-8}$ ($6.551 \times 10^{-9}$, $3.782 \times 10^{-9}$, 7.34%) |
| | 2 | –7.016 | 0.05242 | $9.634 \times 10^{-8}$ | -0.9125 | 0.09871 | C | |
| | 3 | –7.079 | 0.03434 | $8.340 \times 10^{-8}$ | -1.033 | 0.08923 | C | |

See Appendix 4 for graphical representations of the data.

## 4.7 Statistical Analysis

Files were sent to Battelle for analysis. The Statistical report from Battelle is included in this report as Appendix 7.

# 5.0 Discussion and Conclusions

The study goal was to validate a recombinant aromatase assay run with different concentrations of a known inhibitor (4-OH ASDN). Three replicates of the aromatase assay validation were run. Each of these replicates contained full enzyme activity control tubes and background activity control tubes. Half of these controls were run at the beginning of the run, and the other half at the end. In addition, each replicate contained the aromatase inhibitor 4-OH ASDN at six different concentrations.

After assay results were obtained, data was incorporated into spreadsheets provided by Battelle, and Battelle carried out the statistical analysis. The full statistical analysis can be found in Appendix 7 of this report.

A summary of the results, as described in the statistical analysis in Appendix 7, is included here:

1. The concentration response curves were similar across the three replicates.
2. Replicate 2 had a slightly higher estimated $IC_{50}$ than replicates 1 and 3.
3. For the background activity controls, the measurements at the end were less than those at the beginning, in replicates 1 and 2. For the full enzyme activity controls the average percent of control response at the end were lower than at the beginning for all three replicates.
4. For the background activity controls averaged across replicates there were no significant differences between the beginning and the end portions. For the full enzyme activity controls averaged across replicates there was a difference between the beginning and the end portions. The end was lower than the beginning. The variation of portion (end vs. beginning) effects among replicates was estimated to be zero.

# 6.0 References

*Endocrine Disruptor Screening Program Quality Management Plan, Version 2*; Battelle; May 12, 2003.

*Technical Work Plan on Recombinant Aromatase Validation Study*; EPA Contract Number 68-W-01-023, Work Assignment 4-17; Battelle, September 8, 2004.

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

# Appendix 1: Copy of In Vitro Technologies Protocol No. 1132

**In Vitro Technologies, Inc.**
**Protocol No. 1132**
**Version: Final (24 Jan 2005)**

# WA 4-17 Recombinant Aromatase Validation Study–Task 3

| | |
|---|---|
| **Sponsor:** | Battelle Memorial Institute<br>505 King Avenue<br>Columbus, OH 43201-2639 |
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| **Study Director:** | Neil S. Jensen, Ph.D. |
| **EPA Contract Number:** | 68-W-01-023 (Battelle Prime Contractor) |
| **IVT Study Number:** | 270-1132-06 |
| **Experimental Start Date:** | 25 January 2005 |
| **Experimental End Date:** | 28 January 2005 |

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# Objective

The objective of this study is to validate the recombinant aromatase assay with a known inhibitor. This study is part of a multi-laboratory effort for the validation of the recombinant aromatase assay. This protocol is specific to the study to be conducted at In Vitro Technologies, Inc.

# Test Article Identification

- 4-hydroxyandrostenedione (4-OH ASDN, molecular weight 302.4 g/mol; CAS no.: 566-48-3)

Battelle will provide 4-OH ASDN as a stock solution in ethanol. Battelle will be responsible for the preparation, stability, and analysis of the 4-OH ASDN stock.

# Test System Identification

The test system for this study is human recombinant microsomes provided by Battelle.

The route of administration is not applicable since the test system is microsomes. The treatment method will be to mix the microsomes, reagents, and test article in a common reaction vessel so that, microsomal metabolism of androstenedione to estrogen can be used to evaluate enzymatic activity. Each test tube will have a unique label.

# Test System Justification

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 recombinant enzyme enhances its availability.

# Description of Study

In Vitro Technologies will conduct three separate experiments to evaluate the inhibition of recombinant aromatase by 4-OH ASDN.

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# Experimental Methods

## Materials

Battelle will provide the following materials:

- Recombinant microsomes
- Androstenedione (ASDN)
- 4-OH ASDN
- [1B-$^{3}$H] androstenedione ([$^{3}$H] ASDN, 25.3 Ci/mmol, 1 mCi/ml)
- B-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH, Sigma, catalog no. 1630, molecular weight. 833.4 g/mol)
- Ethanol

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

- 0.1 M Phosphate buffer (pH 7.4)
- Propylene glycol (JT Baker, catalog no. 4011-01, molecular weight 137.99 g/mol)
- Liquid scintillation cocktail (Ultima Gold, Packard)
- DC Protein Assay kit (Bio-Rad)

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

## Assays

### 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 six-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 standard or unknown, 125 µL of Bio-Rad DC Protein Kit Reagent A will be added and mixed. Bio-Rad DC Protein Kit Reagent B (1 mL) will be added to each standard or unknown and the samples will be mixed. The samples will be placed at room temperature for at least 15 minutes to allow for color development. The absorbances are stable for approximately 1 hour. Each sample (standards and unknowns) 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 standard curve developed using the protein standards.

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**Aromatase Assay**

The assays will be performed in 13 × 100 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 5 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 2.0 mL of methylene chloride; the tubes will be vortex-mixed for approximately 5 seconds and placed on ice. The tubes will be vortex-mixed an additional 20 to 25 seconds. The tubes will be spun in a centrifuge for 10 minutes at a setting of 1,000 rpm. The methylene chloride layer will be removed and discarded; the aqueous layers will be extracted again with 2 mL of methylene chloride. This extraction procedure will be repeated once more, 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 Recombinant |
| Microsomal Protein (mg/mL)[a] | 0.004 |
| NADPH (mM)[a] | 0.3 |
| [$^3$H]ASDN (nM)[a] | 100 |
| Incubation Time (min) | 15 |

[a] Final concentrations

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

Results will be presented as the activity (velocity) of the enzyme reaction. The amount of estrogen product formed will 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). The activity of the enzyme reaction will be expressed in nmol (mg protein)$^{-1}$min$^{-1}$ and will be calculated by dividing the amount of estrogen formed by the product of mg microsomal protein used times the incubation time (e.g., 15 minutes).

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**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. Each concentration of 4-OH ASDN will be run in triplicate tubes in each study. See Table 2 below 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 will contain all full enzyme activity control assay components except NADPH, and will serve as assay blanks. Four full enzyme activity control samples and four background activity samples will be included with each study and will be treated the same as the other samples. The control sets will be split so that two tubes (of each full enzyme activity control and background activity samples) will be run at the beginning and two at the end of each study set.

The assay will be conducted as described in the Aromatase Assay section above, 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 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 | $1 \times 10^{-4}$ | $1 \times 10^{-6}$ |
| 4-OH ASDN Concentration 2 | 3 | 4-OH ASDN added | $1 \times 10^{-5}$ | $1 \times 10^{-7}$ |
| 4-OH ASDN Concentration 3 | 3 | 4-OH ASDN added | $5 \times 10^{-6}$ | $5 \times 10^{-8}$ |
| 4-OH ASDN Concentration 4 | 3 | 4-OH ASDN added | $2.5 \times 10^{-6}$ | $2.5 \times 10^{-8}$ |

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| | | | | |
|---|---|---|---|---|
| 4-OH ASDN Concentration 5 | 3 | 4-OH ASDN added | $1 \times 10^{-6}$ | $1 \times 10^{-8}$ |
| 4-OH ASDN Concentration 6 | 3 | 4-OH ASDN added | $1 \times 10^{-7}$ | $1 \times 10^{-9}$ |

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

## Description of Data Calculations

In Vitro Technologies will supply all raw data to Battelle in electronic format using Excel spreadsheets and Prism templates (to be developed and provided by Battelle).

### 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 average of the DPMs for the background tubes should be subtracted from the tubes with Total DPM to provide DPM 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 DPM 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) will yield 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 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 (FEAC) 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 FEAC activity and multiplying by 100.

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

$$Y = 100/(1+10^{((LogIC_{50}-X)*HillSlope)})$$

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

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 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 $IC_{50}$ by date, run, technician, assay method

## Statistical Analysis

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.

The statistical analysis described in this section will be carried out by Battelle. The resulting data will be sent to In Vitro Technologies and will be included in the final report.

### Concentration Response Fits for the 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 percent activity values will be compared across daily replicate tests for each test substance.

For each replicate, two repeat tubes of the FEAC 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 FEAC 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).

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For each repetition at each level, the Excel database spreadsheet will include total DPM 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 DPM, 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 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
DAVG = average DPM across the repeat tubes with the same inhibitor concentration
$\beta$ = slope of the concentration response curve ($\beta$ will be negative)
$\mu = \log_{10}IC_{50}$ ($IC_{50}$ is the concentration corresponding to percent of control activity equal to 50%).

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

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

where $\varepsilon$ 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%.

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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 $IC_{50}$ ($10^{\mu}$) and slope ($\beta$). The estimated $IC_{50}$ for an inhibitor compound will be a (weighted) geometric mean across the replicates. The estimated overall standard error will be based on the standard errors within each replicate and the replicate-to-replicate variability. The average value and standard error of $\log_{10}IC_{50}$ or $\beta$ can be calculated based on a one-way random effects analysis of variance model fit.

For each test substance and replicate the estimated $\log_{10}IC_{50}$ ($\mu$), the within replicate standard error of $\mu$, the $IC_{50}$, the slope ($\beta$), the within replicate standard error of $\beta$, 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".

**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.

For each replicate treat ($\beta$, $\mu$) as a random variable with mean ($\beta_{avg}$, $\mu_{avg}$). 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 ($\beta$) and $\log_{10}IC_{50}$ ($\mu$) will also be compared across replicates based on random effects analysis of variance, treating the replicates as random effects. $\beta$ and $\mu$ are estimated,

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separately within each replicate, and plotted along with the average across replicates and associated 95% confidence interval across replicates (including replicate-to-replicate variation).

**Background and Full Enzyme Activity Control Values Across Replicates**

Within each replicate, quadruplicate repetitions will be made of the background activity tubes and the FEAC 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, then 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 FEAC activity. The average of the four background activity samples within a replicate must necessarily be 0 and the average of the four FEAC 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% (FEAC) 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 FEAC tubes and the background activity tubes. The factors in the analysis of variance will be replicate, portion (beginning or end), and 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 insignificant. Note that the replicate effects will necessarily be zero 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 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.

## Reporting of Ambiguities

Ambiguities or unclear directions in the written protocol and a list of all problems which are encountered will be reported to Battelle.

## Criteria for Data Acceptance

The purpose of this study is to develop criteria for data acceptance.

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## Study Report

At completion of Task 3, tabular and graphical summaries of data will be prepared using the Excel spreadsheet and Prism document templates provided by Battelle. These electronic files will be submitted to Battelle within 7 days after completion of the task, 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.

## Data Retention

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

Study records to be maintained will include:

- All records that document the conduct of the laboratory experiments and results obtained, as well as the equipment and chemicals used.
- Protocol and any amendments
- List of any protocol deviations
- List of standard operating procedures
- Quality Assurance Project Plan (QAPP) and any amendments
- List of any QAPP deviations

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# Protocol Approval

This protocol has been reviewed and approved by the following:

## Sponsor Representatives

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

Signature

Date 1/24/05

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

Signature

Date 1-24-05

## Study Director

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

Neil S. Jensen, Ph.D.
Study Director
In Vitro Technologies

Signature

Date 25JAN2005

## Review

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

Signature

Date 1-24-05

Sharon Isbell
Director, Quality Systems
In Vitro Technologies

Signature

Date 25 January 2005

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# Appendix 2: Copy of QAPP for Work Assignment W4-16, Task 3

# 1.0 TITLE AND APPROVAL

## Quality Assurance Project Plan (QAPP)
## For Work Assignment 4-17
## Recombinant Aromatase Validation Study

## Task 3 - Conduct Positive Control Studies in Participating Laboratories

for

## EPA CONTRACT NUMBER 68-W-01-023

December 7, 2004

## SIGNATURE PAGE

**Quality Assurance Project Plan for WA 4-17**
**Recombinant Aromatase Validation Study**
**EPA CONTRACT NUMBER 68-W-01-023**

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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 recombinant microsomes, demonstrate the utility of the microsomal assay to detect known aromatase inhibitors, and compare the performance of the 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, and (2) to test up to 10 reference chemicals with different modes of action in order to test 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 3 and 4 of the work assignment.

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

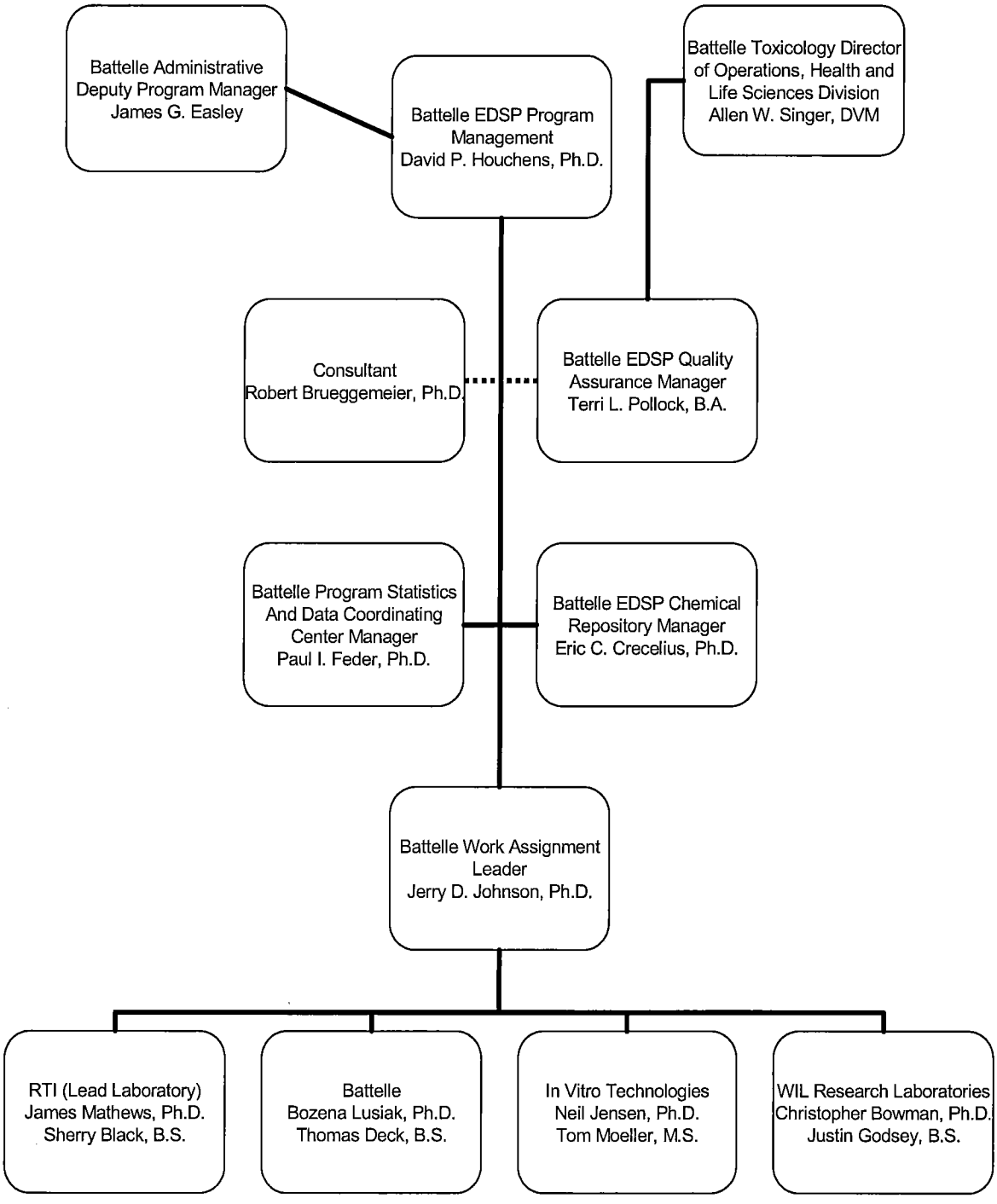


**Figure 4-1. WA 4-17 Organizational Chart**

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 WAL/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 recombinant aromatase assay (WA 2-24) were conducted to optimize the microsomal aromatase assay protocol for recombinant microsomes, demonstrate the utility of the microsomal assay to detect known aromatase inhibitors, and compare the performance of the recombinant assay system and the human placental microsomal assay system.

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 recombinant aromatase assay. A companion work assignment (WA 4-16) has been issued for the conduct of the human placental aromatase assay validation.

The work assignment is comprised of 6 tasks of which two tasks involve experimentation. Only the work in Task 3 is subject to this QAPP. Table 1 summarizes the validation tasks and the laboratory(ies) involved for each experimental task.

**Table 1. WA 4-17 Tasks**

| 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 | Conduct Positive Control Studies in the Participating Laboratories | 3 Participating Laboratories |
| 4 | Conduct Multiple Chemical Studies with Microsomes Supplied by RTI (RTI/Participating Laboratories) | Lead Laboratory + 3 Participating Laboratories |
| 5 | Prepare Study Reports (RTI/Participating Laboratories) | Not an experimental task |
| 6 | Prepare Presentation for EDMVAC | Not an experimental task |

EDMVAC = Endocrine Disruptor Method Validation Committee

## 5.2 Background

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

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

Aromatase is a cytochrome P450 enzyme complex responsible for estrogen biosynthesis and converts androgens, such as testosterone and androstenedione, into the estrogens estradiol and estrone. Aromatase is present in the ovary, placenta, uterus, testis, brain, and extraglandular adipose tissues. Two proteins, cytochrome $P450_{arom}$ 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 recombinant 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, demonstrate the utility of the microsomal assay to detect known aromatase inhibitors, and compare the performance of a recombinant assay system and the human 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 recombinant microsome aromatase assay.

## 6.0 PROJECT/TASK DESCRIPTION

Only Task 3 is under the control by this QAPP. However, this QAPP also addresses the other task in this work assignment and will be reissued prior to the start of the other task together with a finalized task-specific protocol included as an attachment. The Task 3 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 3: Conduct of the 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 recombinant 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) is tested in the aromatase assay at 6 concentrations to construct a dose/response curve from which an $IC_{50}$ may be calculated. Control runs are also 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.

**Task 4: Conduct Studies on Reference Chemicals**

Each participating laboratory will conduct the studies in this task with recombinant microsomes supplied by RTI.

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 must 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 4-17 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 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.

Staff from the participating laboratories will be trained on the performance of the aromatase assay at RTI International as part of Task 3 of WA 4-16. 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 tasks. 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 each task subject to this QAPP will be contained in GLP compliant protocols. A template protocol for Task 3 is attached as an Appendix.

## 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 WA Leader/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 be also 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 is 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 Mamager (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 Recombinant Aromatase Validation Study, EPA Contract Number 68-W-01-023, Work Assignment 4-17. 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 3

EPA Contract No.:
EPA Work Assignment No.:

TITLE: Template Protocol for WA 4-17 Task 3:
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) |
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## 1.0 OBJECTIVES

**Task 3: 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 recombinant 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 recombinant 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 a human recombinant microsome 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 recombinant microsomes will be obtained prior to initiation of the first set of experiments to ensure that sufficient quantities are available to conduct the studies.

### 2.1 Substrate

#### 2.1.1 Substrate Name/Supplier

The substrate for the aromatase assay is androstenedione (ASDN). Non-radiolabeled and radiolabeled ASDN will be used. The non-radiolabeled ASDN and the radiolabeled androstenedione ([1β-$^{3}$H]-androstenedione, [$^{3}$H]ASDN) will be provided to the laboratories by Battelle's Chemical 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.

### 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

Recombinant 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.

Human recombinant microsomes will be obtained from Gentest™ (Woburn, MA; www.gentest.com). The product name is Human CYP19 (Aromatase) Supersomes™ and the catalog number is 456260. The Supersomes™ package size is 0.5 nmoles cytochrome P450 in 0.5 mL. Supplier-provided values for protein concentration, cytochrome c reductase activity, and aromatase activity will be found on the data sheet accompanying each shipment and will be included in the report.

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 controls 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 | $1 \times 10^{-6}$ |
| 4-OH ASDN Concentration 2 | 3 | Complete assay with 4-OH ASDN added | $1 \times 10^{-7}$ |
| 4-OH ASDN Concentration 3 | 3 | Complete assay with 4-OH ASDN added | $5 \times 10^{-8}$ |
| 4-OH ASDN Concentration 4 | 3 | Complete assay with 4-OH ASDN added | $2.5 \times 10^{-8}$ |
| 4-OH ASDN Concentration 5 | 3 | Complete assay with 4-OH ASDN added | $1 \times 10^{-8}$ |
| 4-OH ASDN Concentration 6 | 3 | Complete assay with 4-OH ASDN added | $1 \times 10^{-9}$ |

[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:

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

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.

## 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
$\beta$ ≡ slope of the concentration response curve ($\beta$ 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 $\epsilon$ 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 $IC_{50}$ ($10^{\mu}$) and slope ($\beta$). 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 $\beta$ 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 ($\beta$, $\mu$) as a random variable with mean ($\beta_{avg}$, $\mu_{avg}$) 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 ($\beta$) and $\log_{10}IC_{50}$ ($\mu$) will also be compared across replicates based on random effects analysis of variance, treating the replicates as random effects. $\beta$ and $\mu$ 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 [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.

## 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 3: Excel Spreadsheets for Task 3

# Aromatase Assay Spreadsheet

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Assay Date | 1/25/2005 | Test Chemical ID | 4-OH ASDI | #Concentrations tested | 6 | | |
| Technician ID | TM | Replicate # | 1 | Microsome type | Recombina | Microsome ID Lot 5 | |

| Aliquot # | Weight of aliquot (g) | DPM/Aliq. | DPM/g soln. | |
|---|---|---|---|---|
| 1 | 0.0200 | 36726.48 | 1836324 | |
| 2 | 0.0197 | 39146.39 | 1987126 | |
| 3 | 0.0197 | 39474.38 | 2003776 | |
| 4 | 0.0195 | 41097.34 | 2107556 | |
| 5 | 0.0198 | 41162.06 | 2078892 | |
| | | | Average DPM/g soln | 2002735 |
| | | | SD | 105741 |
| | | | CV | 5.28 |
| | | | µCi/g soln | 0.902 |

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 | 12.6 | 12.6 | | 1000.00 |
| Dilution A | | | 100 | 10.00 |
| Dilution B | | | 10 | 1.00 |

Calculation of concentration nonradiolabeled ASDN in substrate solution

| | |
|---|---|
| Total g substrate solution | 8.0485 g |
| Mass of dilution B used in substrate prep | 4.5545 g |
| Concentration of nonradiolabeled ASDN in substrate soln. | 0.565882 µg/g |

Calculation of Substrate Solution Specific Activity

1) Calculate µg [$^3$H]ASDN/g soln. = 0.01021 µg/g soln.
µg/g soln.

| | |
|---|---|
| a. µCi/g soln | 0.902 |
| b. Specific activity of [$^3$H]ASDN (µCi/mmol) | 25300000 |
| c. Molecular wt of ASDN (mg/mmol) | 286.4 |

Formula=a/bc

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [$^3$H]ASDN/g soln.

= 0.565882 + 0.01021
= 0.576094 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.566 µCi/µg ASDN

995642 dpm/nmol

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Assay Date | 1/25/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | | | | | | |
| Technician ID | TM | Replicate # | 1 | Microsome type | Recombinant | Microsome ID | Lot 5 | | | | |

| | | | | | | | | Protein stock (mg BSA) | Total volume of stock (mL) | Protein stock ID |
|---|---|---|---|---|---|---|---|---|---|---|
| Standards: | 1.5 | 1 | 0.75 | 0.5 | 0.25 | 0.13 | Blank | 26 | 10 | |
| | 0.470 | 0.359 | 0.302 | 0.207 | 0.121 | 0.091 | 0.027 | | | |
| | 0.482 | 0.371 | 0.294 | 0.208 | 0.116 | 0.092 | 0.030 | | | |
| | 0.480 | 0.360 | 0.291 | 0.185 | 0.113 | 0.092 | 0.030 | | | |

| | |
|---|---|
| Samples: | Rep 1 |
| | 0.203 |
| | 0.194 |
| | 0.198 |

| Standard concentration (mg/mL) | Volume of stock used | Final volume of Std | mg Protein per µL | µL Standard Used | mg Protein Measured | $A_{raw}$ | $A_{adj}$ | Curve Output |
|---|---|---|---|---|---|---|---|---|
| 1.5 | 60 | 100 | 0.00156 | 25 | 0.0390 | 0.477 | 0.448 | 0.0373 |
| 1 | 40 | 100 | 0.00104 | 25 | 0.0260 | 0.364 | 0.334 | 0.0272 |
| 0.75 | 30 | 100 | 0.00078 | 25 | 0.0195 | 0.296 | 0.267 | 0.0213 |
| 0.5 | 20 | 100 | 0.00052 | 25 | 0.0130 | 0.200 | 0.171 | 0.0129 |
| 0.25 | 10 | 100 | 0.00026 | 25 | 0.0065 | 0.117 | 0.088 | 0.0055 |
| 0.13 | 5.2 | 100 | 0.00014 | 25 | 0.0034 | 0.091 | 0.062 | 0.0032 |

| | | | | | |
|---|---|---|---|---|---|
| Variables | | Regression results | | | |
| m, b | 0.088 | -0.002 | | | |
| $se_m$, $se_b$ | 0.004 | 0.001 | | | |
| $r^2$, $se_y$ | 0.990 | 0.001 | | | |
| F, df | 400 | 4 | | | |
| $ss_{reg}$, $ss_{resid}$ | 0.001 | 0.000 | | | |

Regression results are calculated using the function LINEST

| | | | | |
|---|---|---|---|---|
| Blank | 0.029 | $r^2$= | 0.990 | |
| | | m= | 0.088 | |
| | | 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 |
|---|---|---|---|---|---|---|---|---|---|
| Rep 1 | 0.203 | 0.174 | 0.013 | 25 | 100 | 1000 | 0.005 | 0.005 | 5.066 |
| Rep 1 | 0.194 | 0.165 | 0.012 | 25 | 100 | 1000 | 0.005 | | |
| Rep 1 | 0.198 | 0.169 | 0.013 | 25 | 100 | 1000 | 0.005 | | |

| Assay Date | 1/25/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | Microsome type | Recombina | Microsome ID | Lot 5 | Technician ID | TM | Replicate # | 1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Microsome Dilution Details | | |
|---|---|---|
| Dilution A | 0.1 | mL microsome Stock used |
| | 1 | mL total volume |
| | 10 | dilution factor |
| Dilution B | 0.5 | mL microsome Dilution A used |
| | 30 | mL total volume |
| | 60 | dilution factor |
| Dilution C (if applicable) | | mL microsome Dilution B used |
| | | mL total volume |
| | NA | dilution factor |
| | 600 | total dilution factor |

| Protein Concentration (stock microsomes, mg/mL): | 5.066 |
|---|---|
| Protein Concentration (dilution added to assay, mg/mL): | 0.008443 |

| 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 | 1/25/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | | 6 Microsome type | Recombinan | Microsome ID | Lot 5 | Technician ID | TM | Replicate # | 1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Sample ID | | Calculate DPM in aqueous portion after extraction | | | | | | | Calculate % turnover | | | Calculate nmol $^3H_2O$ formed | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample type | Replicate/Level | Nominal total volume (mL) | Aliq Volume (mL) | Aliq. # | DPM/aliq | DPM/mL | Ave DPM/mL | Total DPM | Volume of substrate solution used/assay tube (mL) | total DPM in assay tube (initial) | % conversion to product | Total DPM corrected for background (Background Tubes) | nmol $^3H_2O$ formed | Volume diluted microsomes used in assay tube (mL) | Final [protein] in assay (mg/mL) | Incubation time (min) | Aromatase activity (nmo estrogen formed/mg protein/min |
| Full activity control | 1 | 2 | 0.5 | 1 | 6629.104 | 13258.208 | 12610.354 | 25220.708 | 0.1 | 200273 | 12.59 | 24770 | 0.0249 | 1 | 0.004 | 15 | 0.1964 |
| | | | 0.5 | 2 | 5981.25 | 11962.5 | | | 0.1 | | | | | | | | |
| | 2 | 2 | 0.5 | 1 | 5432.922 | 10865.844 | 10835.52 | 21671.04 | 0.1 | 200273 | 10.82 | 21220 | 0.0213 | 1 | 0.004 | 15 | 0.1683 |
| | | | 0.5 | 2 | 5402.598 | 10805.196 | | | 0.1 | | | | | | | | |
| | 3 | 2 | 0.5 | 1 | 5608.824 | 11217.648 | 11226.816 | 22453.632 | 0.1 | 200273 | 11.21 | 22003 | 0.0221 | 1 | 0.004 | 15 | 0.1745 |
| | | | 0.5 | 2 | 5617.992 | 11235.984 | | | 0.1 | | | | | | | | |
| | 4 | 2 | 0.5 | 1 | 5465.008 | 10930.016 | 10976.72 | 21953.44 | 0.1 | 200273 | 10.96 | 21503 | 0.0216 | 1 | 0.004 | 15 | 0.1705 |
| | | | 0.5 | 2 | 5511.712 | 11023.424 | | | 0.1 | | | | | | | | |
| Background control | 1 | 2 | 0.5 | 1 | 131.9847 | 263.9694 | 261.2759 | 522.5518 | 0.1 | 200273 | 0.26 | 72 | 0.0001 | 1 | 0.004 | 15 | 0.0006 |
| | | | 0.5 | 2 | 129.2912 | 258.5824 | | | 0.1 | | | | | | | | |
| | 2 | 2 | 0.5 | 1 | 112.0865 | 224.173 | 212.9687 | 425.9374 | 0.1 | 200273 | 0.21 | -25 | 0.0000 | 1 | 0.004 | 15 | -0.0002 |
| | | | 0.5 | 2 | 100.8822 | 201.7644 | | | 0.1 | | | | | | | | |
| | 3 | 2 | 0.5 | 1 | 134.9695 | 269.939 | 229.94234 | 459.88468 | 0.1 | 200273 | 0.23 | 9 | 0.0000 | 1 | 0.004 | 15 | 0.0001 |
| | | | 0.5 | 2 | 94.97284 | 189.94568 | | | 0.1 | | | | | | | | |
| | 4 | 2 | 0.5 | 1 | 104.6702 | 209.3404 | 196.92953 | 393.85906 | 0.1 | 200273 | 0.20 | -57 | -0.0001 | 1 | 0.004 | 15 | -0.0004 |
| | | | 0.5 | 2 | 92.25933 | 184.51866 | | | 0.1 | | | | | | | | |
| Positive control | 1 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 2 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 3 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 4 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| Negative Control | 1 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 2 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 3 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 4 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| 4-OH ASDN | 1-1 | 2 | 0.5 | 1 | 641.7615 | 1283.523 | 1344.5642 | 2689.1284 | 0.1 | 200273 | 1.34 | 2239 | 0.0022 | 1 | 0.004 | 15 | 0.0178 |
| | | | 0.5 | 2 | 702.8027 | 1405.6054 | | | 0.1 | | | | | | | | |
| | 1-2 | 2 | 0.5 | 1 | 609.6443 | 1219.2886 | 1304.0373 | 2608.0746 | 0.1 | 200273 | 1.30 | 2158 | 0.0022 | 1 | 0.004 | 15 | 0.0171 |
| | | | 0.5 | 2 | 694.393 | 1388.786 | | | 0.1 | | | | | | | | |
| | 1-3 | 2 | 0.5 | 1 | 686.5216 | 1373.0432 | 1375.7567 | 2751.5134 | 0.1 | 200273 | 1.37 | 2301 | 0.0023 | 1 | 0.004 | 15 | 0.0182 |
| | | | 0.5 | 2 | 689.2351 | 1378.4702 | | | 0.1 | | | | | | | | |
| | 2-1 | 2 | 0.5 | 1 | 2900.482 | 5800.964 | 5723.354 | 11446.708 | 0.1 | 200273 | 5.72 | 10996 | 0.0110 | 1 | 0.004 | 15 | 0.0872 |
| | | | 0.5 | 2 | 2822.872 | 5645.744 | | | 0.1 | | | | | | | | |
| | 2-2 | 2 | 0.5 | 1 | 2518.15 | 5036.3 | 5077.051 | 10154.102 | 0.1 | 200273 | 5.07 | 9704 | 0.0097 | 1 | 0.004 | 15 | 0.0770 |
| | | | 0.5 | 2 | 2558.901 | 5117.802 | | | 0.1 | | | | | | | | |
| | 2-3 | 2 | 0.5 | 1 | 2561.79 | 5123.58 | 5035.907 | 10071.814 | 0.1 | 200273 | 5.03 | 9621 | 0.0097 | 1 | 0.004 | 15 | 0.0763 |
| | | | 0.5 | 2 | 2474.117 | 4948.234 | | | 0.1 | | | | | | | | |
| | 3-1 | 2 | 0.5 | 1 | 3696.139 | 7392.278 | 7207.439 | 14414.878 | 0.1 | 200273 | 7.20 | 13964 | 0.0140 | 1 | 0.004 | 15 | 0.1107 |
| | | | 0.5 | 2 | 3511.3 | 7022.6 | | | 0.1 | | | | | | | | |
| | 3-2 | 2 | 0.5 | 1 | 3374.254 | 6748.508 | 6813.413 | 13626.826 | 0.1 | 200273 | 6.80 | 13176 | 0.0132 | 1 | 0.004 | 15 | 0.1045 |
| | | | 0.5 | 2 | 3439.159 | 6878.318 | | | 0.1 | | | | | | | | |
| | 3-3 | 2 | 0.5 | 1 | 3492.305 | 6984.61 | 7022.6 | 14045.2 | 0.1 | 200273 | 7.01 | 13595 | 0.0137 | 1 | 0.004 | 15 | 0.1078 |
| | | | 0.5 | 2 | 3530.295 | 7060.59 | | | 0.1 | | | | | | | | |
| | 4-1 | 2 | 0.5 | 1 | 4205.112 | 8412.224 | 8367.002 | 16734.004 | 0.1 | 200273 | 8.36 | 16283 | 0.0164 | 1 | 0.004 | 15 | 0.1291 |
| | | | 0.5 | 2 | 4160.89 | 8321.78 | | | 0.1 | | | | | | | | |
| | 4-2 | 2 | 0.5 | 1 | 4219.53 | 8439.06 | 8441.774 | 16883.548 | 0.1 | 200273 | 8.43 | 16433 | 0.0165 | 1 | 0.004 | 15 | 0.1303 |
| | | | 0.5 | 2 | 4222.244 | 8444.488 | | | 0.1 | | | | | | | | |
| | 4-3 | 2 | 0.5 | 1 | 4032.579 | 8065.158 | 8204.934 | 16409.868 | 0.1 | 200273 | 8.19 | 15959 | 0.0160 | 1 | 0.004 | 15 | 0.1266 |
| | | | 0.5 | 2 | 4172.355 | 8344.71 | | | 0.1 | | | | | | | | |
| | 5-1 | 2 | 0.5 | 1 | 4983.123 | 9966.246 | 10018.337 | 20036.674 | 0.1 | 200273 | 10.00 | 19586 | 0.0197 | 1 | 0.004 | 15 | 0.1553 |
| | | | 0.5 | 2 | 5035.214 | 10070.428 | | | 0.1 | | | | | | | | |
| | 5-2 | 2 | 0.5 | 1 | 5026.221 | 10052.442 | 9734.599 | 19469.198 | 0.1 | 200273 | 9.72 | 19019 | 0.0191 | 1 | 0.004 | 15 | 0.1508 |
| | | | 0.5 | 2 | 4708.378 | 9416.756 | | | 0.1 | | | | | | | | |
| | 5-3 | 2 | 0.5 | 1 | 4579.056 | 9158.172 | 9159.515 | 18319.03 | 0.1 | 200273 | 9.15 | 17868 | 0.0179 | 1 | 0.004 | 15 | 0.1417 |
| | | | 0.5 | 2 | 4580.429 | 9160.858 | | | 0.1 | | | | | | | | |
| | 6-1 | 2 | 0.5 | 1 | 5618.022 | 11236.044 | 11336.35 | 22672.7 | 0.1 | 200273 | 11.32 | 22222 | 0.0223 | 1 | 0.004 | 15 | 0.1762 |
| | | | 0.5 | 2 | 5718.328 | 11436.656 | | | 0.1 | | | | | | | | |
| | 6-2 | 2 | 0.5 | 1 | 5218.883 | 10437.766 | 10217.221 | 20434.442 | 0.1 | 200273 | 10.20 | 19984 | 0.0201 | 1 | 0.004 | 15 | 0.1585 |
| | | | 0.5 | 2 | 4998.338 | 9996.676 | | | 0.1 | | | | | | | | |
| | 6-3 | 2 | 0.5 | 1 | 5570.354 | 11140.708 | 10978.437 | 21956.874 | 0.1 | 200273 | 10.96 | 21506 | 0.0216 | 1 | 0.004 | 15 | 0.1706 |
| | | | 0.5 | 2 | 5408.083 | 10816.166 | | | 0.1 | | | | | | | | |

| Assay Date | 1/25/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | Microsome 6 type | Recombinant | Microsome ID Lot 5 | Technician ID TM | Replicate # | 1 |
|---|---|---|---|---|---|---|---|---|---|---|

| Control Type | Portion | Average | SD |
|---|---|---|---|
| Full activity | Beginning | 0.1824 | 0.0199 |
| Full activity | End | 0.1725 | 0.0028 |
| Full activity | Overall | 0.1774 | 0.0129 |
| Background | Beginning | 0.0002 | 0.000541775 |
| Background | End | -0.0002 | 0.000370245 |
| Background | Overall | 0.0000 | 0.000436554 |
| 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 | 0.0178 |
| 4-OH ASDN | 1 | 2 | 1.00E-06 | -6.00 | 0.0171 |
| 4-OH ASDN | 1 | 3 | 1.00E-06 | -6.00 | 0.0182 |
| 4-OH ASDN | 2 | 1 | 1.00E-07 | -7.00 | 0.0872 |
| 4-OH ASDN | 2 | 2 | 1.00E-07 | -7.00 | 0.0770 |
| 4-OH ASDN | 2 | 3 | 1.00E-07 | -7.00 | 0.0763 |
| 4-OH ASDN | 3 | 1 | 5.00E-08 | -7.30 | 0.1107 |
| 4-OH ASDN | 3 | 2 | 5.00E-08 | -7.30 | 0.1045 |
| 4-OH ASDN | 3 | 3 | 5.00E-08 | -7.30 | 0.1078 |
| 4-OH ASDN | 4 | 1 | 2.50E-08 | -7.60 | 0.1291 |
| 4-OH ASDN | 4 | 2 | 2.50E-08 | -7.60 | 0.1303 |
| 4-OH ASDN | 4 | 3 | 2.50E-08 | -7.60 | 0.1266 |
| 4-OH ASDN | 5 | 1 | 1.00E-08 | -8.00 | 0.1553 |
| 4-OH ASDN | 5 | 2 | 1.00E-08 | -8.00 | 0.1508 |
| 4-OH ASDN | 5 | 3 | 1.00E-08 | -8.00 | 0.1417 |
| 4-OH ASDN | 6 | 1 | 1.00E-09 | -9.00 | 0.1762 |
| 4-OH ASDN | 6 | 2 | 1.00E-09 | -9.00 | 0.1585 |
| 4-OH ASDN | 6 | 3 | 1.00E-09 | -9.00 | 0.1706 |

| Percent of control values | | | | |
|---|---|---|---|---|
| | Log[test | Replicate | | |
| Level | substance] | 1 | 2 | 3 |
| 1 | -6.00 | 10.01 | 9.64 | 10.28 |
| 2 | -7.00 | 49.15 | 43.37 | 43.00 |
| 3 | -7.30 | 62.41 | 58.89 | 60.76 |
| 4 | -7.60 | 72.78 | 73.45 | 71.33 |
| 5 | -8.00 | 87.54 | 85.00 | 79.86 |
| 6 | -9.00 | 99.32 | 89.32 | 96.12 |

# Aromatase Assay Spreadsheet

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Assay Date | 1/26/2005 | Test Chemical ID | 4-OH ASD | # Concentrations tested | 6 | | |
| Technician ID | TM | Replicate # | 2 | Microsome type | Recombina | Microsome ID Lot 5 | |

| Aliquot # | Weight of aliquot (g) | DPM/Aliq. | DPM/g soln. | |
|---|---|---|---|---|
| 1 | 0.0202 | 36849.16 | 1824216 | |
| 2 | 0.0199 | 36562.97 | 1837335 | |
| 3 | 0.0200 | 38768.66 | 1938433 | |
| 4 | 0.0201 | 39589.54 | 1969629 | |
| 5 | 0.0202 | 41676.63 | 2063200 | |
| | | | Average DPM/g soln | 1926562 |
| | | | SD | 98871 |
| | | | CV | 5.13 |
| | | | µCi/g soln | 0.868 |

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 | 13.7 | 13.7 | | 1000.00 |
| Dilution A | | | 100 | 10.00 |
| Dilution B | | | 10 | 1.00 |

Calculation of concentration nonradiolabeled ASDN in substrate solution

| | |
|---|---|
| Total g substrate solution | 8.0451 g |
| Mass of dilution B used in substrate prep | 4.5441 g |
| Concentration of nonradiolabeled ASDN in substrate soln. | 0.564828 µg/g |

Calculation of Substrate Solution Specific Activity

1) Calculate µg [$^3$H]ASDN/g soln. = 0.00982 µg/g soln.
µg/g soln.

a. µCi/g soln 0.868
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.

= 0.564828 + 0.00982
= 0.574652 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.510 µCi/µg ASDN

960177 dpm/nmol

| | | | | | |
|---|---|---|---|---|---|
| Assay Date | 1/26/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 |
| Technician ID | TM | Replicate # | 2 | Microsome type | Recombinant |

Microsome ID Lot 5

| | 1.5 | 1 | 0.75 | 0.5 | 0.25 | 0.13 | Blank | Protein stock (mg BSA) | Total volume of stock (mL) | Protein stock ID |
|---|---|---|---|---|---|---|---|---|---|---|
| Standards: | 0.415 | 0.332 | 0.262 | 0.189 | 0.120 | 0.065 | 0.030 | 26 | 10 | |
| | 0.427 | 0.331 | 0.264 | 0.178 | 0.127 | 0.067 | 0.028 | | | |
| | 0.418 | 0.335 | 0.255 | 0.178 | 0.126 | 0.069 | 0.028 | | | |

| Samples: | Rep 2 |
|---|---|
| | 0.196 |
| | 0.189 |
| | 0.185 |

| Standard concentration (mg/mL) | Volume of stock used | Final volume of Std | mg Protein per µL | µL Standard Used | mg Protein Measured | $A_{raw}$ | $A_{adj}$ | Curve Output |
|---|---|---|---|---|---|---|---|---|
| 1.5 | 60 | 100 | 0.00156 | 25 | 0.0390 | 0.420 | 0.391 | 0.0366 |
| 1 | 40 | 100 | 0.00104 | 25 | 0.0260 | 0.333 | 0.304 | 0.0280 |
| 0.75 | 30 | 100 | 0.00078 | 25 | 0.0195 | 0.260 | 0.232 | 0.0208 |
| 0.5 | 20 | 100 | 0.00052 | 25 | 0.0130 | 0.181 | 0.153 | 0.0130 |
| 0.25 | 10 | 100 | 0.00026 | 25 | 0.0065 | 0.124 | 0.096 | 0.0073 |
| 0.13 | 5.2 | 100 | 0.00014 | 25 | 0.0034 | 0.067 | 0.039 | 0.0016 |

| | | | |
|---|---|---|---|
| Blank | 0.028 | $r^2$= | 0.983 |
| | | m= | 0.099 |
| | | b= | -0.002 |

| Variables | Regression results | |
|---|---|---|
| m, b | 0.099 | -0.002 |
| $se_m$, $se_b$ | 0.007 | 0.002 |
| $r^2$, $se_y$ | 0.983 | 0.002 |
| F, df | 229 | 4 |
| $ss_{reg}$, $ss_{resid}$ | 0.001 | 0.000 |

Regression results are calculated using the function LINEST

| | $A_{raw}$ | $A_{adj.}$ | mg protein measured | µL diluted µSOMES | Vol usome prep. (µL) | Final vol. Diluted usomes (µL) | mg protein/µL Prep. | average mg/µL | mg/mL |
|---|---|---|---|---|---|---|---|---|---|
| Rep 2 | 0.196 | 0.168 | 0.014 | 25 | 100 | 1000 | 0.006 | 0.006 | 5.533 |
| Rep 2 | 0.189 | 0.160 | 0.014 | 25 | 100 | 1000 | 0.005 | | |
| Rep 2 | 0.185 | 0.156 | 0.013 | 25 | 100 | 1000 | 0.005 | | |

| Assay Date | 1/26/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | Microsome type | Recombina | Microsome ID | Lot 5 | Technician ID | TM | Replicate # | 2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Microsome Dilution Details | | |
|---|---|---|
| Dilution A | 0.1 | mL microsome Stock used |
| | 1 | mL total volume |
| | 10 | dilution factor |
| Dilution B | 0.5 | mL microsome Dilution A used |
| | 30 | mL total volume |
| | 60 | dilution factor |
| Dilution C (if applicable) | | mL microsome Dilution B used |
| | | mL total volume |
| | NA | dilution factor |
| | 600 | total dilution factor |

| Protein Concentration (stock microsomes, mg/mL): | 5.533 |
|---|---|
| Protein Concentration (dilution added to assay, mg/mL): | 0.009222 |

| 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 | 1/26/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | Microsome type | Recombinan | Microsome ID | Lot 5 | Technician ID | TM | Replicate # | 2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Sample ID | | Calculate DPM in aqueous portion after extraction | | | | | | | Calculate % turnover | | | Calculate nmol $^3H_2O$ formed | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample type | Replicate/Level | Nominal total volume (mL) | Aliq Volume (mL) | Aliq. # | DPM/aliq | DPM/mL | Ave DPM/mL | Total DPM | Volume of substrate solution used/assay tube (mL) | total DPM in assay tube (initial) | % conversion to product | Total DPM corrected for background (Background Tubes) | nmol $^3H_2O$ formed | Volume diluted microsomes used in assay tube (mL) | Final [protein] in assay (mg/mL) | Incubation time (min) | Aromatase activity (nmo estrogen formed/mg protein/min |
| Full activity control | 1 | 2 | 0.5 | 1 | 6701.592 | 13403.184 | 13384.329 | 26768.658 | 0.1 | 192656 | 13.69 | 26318 | 0.0274 | 1 | 0.005 | 15 | 0.1982 |
| | | | 0.5 | 2 | 6682.737 | 13365.474 | | | 0.1 | | | | | | | | |
| | 2 | 2 | 0.5 | 1 | 6148.353 | 12296.706 | 12176.418 | 24352.836 | 0.1 | 192656 | 12.64 | 23902 | 0.0249 | 1 | 0.005 | 15 | 0.1800 |
| | | | 0.5 | 2 | 6028.065 | 12056.13 | | | 0.1 | | | | | | | | |
| | 3 | 2 | 0.5 | 1 | 5867.385 | 11734.77 | 11523.651 | 23047.302 | 0.1 | 192656 | 11.96 | 22697 | 0.0235 | 1 | 0.005 | 15 | 0.1701 |
| | | | 0.5 | 2 | 5656.266 | 11312.532 | | | 0.1 | | | | | | | | |
| | 4 | 2 | 0.5 | 1 | 5721.877 | 11443.754 | 11495.697 | 22991.394 | 0.1 | 192656 | 11.93 | 22541 | 0.0235 | 1 | 0.005 | 15 | 0.1697 |
| | | | 0.5 | 2 | 5773.82 | 11547.64 | | | 0.1 | | | | | | | | |
| Background control | 1 | 2 | 0.5 | 1 | 123.0036 | 246.0072 | 246.0072 | 492.0144 | 0.1 | 192656 | 0.26 | 41 | 0.0000 | 1 | 0.005 | 15 | 0.0003 |
| | | | 0.5 | 2 | 123.0036 | 246.0072 | | | 0.1 | | | | | | | | |
| | 2 | 2 | 0.5 | 1 | 128.4894 | 256.9788 | 229.6406 | 459.2812 | 0.1 | 192656 | 0.24 | 9 | 0.0000 | 1 | 0.005 | 15 | 0.0001 |
| | | | 0.5 | 2 | 101.1512 | 202.3024 | | | 0.1 | | | | | | | | |
| | 3 | 2 | 0.5 | 1 | 98.41735 | 196.8347 | 206.95775 | 413.9155 | 0.1 | 192656 | 0.21 | -37 | 0.0000 | 1 | 0.005 | 15 | -0.0003 |
| | | | 0.5 | 2 | 108.5404 | 217.0808 | | | 0.1 | | | | | | | | |
| | 4 | 2 | 0.5 | 1 | 102.3555 | 204.711 | 218.9261 | 437.8522 | 0.1 | 192656 | 0.23 | -13 | 0.0000 | 1 | 0.005 | 15 | -0.0001 |
| | | | 0.5 | 2 | 116.5706 | 233.1412 | | | 0.1 | | | | | | | | |
| Positive control | 1 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 2 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 3 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 4 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| Negative Control | 1 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 2 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 3 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 4 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| 4-OH ASDN | 1-1 | 2 | 0.5 | 1 | 719.4529 | 1438.9058 | 1332.7093 | 2665.4186 | 0.1 | 192656 | 1.38 | 2215 | 0.0023 | 1 | 0.005 | 15 | 0.0167 |
| | | | 0.5 | 2 | 613.2584 | 1226.5128 | | | 0.1 | | | | | | | | |
| | 1-2 | 2 | 0.5 | 1 | 732.6667 | 1465.3334 | 1364.9178 | 2729.8356 | 0.1 | 192656 | 1.42 | 2279 | 0.0024 | 1 | 0.005 | 15 | 0.0172 |
| | | | 0.5 | 2 | 632.2511 | 1264.5022 | | | 0.1 | | | | | | | | |
| | 1-3 | 2 | 0.5 | 1 | 549.5 | 1099 | 1170.897 | 2341.794 | 0.1 | 192656 | 1.22 | 1891 | 0.0020 | 1 | 0.005 | 15 | 0.0142 |
| | | | 0.5 | 2 | 621.397 | 1242.794 | | | 0.1 | | | | | | | | |
| | 2-1 | 2 | 0.5 | 1 | 3011.423 | 6022.846 | 5926.09 | 11852.18 | 0.1 | 192656 | 6.15 | 11401 | 0.0119 | 1 | 0.005 | 15 | 0.0858 |
| | | | 0.5 | 2 | 2914.667 | 5829.334 | | | 0.1 | | | | | | | | |
| | 2-2 | 2 | 0.5 | 1 | 2849.2 | 5698.4 | 5684.833 | 11369.666 | 0.1 | 192656 | 5.90 | 10919 | 0.0114 | 1 | 0.005 | 15 | 0.0822 |
| | | | 0.5 | 2 | 2835.633 | 5671.266 | | | 0.1 | | | | | | | | |
| | 2-3 | 2 | 0.5 | 1 | 2724.378 | 5448.756 | 5467.751 | 10935.502 | 0.1 | 192656 | 5.68 | 10485 | 0.0109 | 1 | 0.005 | 15 | 0.0789 |
| | | | 0.5 | 2 | 2743.373 | 5486.746 | | | 0.1 | | | | | | | | |
| | 3-1 | 2 | 0.5 | 1 | 3585.842 | 7171.684 | 7246.442 | 14492.884 | 0.1 | 192656 | 7.52 | 14042 | 0.0146 | 1 | 0.005 | 15 | 0.1057 |
| | | | 0.5 | 2 | 3660.6 | 7321.2 | | | 0.1 | | | | | | | | |
| | 3-2 | 2 | 0.5 | 1 | 3535.722 | 7071.444 | 6920.334 | 13840.668 | 0.1 | 192656 | 7.18 | 13390 | 0.0139 | 1 | 0.005 | 15 | 0.1008 |
| | | | 0.5 | 2 | 3384.612 | 6769.224 | | | 0.1 | | | | | | | | |
| | 3-3 | 2 | 0.5 | 1 | 3393.536 | 6787.072 | 6833.238 | 13666.476 | 0.1 | 192656 | 7.09 | 13216 | 0.0136 | 1 | 0.005 | 15 | 0.0995 |
| | | | 0.5 | 2 | 3439.702 | 6879.404 | | | 0.1 | | | | | | | | |
| | 4-1 | 2 | 0.5 | 1 | 4502.619 | 9005.238 | 9048.979 | 18097.958 | 0.1 | 192656 | 9.39 | 17647 | 0.0184 | 1 | 0.005 | 15 | 0.1329 |
| | | | 0.5 | 2 | 4546.36 | 9092.72 | | | 0.1 | | | | | | | | |
| | 4-2 | 2 | 0.5 | 1 | 4311.251 | 8622.502 | 8790.675 | 17581.35 | 0.1 | 192656 | 9.13 | 17131 | 0.0176 | 1 | 0.005 | 15 | 0.1290 |
| | | | 0.5 | 2 | 4479.424 | 8958.848 | | | 0.1 | | | | | | | | |
| | 4-3 | 2 | 0.5 | 1 | 4306.363 | 8612.726 | 8705.537 | 17411.074 | 0.1 | 192656 | 9.04 | 16960 | 0.0177 | 1 | 0.005 | 15 | 0.1277 |
| | | | 0.5 | 2 | 4399.174 | 8798.348 | | | 0.1 | | | | | | | | |
| | 5-1 | 2 | 0.5 | 1 | 5273.14 | 10546.28 | 10280.505 | 20561.01 | 0.1 | 192656 | 10.67 | 20110 | 0.0209 | 1 | 0.005 | 15 | 0.1514 |
| | | | 0.5 | 2 | 5007.365 | 10014.73 | | | 0.1 | | | | | | | | |
| | 5-2 | 2 | 0.5 | 1 | 5201.304 | 10402.608 | 10004.242 | 20008.484 | 0.1 | 192656 | 10.39 | 19558 | 0.0204 | 1 | 0.005 | 15 | 0.1473 |
| | | | 0.5 | 2 | 4802.938 | 9605.876 | | | 0.1 | | | | | | | | |
| | 5-3 | 2 | 0.5 | 1 | 4837.277 | 9674.554 | 9553.736 | 19107.472 | 0.1 | 192656 | 9.92 | 18657 | 0.0194 | 1 | 0.005 | 15 | 0.1405 |
| | | | 0.5 | 2 | 4716.459 | 9432.918 | | | 0.1 | | | | | | | | |
| | 6-1 | 2 | 0.5 | 1 | 5585.216 | 11170.432 | 11352.714 | 22705.428 | 0.1 | 192656 | 11.79 | 22255 | 0.0232 | 1 | 0.005 | 15 | 0.1676 |
| | | | 0.5 | 2 | 5767.498 | 11534.996 | | | 0.1 | | | | | | | | |
| | 6-2 | 2 | 0.5 | 1 | 5410.251 | 10820.502 | 10617.504 | 21235.008 | 0.1 | 192656 | 11.02 | 20784 | 0.0216 | 1 | 0.005 | 15 | 0.1565 |
| | | | 0.5 | 2 | 5207.253 | 10414.506 | | | 0.1 | | | | | | | | |
| | 6-3 | 2 | 0.5 | 1 | 5567.66 | 11135.32 | 11022.189 | 22044.378 | 0.1 | 192656 | 11.44 | 21594 | 0.0225 | 1 | 0.005 | 15 | 0.1626 |
| | | | 0.5 | 2 | 5454.529 | 10909.058 | | | 0.1 | | | | | | | | |

| Assay Date | 1/26/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | | Microsome 6 type | Recombinant | Microsome ID Lot 5 | Technician ID TM | Replicate # | 2 |
|---|---|---|---|---|---|---|---|---|---|---|---|

| Control Type | Portion | Average | SD |
|---|---|---|---|
| Full activity | Beginning | 0.1891 | 0.0129 |
| Full activity | End | 0.1699 | 0.0003 |
| Full activity | Overall | 0.1795 | 0.0133 |
| Background | Beginning | 0.0002 | 0.00017427 |
| Background | End | -0.0002 | 0.000127438 |
| Background | Overall | 0.0000 | 0.000249664 |
| 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 | 0.0167 |
| 4-OH ASDN | 1 | 2 | 1.00E-06 | -6.00 | 0.0172 |
| 4-OH ASDN | 1 | 3 | 1.00E-06 | -6.00 | 0.0142 |
| 4-OH ASDN | 2 | 1 | 1.00E-07 | -7.00 | 0.0858 |
| 4-OH ASDN | 2 | 2 | 1.00E-07 | -7.00 | 0.0822 |
| 4-OH ASDN | 2 | 3 | 1.00E-07 | -7.00 | 0.0789 |
| 4-OH ASDN | 3 | 1 | 5.00E-08 | -7.30 | 0.1057 |
| 4-OH ASDN | 3 | 2 | 5.00E-08 | -7.30 | 0.1008 |
| 4-OH ASDN | 3 | 3 | 5.00E-08 | -7.30 | 0.0995 |
| 4-OH ASDN | 4 | 1 | 2.50E-08 | -7.60 | 0.1329 |
| 4-OH ASDN | 4 | 2 | 2.50E-08 | -7.60 | 0.1290 |
| 4-OH ASDN | 4 | 3 | 2.50E-08 | -7.60 | 0.1277 |
| 4-OH ASDN | 5 | 1 | 1.00E-08 | -8.00 | 0.1514 |
| 4-OH ASDN | 5 | 2 | 1.00E-08 | -8.00 | 0.1473 |
| 4-OH ASDN | 5 | 3 | 1.00E-08 | -8.00 | 0.1405 |
| 4-OH ASDN | 6 | 1 | 1.00E-09 | -9.00 | 0.1676 |
| 4-OH ASDN | 6 | 2 | 1.00E-09 | -9.00 | 0.1565 |
| 4-OH ASDN | 6 | 3 | 1.00E-09 | -9.00 | 0.1626 |

| Percent of control values | | | | |
|---|---|---|---|---|
| | Log[test | Replicate | | |
| Level | substance] | 1 | 2 | 3 |
| 1 | -6.00 | 9.29 | 9.56 | 7.93 |
| 2 | -7.00 | 47.83 | 45.80 | 43.98 |
| 3 | -7.30 | 58.90 | 56.17 | 55.44 |
| 4 | -7.60 | 74.03 | 71.86 | 71.14 |
| 5 | -8.00 | 84.36 | 82.04 | 78.26 |
| 6 | -9.00 | 93.35 | 87.18 | 90.58 |

# Aromatase Assay Spreadsheet

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Assay Date | 1/27/2005 | Test Chemical ID | 4-OH ASDI | #Concentrations tested | 6 | | |
| Technician ID | TM | Replicate # | 3 | Microsome type | Recombina | Microsome ID | Lot 5 |

| Aliquot # | Weight of aliquot (g) | DPM/Aliq. | DPM/g soln. | |
|---|---|---|---|---|
| 1 | 0.0200 | 35258.83 | 1762942 | |
| 2 | 0.0199 | 37266.5 | 1872688 | |
| 3 | 0.0198 | 39263.84 | 1983022 | |
| 4 | 0.0198 | 37263.97 | 1882019 | |
| 5 | 0.0201 | 37867.74 | 1883967 | |
| | | | Average DPM/g soln | 1876928 |
| | | | SD | 78010 |
| | | | CV | 4.16 |
| | | | µCi/g soln | 0.845 |

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 | 12.7 | 12.7 | | 1000.00 |
| Dilution A | | | 100 | 10.00 |
| Dilution B | | | 10 | 1.00 |

Calculation of concentration nonradiolabeled ASDN in substrate solution

| | |
|---|---|
| Total g substrate solution | 8.0695 g |
| Mass of dilution B used in substrate prep | 4.5562 g |
| Concentration of nonradiolabeled ASDN in substrate soln. | 0.56462 µg/g |

Calculation of Substrate Solution Specific Activity

1) Calculate µg [$^3$H]ASDN/g soln. = 0.00957 µg/g soln.
µg/g soln.

a. µCi/g soln 0.845
b. Specific activity of [$^3$H]ASDN (µCi/mmol) 25300000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/bc

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [$^3$H]ASDN/g soln.

= 0.564620 + 0.00957
= 0.574191 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.472 µCi/µg ASDN

936191 dpm/nmol

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Assay Date | 1/27/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | | | | |
| Technician ID | TM | Replicate # | 3 | Microsome type | Recombinant | Microsome ID | Lot 5 | | |

| | | | | | | | | Protein stock (mg BSA) | Total volume of stock (mL) | Protein stock ID |
|---|---|---|---|---|---|---|---|---|---|---|
| Standards: | 1.5 | 1 | 0.75 | 0.5 | 0.25 | 0.13 | Blank | 26 | 10 | |
| | 0.474 | 0.357 | 0.276 | 0.218 | 0.123 | 0.078 | 0.028 | | | |
| | 0.478 | 0.356 | 0.295 | 0.222 | 0.130 | 0.085 | 0.030 | | | |
| | 0.471 | 0.363 | 0.291 | 0.226 | 0.130 | 0.089 | 0.033 | | | |
| Samples: | Rep 3 | | | | | | | | | |
| | 0.162 | | | | | | | | | |
| | 0.165 | | | | | | | | | |
| | 0.164 | | | | | | | | | |

| Standard concentration (mg/mL) | Volume of stock used | Final volume of Std | mg Protein per µL | µL Standard Used | mg Protein Measured | $A_{raw}$ | $A_{adj}$ | Curve Output |
|---|---|---|---|---|---|---|---|---|
| 1.5 | 60 | 100 | 0.00156 | 25 | 0.0390 | 0.474 | 0.444 | 0.0374 |
| 1 | 40 | 100 | 0.00104 | 25 | 0.0260 | 0.359 | 0.328 | 0.0269 |
| 0.75 | 30 | 100 | 0.00078 | 25 | 0.0195 | 0.287 | 0.257 | 0.0205 |
| 0.5 | 20 | 100 | 0.00052 | 25 | 0.0130 | 0.222 | 0.192 | 0.0146 |
| 0.25 | 10 | 100 | 0.00026 | 25 | 0.0065 | 0.128 | 0.097 | 0.0060 |
| 0.13 | 5.2 | 100 | 0.00014 | 25 | 0.0034 | 0.084 | 0.054 | 0.0021 |
| | | | Blank | 0.030 | | | | |

| Variables | Regression results | |
|---|---|---|
| m, b | 0.090 | -0.003 |
| $se_m$, $se_b$ | 0.005 | 0.001 |
| $r^2$, $se_y$ | 0.990 | 0.001 |
| F, df | 398 | 4 |
| $ss_{reg}$, $ss_{resid}$ | 0.001 | 0.000 |

$r^2$= 0.990
m= 0.090
b= -0.003

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 |
|---|---|---|---|---|---|---|---|---|---|
| Rep 3 | 0.162 | 0.132 | 0.009 | 25 | 100 | 1000 | 0.004 | 0.004 | 3.703 |
| Rep 3 | 0.165 | 0.134 | 0.009 | 25 | 100 | 1000 | 0.004 | | |
| Rep 3 | 0.164 | 0.133 | 0.009 | 25 | 100 | 1000 | 0.004 | | |

| Assay Date | 1/27/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | Microsome type | Recombina | Microsome ID | Lot 5 | Technician ID | TM | Replicate # | 3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Microsome Dilution Details | | |
|---|---|---|
| Dilution A | 0.1 | mL microsome Stock used |
| | 1 | mL total volume |
| | 10 | dilution factor |
| Dilution B | 0.5 | mL microsome Dilution A used |
| | 30 | mL total volume |
| | 60 | dilution factor |
| Dilution C (if applicable) | | mL microsome Dilution B used |
| | | mL total volume |
| | NA | dilution factor |
| | 600 | total dilution factor |

| Protein Concentration (stock microsomes, mg/mL): | 3.702829 |
|---|---|
| Protein Concentration (dilution added to assay, mg/mL): | 0.006171 |

| 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 | 1/27/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | | 6 Microsome type | Recombinan | Microsome ID | Lot 5 | Technician ID | TM | Replicate # | 3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Sample ID | | Calculate DPM in aqueous portion after extraction | | | | | | | Calculate % turnover | | | Calculate nmol $^3H_2O$ formed | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample type | Replicate/Level | Nominal total volume (mL) | Aliq Volume (mL) | Aliq. # | DPM/aliq | DPM/mL | Ave DPM/mL | Total DPM | Volume of substrate solution used/assay tube (mL) | total DPM in assay tube (initial) | % conversion to product | Total DPM corrected for background (Background Tubes) | nmol $^3H_2O$ formed | Volume diluted microsomes used in assay tube (mL) | Final [protein] in assay (mg/mL) | Incubation time (min) | Aromatase activity (nmo estrogen formed/mg protein/min |
| Full activity control | 1 | 2 | 0.5 | 1 | 6699.931 | 13399.862 | 13449.998 | 26899.996 | 0.1 | 187693 | 14.33 | 26469 | 0.0283 | 1 | 0.003 | 15 | 0.3054 |
| | | | 0.5 | 2 | 6750.067 | 13500.134 | | | 0.1 | | | | | | | | |
| | 2 | 2 | 0.5 | 1 | 6924.46 | 13848.92 | 13685.741 | 27371.482 | 0.1 | 187693 | 14.58 | 26940 | 0.0288 | 1 | 0.003 | 15 | 0.3109 |
| | | | 0.5 | 2 | 6761.281 | 13522.562 | | | 0.1 | | | | | | | | |
| | 3 | 2 | 0.5 | 1 | 6216.673 | 12433.346 | 12333.87 | 24667.74 | 0.1 | 187693 | 13.14 | 24237 | 0.0259 | 1 | 0.003 | 15 | 0.2797 |
| | | | 0.5 | 2 | 6117.197 | 12234.394 | | | 0.1 | | | | | | | | |
| | 4 | 2 | 0.5 | 1 | 6046.532 | 12093.064 | 11814.411 | 23628.822 | 0.1 | 187693 | 12.59 | 23198 | 0.0248 | 1 | 0.003 | 15 | 0.2677 |
| | | | 0.5 | 2 | 5767.879 | 11535.758 | | | 0.1 | | | | | | | | |
| Background control | 1 | 2 | 0.5 | 1 | 116.1838 | 232.3676 | 227.9626 | 455.9252 | 0.1 | 187693 | 0.24 | 25 | 0.0000 | 1 | 0.003 | 15 | 0.0003 |
| | | | 0.5 | 2 | 111.7788 | 223.5576 | | | 0.1 | | | | | | | | |
| | 2 | 2 | 0.5 | 1 | 81.14394 | 162.28788 | 181.83654 | 363.67308 | 0.1 | 187693 | 0.19 | -67 | -0.0001 | 1 | 0.003 | 15 | -0.0008 |
| | | | 0.5 | 2 | 100.6926 | 201.3852 | | | 0.1 | | | | | | | | |
| | 3 | 2 | 0.5 | 1 | 116.1838 | 232.3676 | 197.71246 | 395.42492 | 0.1 | 187693 | 0.21 | -36 | 0.0000 | 1 | 0.003 | 15 | -0.0004 |
| | | | 0.5 | 2 | 81.52866 | 163.05732 | | | 0.1 | | | | | | | | |
| | 4 | 2 | 0.5 | 1 | 125.6661 | 251.3322 | 254.7592 | 509.5184 | 0.1 | 187693 | 0.27 | 78 | 0.0001 | 1 | 0.003 | 15 | 0.0009 |
| | | | 0.5 | 2 | 129.0931 | 258.1862 | | | 0.1 | | | | | | | | |
| Positive control | 1 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 2 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 3 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 4 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| Negative Control | 1 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 2 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 3 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| | 4 | 2 | | 1 | | | | | | 0 | | #VALUE! | #VALUE! | | 0.000 | | #VALUE! |
| | | | | 2 | | | | | | | | | | | | | |
| 4-OH ASDN | 1-1 | 2 | 0.5 | 1 | 751.4362 | 1502.8724 | 1476.9432 | 2953.8864 | 0.1 | 187693 | 1.57 | 2523 | 0.0027 | 1 | 0.003 | 15 | 0.0291 |
| | | | 0.5 | 2 | 725.507 | 1451.014 | | | 0.1 | | | | | | | | |
| | 1-2 | 2 | 0.5 | 1 | 636.0279 | 1272.0558 | 1359.2532 | 2718.5064 | 0.1 | 187693 | 1.45 | 2287 | 0.0024 | 1 | 0.003 | 15 | 0.0264 |
| | | | 0.5 | 2 | 723.2253 | 1446.4506 | | | 0.1 | | | | | | | | |
| | 1-3 | 2 | 0.5 | 1 | 633.4633 | 1266.9266 | 1272.0559 | 2544.1118 | 0.1 | 187693 | 1.36 | 2113 | 0.0023 | 1 | 0.003 | 15 | 0.0244 |
| | | | 0.5 | 2 | 638.5926 | 1277.1852 | | | 0.1 | | | | | | | | |
| | 2-1 | 2 | 0.5 | 1 | 3049.698 | 6099.396 | 6096.336 | 12192.672 | 0.1 | 187693 | 6.50 | 11762 | 0.0126 | 1 | 0.003 | 15 | 0.1357 |
| | | | 0.5 | 2 | 3046.638 | 6093.276 | | | 0.1 | | | | | | | | |
| | 2-2 | 2 | 0.5 | 1 | 2860.718 | 5721.436 | 5704.891 | 11409.782 | 0.1 | 187693 | 6.08 | 10979 | 0.0117 | 1 | 0.003 | 15 | 0.1267 |
| | | | 0.5 | 2 | 2844.173 | 5688.346 | | | 0.1 | | | | | | | | |
| | 2-3 | 2 | 0.5 | 1 | 3015.63 | 6031.26 | 5731.566 | 11463.136 | 0.1 | 187693 | 6.11 | 11032 | 0.0118 | 1 | 0.003 | 15 | 0.1273 |
| | | | 0.5 | 2 | 2715.938 | 5431.876 | | | 0.1 | | | | | | | | |
| | 3-1 | 2 | 0.5 | 1 | 4283.485 | 8566.97 | 8237.355 | 16474.71 | 0.1 | 187693 | 8.78 | 16044 | 0.0171 | 1 | 0.003 | 15 | 0.1851 |
| | | | 0.5 | 2 | 3953.87 | 7907.74 | | | 0.1 | | | | | | | | |
| | 3-2 | 2 | 0.5 | 1 | 4117.64 | 8235.28 | 8096.331 | 16192.662 | 0.1 | 187693 | 8.63 | 15762 | 0.0168 | 1 | 0.003 | 15 | 0.1819 |
| | | | 0.5 | 2 | 3978.691 | 7957.382 | | | 0.1 | | | | | | | | |
| | 3-3 | 2 | 0.5 | 1 | 3896.681 | 7793.362 | 7789.695 | 15579.39 | 0.1 | 187693 | 8.30 | 15148 | 0.0162 | 1 | 0.003 | 15 | 0.1748 |
| | | | 0.5 | 2 | 3893.014 | 7786.028 | | | 0.1 | | | | | | | | |
| | 4-1 | 2 | 0.5 | 1 | 5048.268 | 10096.536 | 9911.97 | 19823.94 | 0.1 | 187693 | 10.56 | 19393 | 0.0207 | 1 | 0.003 | 15 | 0.2236 |
| | | | 0.5 | 2 | 4863.702 | 9727.404 | | | 0.1 | | | | | | | | |
| | 4-2 | 2 | 0.5 | 1 | 4783.032 | 9566.064 | 9366.496 | 18732.992 | 0.1 | 187693 | 9.98 | 18302 | 0.0195 | 1 | 0.003 | 15 | 0.2112 |
| | | | 0.5 | 2 | 4583.464 | 9166.928 | | | 0.1 | | | | | | | | |
| | 4-3 | 2 | 0.5 | 1 | 4731.126 | 9462.252 | 9563.634 | 19127.268 | 0.1 | 187693 | 10.19 | 18696 | 0.0200 | 1 | 0.003 | 15 | 0.2157 |
| | | | 0.5 | 2 | 4832.508 | 9665.016 | | | 0.1 | | | | | | | | |
| | 5-1 | 2 | 0.5 | 1 | 5541.431 | 11082.862 | 10888.494 | 21776.988 | 0.1 | 187693 | 11.60 | 21346 | 0.0228 | 1 | 0.003 | 15 | 0.2463 |
| | | | 0.5 | 2 | 5347.063 | 10694.126 | | | 0.1 | | | | | | | | |
| | 5-2 | 2 | 0.5 | 1 | 5448.594 | 10897.188 | 10798.409 | 21596.818 | 0.1 | 187693 | 11.51 | 21166 | 0.0226 | 1 | 0.003 | 15 | 0.2442 |
| | | | 0.5 | 2 | 5349.815 | 10699.63 | | | 0.1 | | | | | | | | |
| | 5-3 | 2 | 0.5 | 1 | 6010.223 | 12020.446 | 11857.388 | 23714.776 | 0.1 | 187693 | 12.63 | 23284 | 0.0249 | 1 | 0.003 | 15 | 0.2687 |
| | | | 0.5 | 2 | 5847.165 | 11694.33 | | | 0.1 | | | | | | | | |
| | 6-1 | 2 | 0.5 | 1 | 6131.954 | 12263.908 | 12105.919 | 24211.838 | 0.1 | 187693 | 12.90 | 23781 | 0.0254 | 1 | 0.003 | 15 | 0.2744 |
| | | | 0.5 | 2 | 5973.965 | 11947.93 | | | 0.1 | | | | | | | | |
| | 6-2 | 2 | 0.5 | 1 | 6006.36 | 12012.72 | 12112.741 | 24225.482 | 0.1 | 187693 | 12.91 | 23794 | 0.0254 | 1 | 0.003 | 15 | 0.2746 |
| | | | 0.5 | 2 | 6106.381 | 12212.762 | | | 0.1 | | | | | | | | |
| | 6-3 | 2 | 0.5 | 1 | 6275.226 | 12550.452 | 12458.85 | 24917.7 | 0.1 | 187693 | 13.28 | 24487 | 0.0262 | 1 | 0.003 | 15 | 0.2825 |
| | | | 0.5 | 2 | 6183.624 | 12367.248 | | | 0.1 | | | | | | | | |

| Assay Date | 1/27/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | Microsome type | Recombinant | Microsome ID Lot 5 | Technician ID TM | Replicate # | 3 |
|---|---|---|---|---|---|---|---|---|---|---|---|

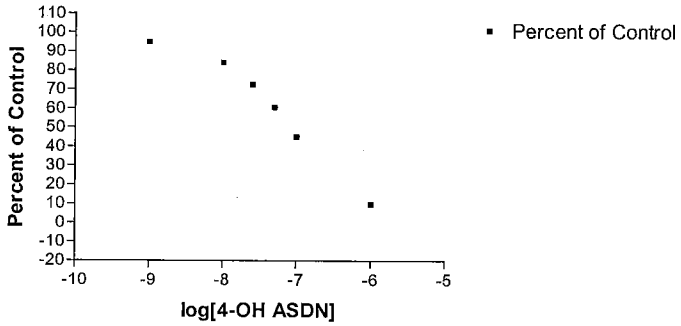
| Control Type | Portion | Average | SD |
|---|---|---|---|
| Full activity | Beginning | 0.3081 | 0.0038 |
| Full activity | End | 0.2737 | 0.0085 |
| Full activity | Overall | 0.2909 | 0.0206 |
| Background | Beginning | -0.0002 | 0.000752702 |
| Background | End | 0.0002 | 0.00093091 |
| Background | Overall | 0.0000 | 0.000747352 |
| 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 | 0.0291 |
| 4-OH ASDN | 1 | 2 | 1.00E-06 | -6.00 | 0.0264 |
| 4-OH ASDN | 1 | 3 | 1.00E-06 | -6.00 | 0.0244 |
| 4-OH ASDN | 2 | 1 | 1.00E-07 | -7.00 | 0.1357 |
| 4-OH ASDN | 2 | 2 | 1.00E-07 | -7.00 | 0.1267 |
| 4-OH ASDN | 2 | 3 | 1.00E-07 | -7.00 | 0.1273 |
| 4-OH ASDN | 3 | 1 | 5.00E-08 | -7.30 | 0.1851 |
| 4-OH ASDN | 3 | 2 | 5.00E-08 | -7.30 | 0.1819 |
| 4-OH ASDN | 3 | 3 | 5.00E-08 | -7.30 | 0.1748 |
| 4-OH ASDN | 4 | 1 | 2.50E-08 | -7.60 | 0.2238 |
| 4-OH ASDN | 4 | 2 | 2.50E-08 | -7.60 | 0.2112 |
| 4-OH ASDN | 4 | 3 | 2.50E-08 | -7.60 | 0.2157 |
| 4-OH ASDN | 5 | 1 | 1.00E-08 | -8.00 | 0.2463 |
| 4-OH ASDN | 5 | 2 | 1.00E-08 | -8.00 | 0.2442 |
| 4-OH ASDN | 5 | 3 | 1.00E-08 | -8.00 | 0.2687 |
| 4-OH ASDN | 6 | 1 | 1.00E-09 | -9.00 | 0.2744 |
| 4-OH ASDN | 6 | 2 | 1.00E-09 | -9.00 | 0.2746 |
| 4-OH ASDN | 6 | 3 | 1.00E-09 | -9.00 | 0.2825 |

| Percent of control values | | | | |
|---|---|---|---|---|
| | Log[test | Replicate | | |
| Level | substance] | 1 | 2 | 3 |
| 1 | -6.00 | 10.01 | 9.07 | 8.38 |
| 2 | -7.00 | 46.65 | 43.55 | 43.76 |
| 3 | -7.30 | 63.64 | 62.52 | 60.09 |
| 4 | -7.60 | 76.92 | 72.60 | 74.16 |
| 5 | -8.00 | 84.67 | 83.95 | 92.36 |
| 6 | -9.00 | 94.33 | 94.38 | 97.13 |

# Appendix 4: Prism Output for Task 3

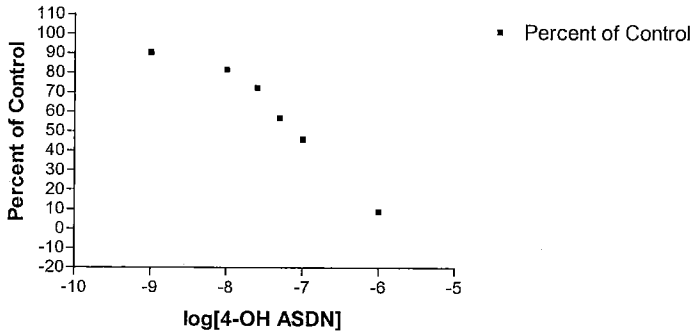
**IVT 4-17 Task 3 Replicate 1**




| log[4-OH ASDN] | Percent of Control | | |
|---|---|---|---|
| | Y1 | Y2 | Y3 |
| -6.00 | 10.01 | 9.64 | 10.28 |
| -7.00 | 49.15 | 43.37 | 43.00 |
| -7.30 | 62.41 | 58.89 | 60.76 |
| -7.60 | 72.78 | 73.45 | 71.33 |
| -8.00 | 87.54 | 85.00 | 79.86 |
| -9.00 | 99.32 | 89.32 | 96.12 |

| | Percent of Control |
|---|---|
| Sigmoidal dose-response (variable slope) | |
| Best-fit values | |
| BOTTOM | -0.09565 |
| TOP | 96.59 |
| LOGEC50 | -7.055 |
| HILLSLOPE | -0.8874 |
| EC50 | 8.809e-008 |
| Std. Error | |
| BOTTOM | 4.586 |
| TOP | 2.233 |
| LOGEC50 | 0.05859 |
| HILLSLOPE | 0.1088 |
| 95% Confidence Intervals | |
| BOTTOM | -9.934 to 9.742 |
| TOP | 91.80 to 101.4 |
| LOGEC50 | -7.181 to -6.929 |
| HILLSLOPE | -1.121 to -0.6539 |
| EC50 | 6.595e-008 to 1.176e-007 |
| Goodness of Fit | |
| Degrees of Freedom | 14 |
| $R^2$ | 0.9917 |
| Absolute Sum of Squares | 117.0 |
| Sy.x | 2.891 |
| Data | |
| Number of X values | 6 |
| Number of Y replicates | 3 |
| Total number of values | 18 |
| Number of missing values | 0 |

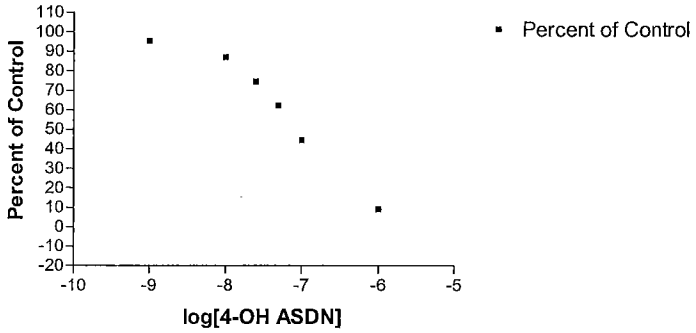
**IVT 4-17 Task 3 Replicate 2**




| log[4-OH ASDN] | Percent of Control | | |
|---|---|---|---|
| | Y | Y | Y |
| -6.0 | 9.3 | 9.6 | 7.9 |
| -7.0 | 47.8 | 45.8 | 44.0 |
| -7.3 | 58.9 | 56.2 | 55.4 |
| -7.6 | 74.0 | 71.9 | 71.1 |
| -8.0 | 84.4 | 82.0 | 78.3 |
| -9.0 | 93.3 | 87.2 | 90.6 |

| | Percent of Control |
|---|---|
| Sigmoidal dose-response (variable slope) | |
| Best-fit values | |
| BOTTOM | -0.7924 |
| TOP | 91.98 |
| LOGEC50 | -7.016 |
| HILLSLOPE | -0.9125 |
| EC50 | 9.634e-008 |
| Std. Error | |
| BOTTOM | 3.937 |
| TOP | 1.791 |
| LOGEC50 | 0.05242 |
| HILLSLOPE | 0.09871 |
| 95% Confidence Intervals | |
| BOTTOM | -9.238 to 7.653 |
| TOP | 88.13 to 95.82 |
| LOGEC50 | -7.129 to -6.904 |
| HILLSLOPE | -1.124 to -0.7008 |
| EC50 | 7.437e-008 to 1.248e-007 |
| Goodness of Fit | |
| Degrees of Freedom | 14 |
| $R^2$ | 0.9937 |
| Absolute Sum of Squares | 82.03 |
| Sy.x | 2.421 |
| Data | |
| Number of X values | 6 |
| Number of Y replicates | 3 |
| Total number of values | 18 |
| Number of missing values | 0 |

**IVT 4-17 Task 3 Replicate 3**




| log[4-OH ASDN] | Percent of Control | | |
|---|---|---|---|
| | Y1 | Y2 | Y3 |
| -6.0 | 10.0 | 9.1 | 8.4 |
| -7.0 | 46.7 | 43.5 | 43.8 |
| -7.3 | 63.6 | 62.5 | 60.1 |
| -7.6 | 76.9 | 72.6 | 74.2 |
| -8.0 | 84.7 | 83.9 | 92.4 |
| -9.0 | 94.3 | 94.4 | 97.1 |

| | Percent of Control |
|---|---|
| Sigmoidal dose-response (variable slope) | |
| Best-fit values | |
| BOTTOM | 2.428 |
| TOP | 96.24 |
| LOGEC50 | -7.079 |
| HILLSLOPE | -1.033 |
| EC50 | 8.340e-008 |
| Std. Error | |
| BOTTOM | 2.679 |
| TOP | 1.538 |
| LOGEC50 | 0.03434 |
| HILLSLOPE | 0.08923 |
| 95% Confidence Intervals | |
| BOTTOM | -3.319 to 8.174 |
| TOP | 92.94 to 99.54 |
| LOGEC50 | -7.152 to -7.005 |
| HILLSLOPE | -1.224 to -0.8413 |
| EC50 | 7.039e-008 to 9.881e-008 |
| Goodness of Fit | |
| Degrees of Freedom | 14 |
| $R^2$ | 0.9950 |
| Absolute Sum of Squares | 75.04 |
| Sy.x | 2.315 |
| Data | |
| Number of X values | 6 |
| Number of Y replicates | 3 |
| Total number of values | 18 |
| Number of missing values | 0 |

# Appendix 5: Copy of Battelle Chemistry Report

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.

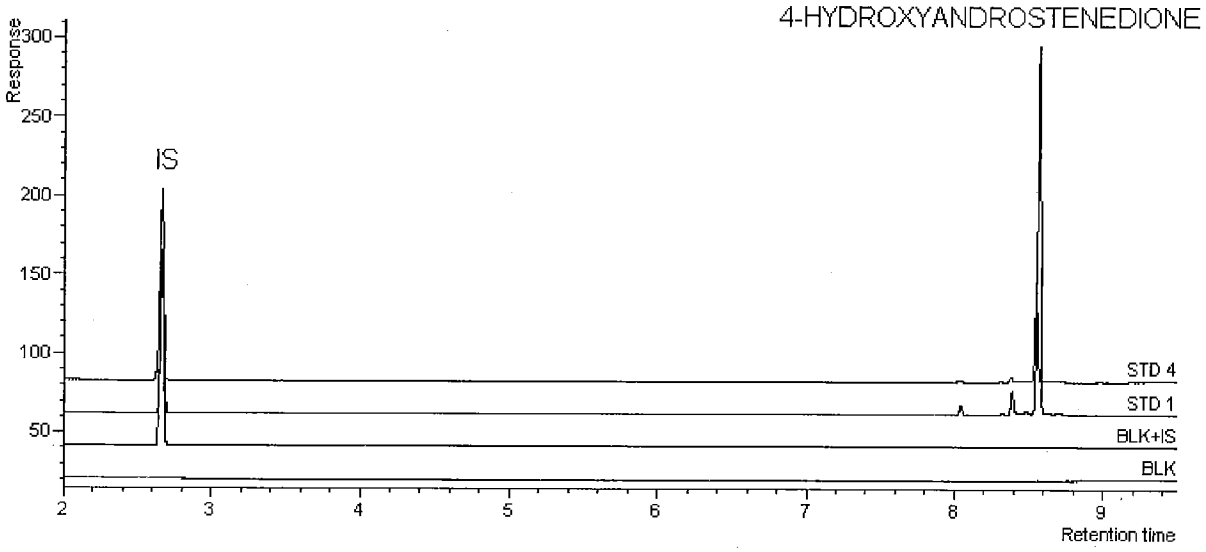



**Figure 2 – Representative Overlaid Chromatograms from a High and Low Vehicle/Calibration Standard, Blank with Internal Standard, and Blank from the Validation (Shown Top to Bottom)**

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

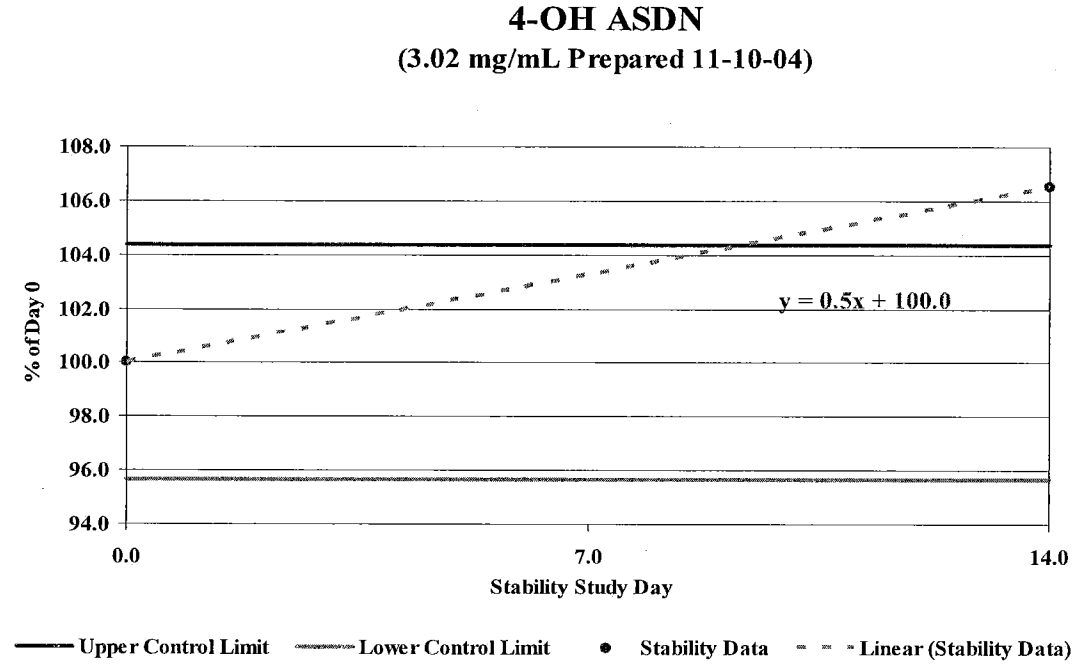
The results from the storage stability study are shown in Table 5 and presented in control chart format in Figure 3.

**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.



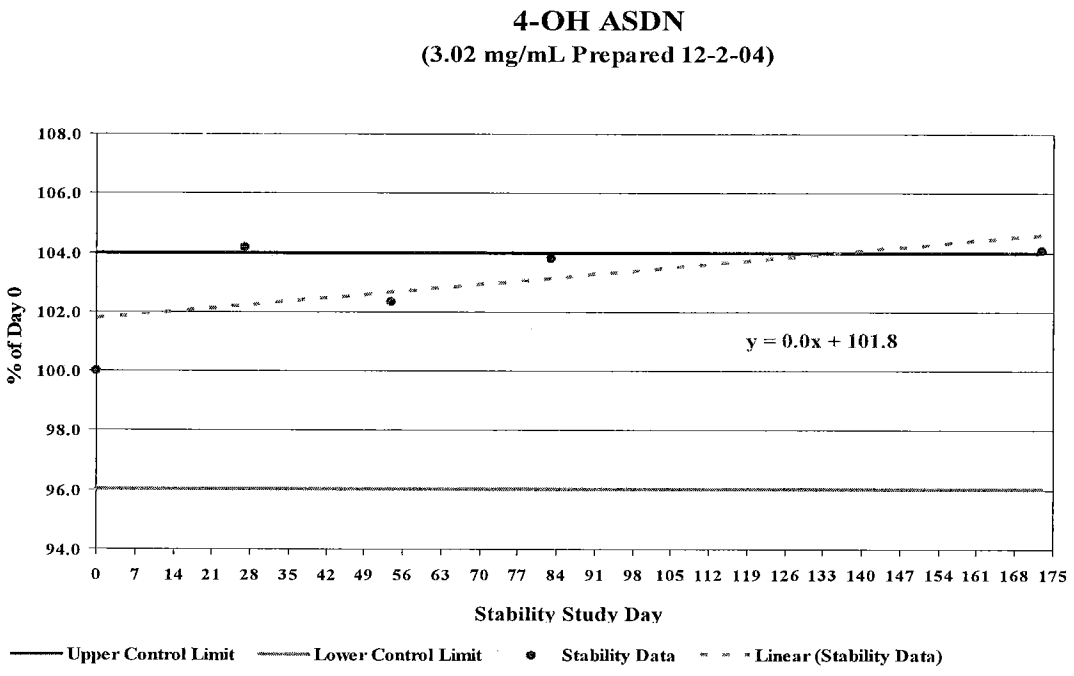




**Figure 3 – Control Charts for the Storage Stability Studies**

## 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.

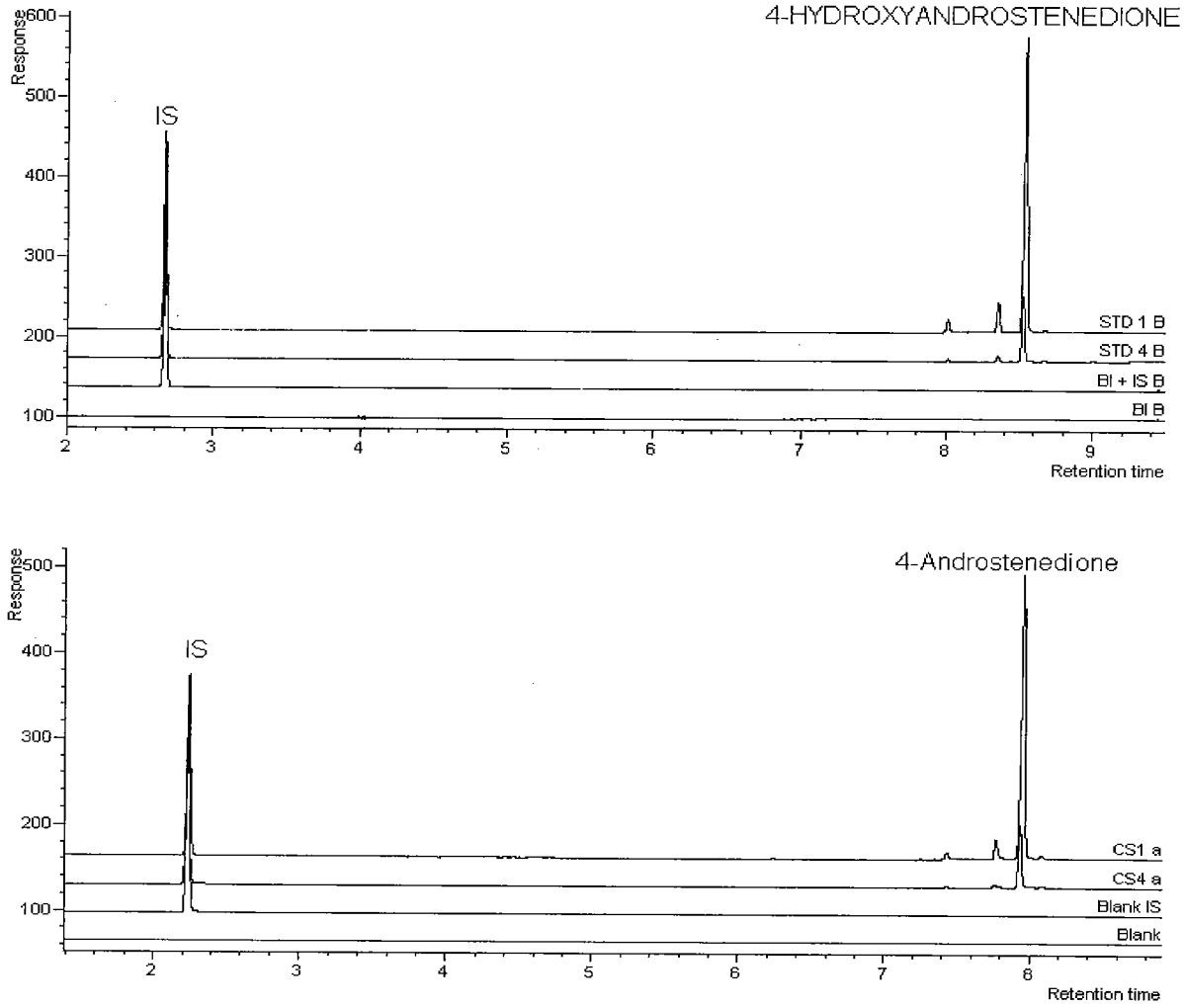



**Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from Formulation Analysis Batch 1-ASDN and Batch 2-ASDN (Shown Top to Bottom)**

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 $\leq$ 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 6: Copy of RTI [$^3$H]ASDN Purity Assessment Report

# FINAL ANALYSIS REPORT

# PLACENTAL AROMATASE VALIDATION STUDY

## [$^3$H]ASDN Radiochemical Purity Determination

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

**Sponsor:**

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

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

RTI INTERNATIONAL

FINAL REPORT

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

Author:

Sherry J. Black 9/28/05

Sherry Black Date
Research Chemist

Approved:

J. Mathews 9-28-05

James Mathews, Ph. D, DABT Date
Study Director

RTI INTERNATIONAL

# Quality Assurance Statement

| | |
|---|---|
| **Study Title:** | **[$^3$H] ASDN Radiochemical Purity Determination WA 4-16 and WA 4-17** |
| **Sponsor:** | **Battelle Memorial Institute** |
| **Study Code:** | **An05-928** |
| **Protocol Number:** | **RTI-928-AN** |

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

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

K. Collier
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).

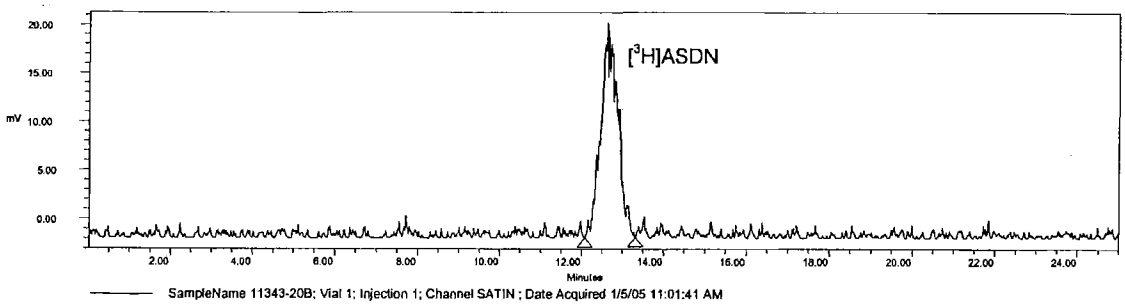
The radiochemical purity of the [$^3$H]ASDN (1:100 dilution in ethanol) was determined using high performance liquid chromatography (HPLC) and liquid scintillation counting. The HPLC system consists of a Waters 2690 Separations Module, a Waters 2487 Dual $\lambda$ Absorbance Detector and a $\beta$-RAM 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 7: Copy of Statistician's Report

DRAFT REPORT

# RECOMBINANT AROMATASE VALIDATION STUDY
# 4-OH ASDN POSITIVE CONTROL INHIBITOR STUDY

## INTRALABORATORY STATISTICAL ANALYSIS OF IN VITRO TECHNOLOGIES DATA

EPA CONTRACT NUMBER 68-W-01-023
WORK ASSIGNMENT 4-17, TASK 3

October 25, 2005

Prepared for

U.S. ENVIRONMENTAL PROTECTION AGENCY
ENDOCRINE DISRUPTOR SCREENING PROGRAM
WASHINGTON, D.C.

Prepared by

BATTELLE
505 King Avenue
Columbus, Ohio 43201

**Recombinant Aromatase Validation Study**
**4-OH ASDN Positive Control Inhibitor Study**
**Intralaboratory Statistical Analysis of In Vitro Technologies Data**

**EPA CONTRACT NUMBER 68-W-01-023**
**WORK ASSIGNMENT 4-17, TASK 3**


Ying-Liang Chou, Author _____ Date _____

Paul I. Feder, Reviewer _____ Date _____

# Offsite Quality Assurance Statement

**Study Number:**270113206

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

| Phase Inspected | Inspection Date | Date Reported to Battelle Task Leader/ Battelle Management | Date Reported to Offsite Study Director/ Management |
|---|---|---|---|
| Audit study file | 10/14/2005 | 10/14/2005 | 10/14/2005 |
| Audit draft report | 10/14/2005 | 10/14/2005 | 10/14/2005 |

**Quality Assurance Unit** **Date**

Hillary Flory 10-25-05

This report discusses the methods and results of the intralaboratory statistical analysis on the data collected at In Vitro with the recombinant aromatase assay in the 4-OH ASDN positive control inhibitor study.

## Summary and Conclusions

Statistical analyses were carried out on the percent of control responses for aromatase activity in three independent replicates. Within each replicate three repeat tubes were run at each of six graded concentrations of the positive control inhibitor 4-OH ASDN. Additionally two full enzyme activity control tubes and two background activity control tubes were run at the beginning of each replicate and two full enzyme activity controls and two background activity controls were run at the end.

Concentration response curves were fitted within each replicate to describe the relation between 4-OH ASDN concentration and extent of inhibition. The concentration response curves were summarized by the $IC_{50}$ (concentration corresponding to 50 percent inhibition) and slope. Results were compared across replicates. In addition full enzyme activity control and background activity control tube responses were compared between beginning and end of each replicate to identify differences within replicates and differences across replicates.

The following results were obtained:

1. The concentration response curves were similar across the three replicates.
2. Replicate 2 had a slightly lower estimated $IC_{50}$ than replicates 1 and 3.
3. For the background activity controls, the measurements at the end were less than those at the beginning, in replicates 1 and 2. For the full enzyme activity controls the average percent of control response at the end were lower than at the beginning for all three replicates.
4. For the background activity controls averaged across replicates there were not significant differences between the beginning and the end portions. For the full enzyme activity controls averaged across replicates there was a highly significant difference between the beginning and the end portions. The end was lower than the beginning. The variation of portion (end vs. beginning) effects among replicates was estimated to be zero.

## Introduction and Background

Task 3 of the Recombinant Aromatase Validation Study involves the individual laboratories independently carrying out the recombinant aromatase assay with positive control inhibitor 4-OH ASDN according to a common protocol. This report discusses the methods and results of the intralaboratory statistical analysis performed on the experimental data collected by In Vitro. Aromatase activity levels were determined for the full enzyme activity control[1], the

[1] Full enzyme activity control. Full assay with no inhibitor substance. Ethyl alcohol vehicle is included.

background activity control[2], and for six graded concentrations of positive control inhibitor 4-OH ASDN.

Three replicates of the positive control inhibitor study were carried out. Within each replicate three repetitions were run at each of the 4-OH ASDN log (base 10) concentrations -6, -7, -7.3, -7.6, -8, and -9. In addition two repeat tubes of the full enzyme activity control and background activity control controls were run prior to the 4-OH ASDN runs and two repeat tubes of the full enzyme activity control and background activity control were run following the 4-OH ASDN runs.

Statistical analyses were carried out on the "percent of control" responses. Percent of control is defined as the ratio of the (background corrected) aromatase activity in the tube under consideration to the average (background corrected) aromatase activity among the four full enzyme activity control tubes within the replicate, times 100. The average percent of control among the four full enzyme activity control tubes is necessarily 100 percent within each replicate. The average percent of control among the four background activity control tubes is necessarily 0 percent.

Nominally for an inhibitor the percent of control activity values vary between approximately 0% near the high inhibition concentrations and approximately 100% near the low inhibition concentrations, but this may vary with the inhibitor.

## Objectives

The primary objectives of the statistical analysis are:

1. Fit concentration curves within each replicate to describe the trend in the percent of control activity across varying inhibitor concentrations of test substance 4-OH ASDN.
2. Estimate the $IC_{50}$ concentration, slope, and associated standard errors within each replicate.
3. Combine results across replicates to determine the average $IC_{50}$ concentration, average slope, and associated standard errors.
4. Determine whether there are differences between the full enzyme activity control and background activity control obtained at the beginning and those obtained at the end of each replicate.
5. Assess the consistency of conditions within replicates and across replicates based on the full enzyme activity control and background activity control values.

[2]Background activity control. NADPH cofactor is omitted from the assay. Only nonspecific background response should occur. Ethyl alcohol vehicle is included.

## Statistical Analysis Methods

Concentration Response Trend Curves

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

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

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

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

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

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

The response curve was fitted by weighted least squares nonlinear regression analysis with weights equal to 1/Y. This weighting system gives greater weight to the lower end of the concentration response curve, where greater inhibition occurs.

Model fits were carried out using PRISM software (Version 4). Observed percent of control values above 100% were set to 99.5%. Observed percent of control values below 0% were set to 0.5%. This adjustment tacitly assumes an upper bound of 100% on the concentration response curve and a lower bound of 0%.

For each replicate the estimated $\log_{10}IC_{50}$ ($\mu$) and its associated standard error, the $IC_{50}$ and its associated geometric standard error, the slope ($\beta$) and its associated standard error, and the "Status" of each response curve are reported. The "Status" of each response curve is indicated as "C", complete, if the concentration response curve inhibition ranges from essentially 0 percent to 100 percent of control. Otherwise it is indicated as "II", incomplete but can interpolate to $\log_{10}IC_{50}$ or "IX", incomplete but must extrapolate to $\log_{10}IC_{50}$.

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

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

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

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

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

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

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

where $\beta_{avg}$ and $\mu_{avg}$ were estimated across the three replicates, based on the random effects one-way analysis of variance model discussed above.

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

multiple comparisons were carried out using PRISM and the SAS statistical analysis system-Version 9.

Analysis of Variance of Full Enzyme Activity Controls and Background Activity Controls Across Replicates

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

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

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

This analysis was carried out using the SAS statistical analysis system, Version 9.

## Statistical Analysis Results

The percent of control responses are displayed in Table A-1 for each replicate and for each 4-OH ASDN ($\log_{10}$) concentration. The percent of control responses for full enzyme activity background activity control and background activity control are displayed in Table A-2, sorted by replicate and beginning and end within replicate.

Concentration response curves were fitted separately to the repeat tubes data within each replicate and to the averages of the repetitions within each replicate (Table A-1). The parameters of these fitted concentration response curves are displayed in Table 1. The individual repetition data within each replicate are plotted in Figure A-1 through Figure A-3 with the corresponding fitted concentration response curves superimposed in each figure. Figure 1 displays the three concentration response curves fitted to the averages of the three repetitions within each replicate.

All three concentration curves were similar. (Table 1 and Figure 1).

The parameters of the average concentration response curve, based on random effects analysis of variance model fits with replicate as a random effect are displayed in Table 1. The average concentration response curve, along with the averages of three repetitions within each replicate, are plotted together in Figure 2.

The parameter estimates for each replicate and the average parameter estimates across replicates and the associated 95% confidence intervals are displayed in Table 2, and graphed in Figure 3 for $\log_{10}IC_{50}$ ($\mu$) and Figure 4 for slope ($\beta$). The estimated values for the $\log_{10}IC_{50}$s and the slopes ($\beta$) for the three replicates are all close to the averages.

The results of analyses of variance for these estimates are presented in Table 3. For each replicate the squares of the standard errors associated with each parameter are given. These estimates include only within replicate variation. Across replicates, the replicate-to-replicate variation and the square of the standard error of the overall average are displayed. These estimates include both within replicate variation and replicate-to-replicate variation.

For both $\log_{10}IC_{50}$ and slope ($\beta$) the replicate-to-replicate variation is within the range of the individual replicate within-replicate variances.

The background activity control and full enzyme activity control responses for each replicate are displayed in Table A-2. These data are plotted by replicate in Figures 5 and 6, with plotting symbol distinguishing between beginning and end of the replicate. Figures 7 and 8 show the differences between the averages at the beginning and at the end within each replicate (end minus beginning). For background activity controls the averages of the two measurements at the end are approximately 0.2% lower than those at the beginning for replicates 1 and 2 and 0.2% higher than those at the beginning for replicate 3 (Figure 7 (end minus beginning) ). The average standard error of the background activity control differences is about 0.12%, so the beginning and the end are about the same. For full enzyme activity control, the averages of the two percent of controls measurements at the end are 5-12% lower than those for at the beginning for all three replicates (Figure 8 (end minus beginning)). The average standard error of the full enzyme activity control differences is about 2.7%, so all three replicates appear to be lower at the end.

Mixed effects analysis of variance models were fitted to the background activity control and full enzyme activity control data with portion as a fixed effect and with replicate and replicate by portion interaction as random effects. The component of variation due to replicate is constrained to be 0 by the definitions of the background and full enzyme activity control responses. The results are displayed in Table 4 (beginning minus end). The left panel of the table displays the results of the tests for the differences between the responses collected at the beginning and at the end of a replicate. The right panel displays the estimated variance components. No significant differences between the beginning and the end, averaged across replicates, were observed for background activity controls. A highly significant difference between the beginning and the end occurs for full enzyme activity controls. The end values are

lower than the beginning values. The estimated variance for the portion by replicate interaction is zero.

**Table 1. Estimated Parameters of the Concentration Response Curve Fits by Replicate and Averaged Across Replicates. Percent of Control Activity. Recombinant Aromatase Assay**

| Replicate | $Log_{10}IC_{50}$ (SE) | $IC_{50}$ (GSE)[d] | Slope (SE) | Status |
|---|---|---|---|---|
| **Individual Values[a]** | | | | |
| 1 | -7.104 (0.01696) | $7.863x10^{-8}$ (1.03982) | -0.8548 (0.02434) | C |
| 2 | -7.142 (0.02305) | $7.218x10^{-8}$ (1.05451) | -0.8514 (0.03245) | C |
| 3 | -7.091 (0.01372) | $8.114x10^{-8}$ (1.03210) | -0.9153 (0.02193) | C |
| **Mean[c]** | -7.107 (0.01329) | $7.811x10^{-8}$ (1.03106) | -0.8771 (0.02211) | -- |
| **Averages Values[b]** | | | | |
| 1 | -7.102 (0.01476) | $7.898x10^{-8}$ (1.03457) | -0.8557 (0.02119) | C |
| 2 | -7.140 (0.03930) | $7.242x10^{-8}$ (1.09471) | -0.8501 (0.05520) | C |
| 3 | -7.089 (0.01403) | $8.139x10^{-8}$ (1.03283) | -0.9150 (0.02242) | C |

a. Concentration response curve fitted to the data collected within each replicate, with three repetitions at each 4-OH ASDN concentration level.
b. Concentration response curve fitted to the averages of the three repetitions at each 4-OH ASDN concentration level within each replicate.
c. Weighted averages of the parameter estimates across the three replicates.
d. 10 to the power of $log_{10}IC_{50}$ and 10 to the power of its associated standard error.

**Table 2. Estimated Parameters of the Concentration Response Curve Fits by Replicate and Averaged Across Replicates. Percent of Control Activity. Recombinant Aromatase Assay**

| Parameter | Estimate (95% CI) | | | |
|---|---|---|---|---|
| | Replicate 1[a] | Replicate 2[a] | Replicate 3[a] | Mean[b] |
| $Log_{10}IC_{50}$ | -7.104 (-7.140, -7.068) | -7.142 (-7.191, -7.093) | -7.091 (-7.120, -7.062) | -7.107 (-7.177, -7.038) |
| Slope | -0.8548 (-0.9064, -0.8032) | -0.8514 (-0.9202, -0.7826) | -0.9153 (-0.9618, -0.8688) | -0.8771 (-0.9601, -0.7940) |

a. Parameter estimates and their associated 95% confidence intervals for each replicate, based on the concentration response curves fitted to the individual values within replicates.
b. Mean and its associated 95% confidence interval, based on a heterogeneous variance one-way analysis of variance model with replicate treated as a random effect.

**Table 3. Variances Associated with Estimated Parameters of Concentration Response Curves. Percent of Control Activity. Recombinant Aromatase Assay**

| Parameter | Variance/Degree of Freedom[a,b] | | | | |
|---|---|---|---|---|---|
| | | | | Overall | |
| | Replicate 1 | Replicate 2 | Replicate 3 | Random Replicate (p-value)[d] | Variance of Mean[c] |
| $Log_{10}IC_{50}$ | 0.000288 /df=16 | 0.000531 /df=16 | 0.000188 /df=16 | 0.000227 /df=2 (p=0.3549) | 0.000177 /df=1.673 |
| Slope | 0.000592 /df=16 | 0.001053 /df=16 | 0.000481 /df=16 | 0.000795 /df=2 (p=0.2826) | 0.000489 /df=2.339 |

a. The variance estimates for each replicate were based on the concentration response curves fitted to the individual results within each concentration level.

b. Variance estimates for the random replicate were estimated based on a one-way random effects analysis of variance. The variances for each replicate were fixed at their reported values.

c. Degrees of freedom for the variance of mean were estimated by $2*((1/K)* \sum(S_r^2 + S_i^2))^2/(var(S_r^2)+(2/K^2)* \sum(S_i^4 /d\ f_i))$, where $S_r^2$ is random replicate variance, $S_i^2$ and $df_i$ are estimated variance and degree of freedom for a given replicate, $var(S_r^2)$ is the variance associated with the estimation of $S_r^2$ and K is the number of replicates (Hartung and Makambi, 2001).

d. p-value is based on the Wald Z-test result.

**Table 4. Variance Components of Full Enzyme Activity Control and Background Activity Control of Percent of Control Across Replicates**

| Parameter | Difference Between Beginning and End Portion (Beginning Minus End) | | Variance Components | | | |
|---|---|---|---|---|---|---|
| | Estimate (%) (Std. Error) | p-Value/ Degree of Freedom | Replicate[a] | Portion* Replicate | Residual (Repetition) | Total Variance |
| Background Activity Control | 0.0837( 0.1178) | 0.4936/df=10 | 0 | 0 | 0.0417 | 0.0417 |
| Full Enzyme Activity Control | 9.3543 (2.6605) | 0.0056/df=10 | 0 | 0 | 21.2350 | 21.2350 |

a. The replicate component of variation is constrained to be 0, by definition of background activity and full enzyme activity control responses.

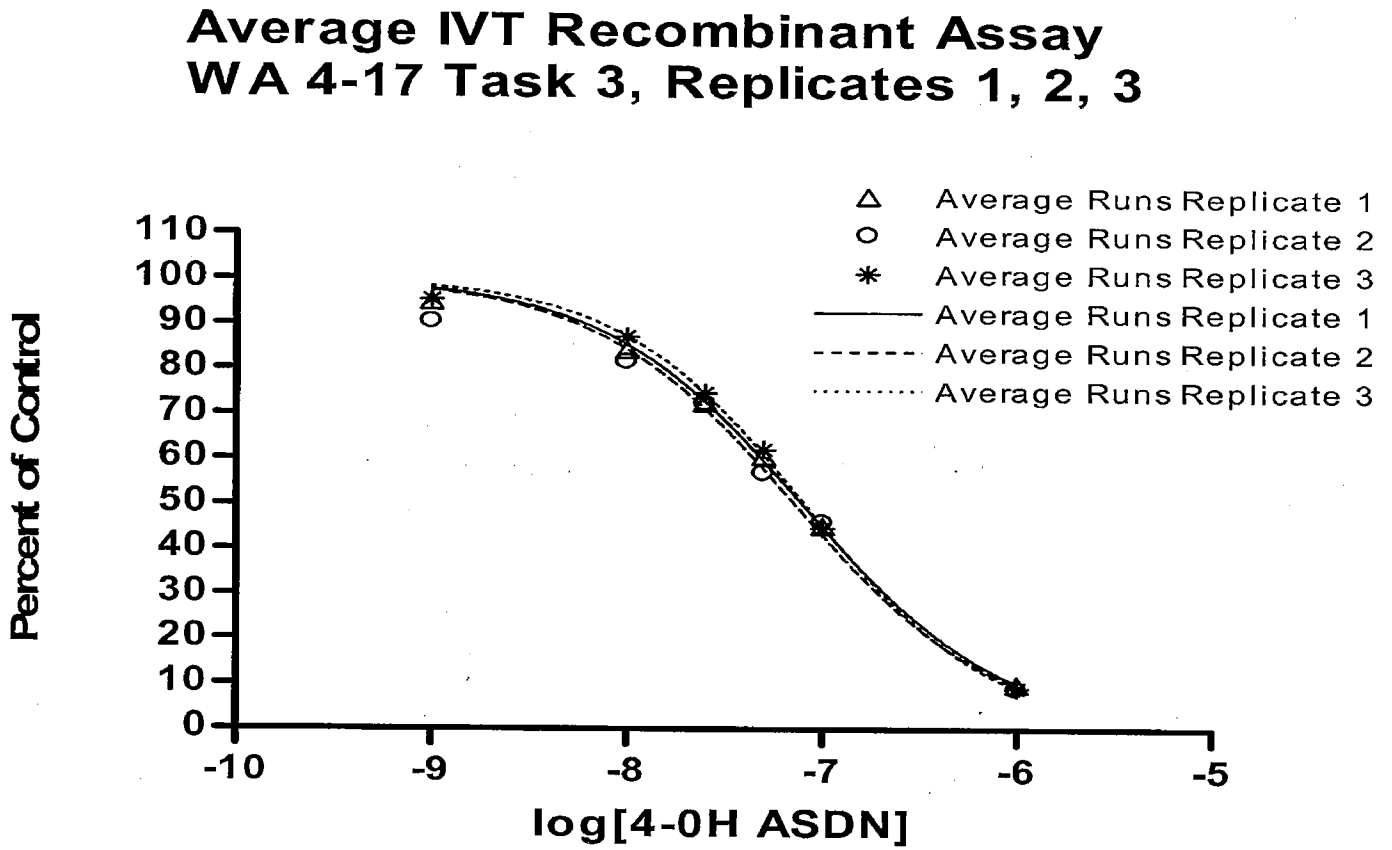



**Figure 1. Concentration Response Curves and Averages of Repetitions Within 4-OH ASDN Concentrations. Recombinant Aromatase Assay. By Replicate**

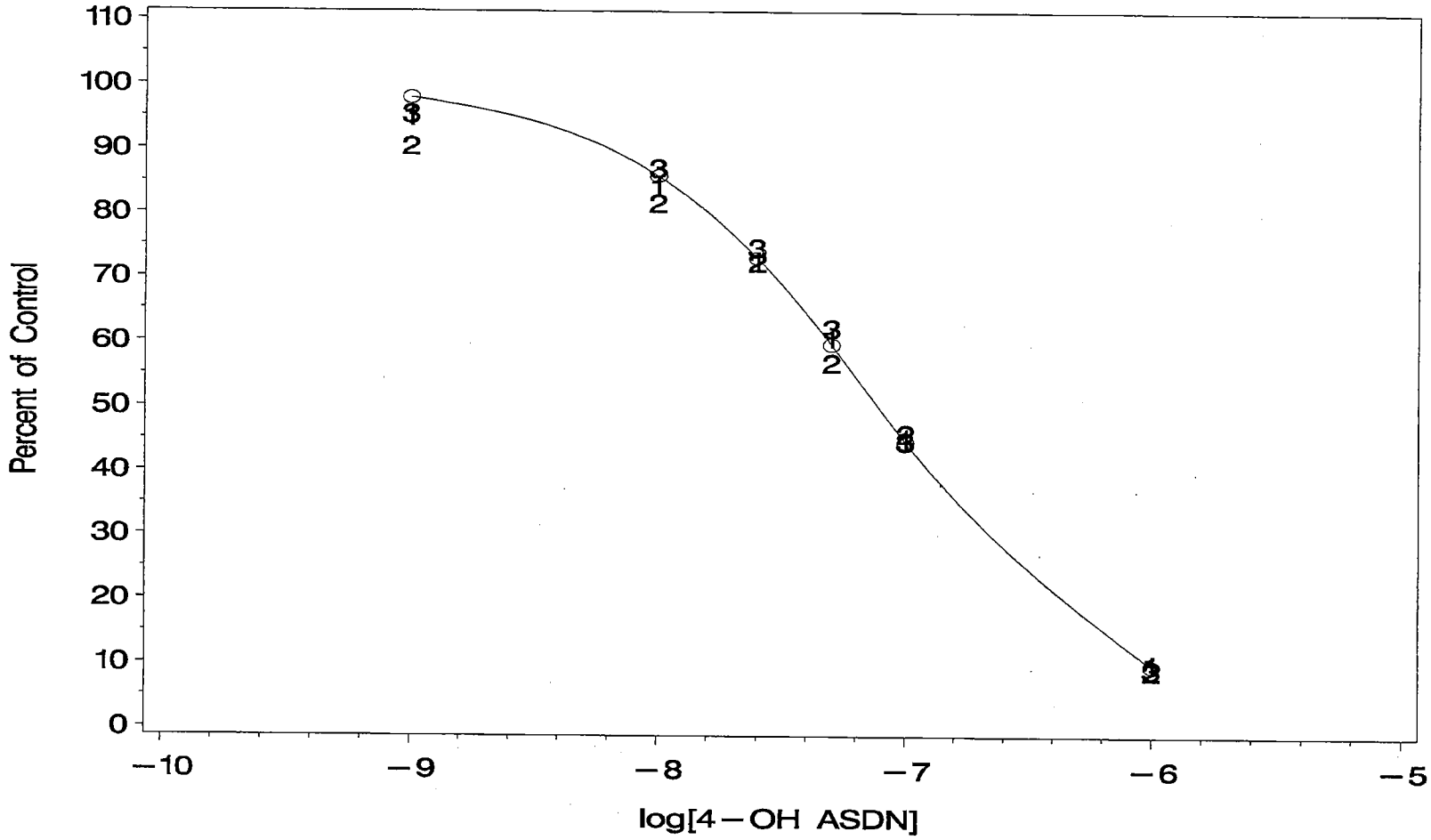



**Figure 2. Overall Average Concentration Response Curve Across Replicates and Average Responses Across Repetitions Within 4-OH ASDN Concentrations. Recombinant Aromatase Assay. Parameters of Average Curve Based on One-Way Analysis of Variance Across Replicate Parameter Values**

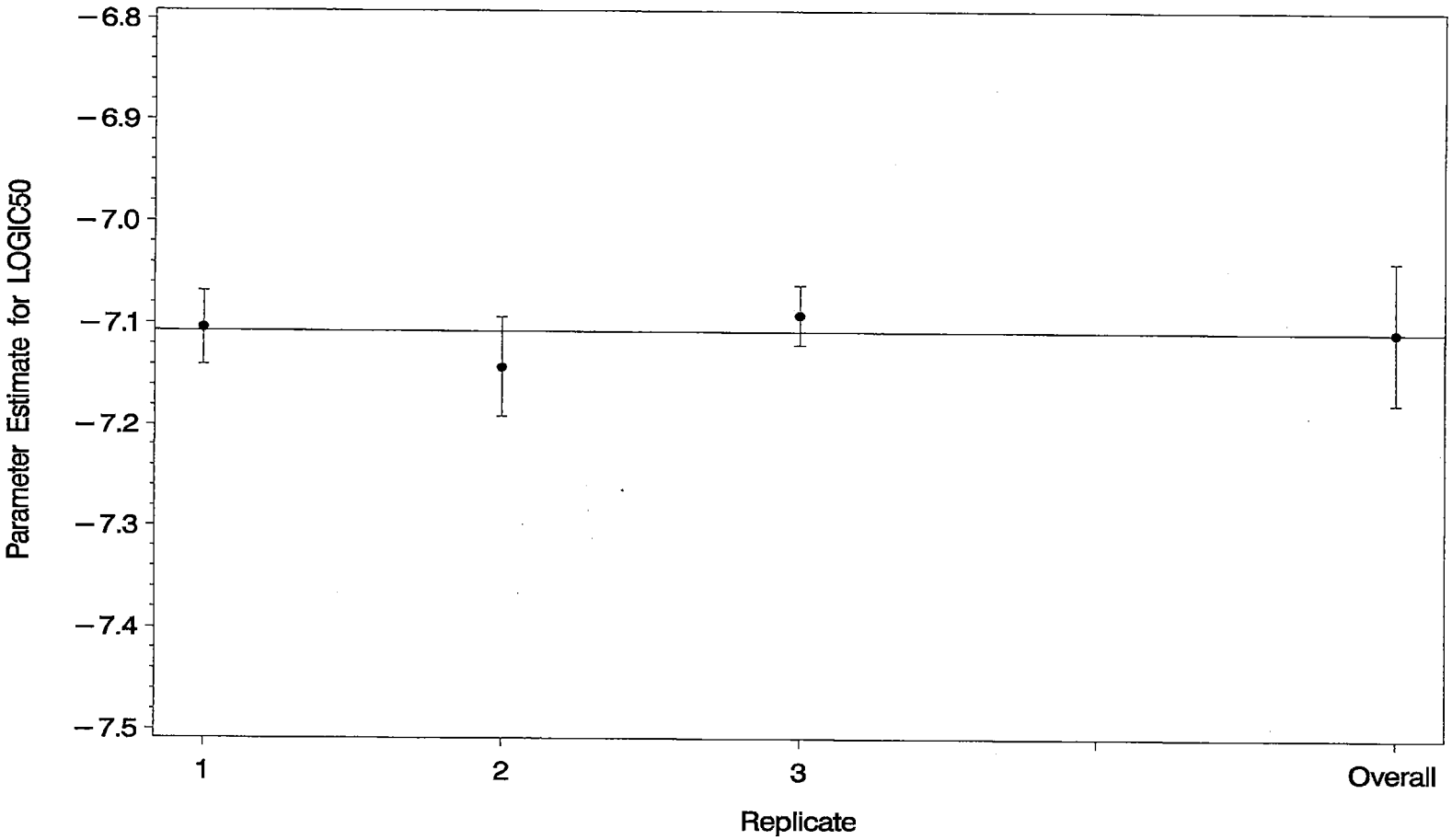



**Figure 3. $Log_{10}IC_{50}$ Parameter Estimates and Their Associated 95% Confidence Intervals for Each Replicate and Across Replicates. Recombinant Aromatase Assay. The Solid Reference Line Corresponds to the Average Across Replicates**

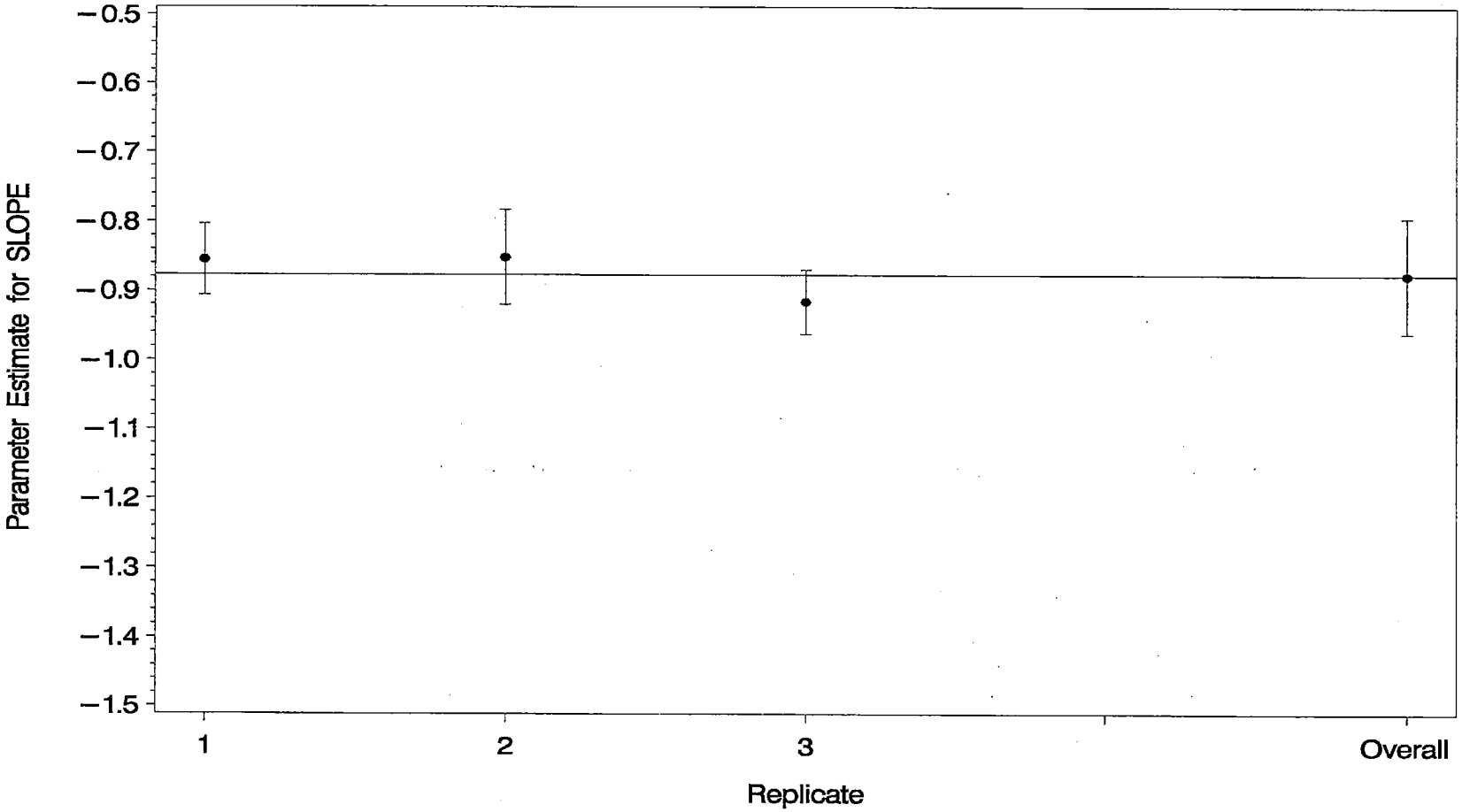



**Figure 4. Slope (β) Parameter Estimates and Their Associated 95% Confidence Intervals for Each Replicate and Across Replicates. Recombinant Aromatase Assay. The Solid Reference Line Corresponds to the Average Across Replicates**

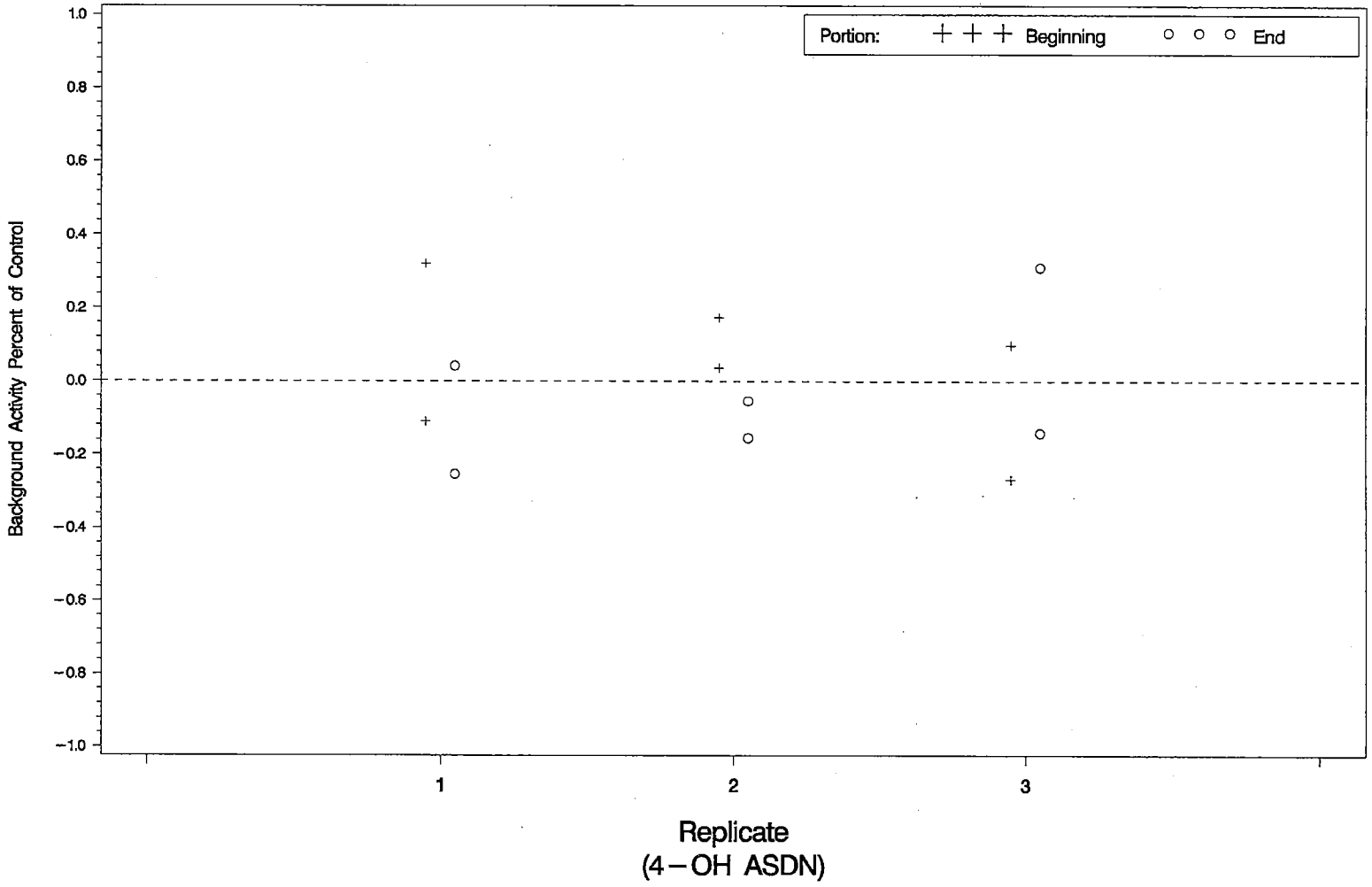



**Figure 5. Background Activity Controls. Percent of Control by Replicate and Portion of Replicate (Beginning or End). Recombinant Aromatase Assay. Reference Line at 0%**

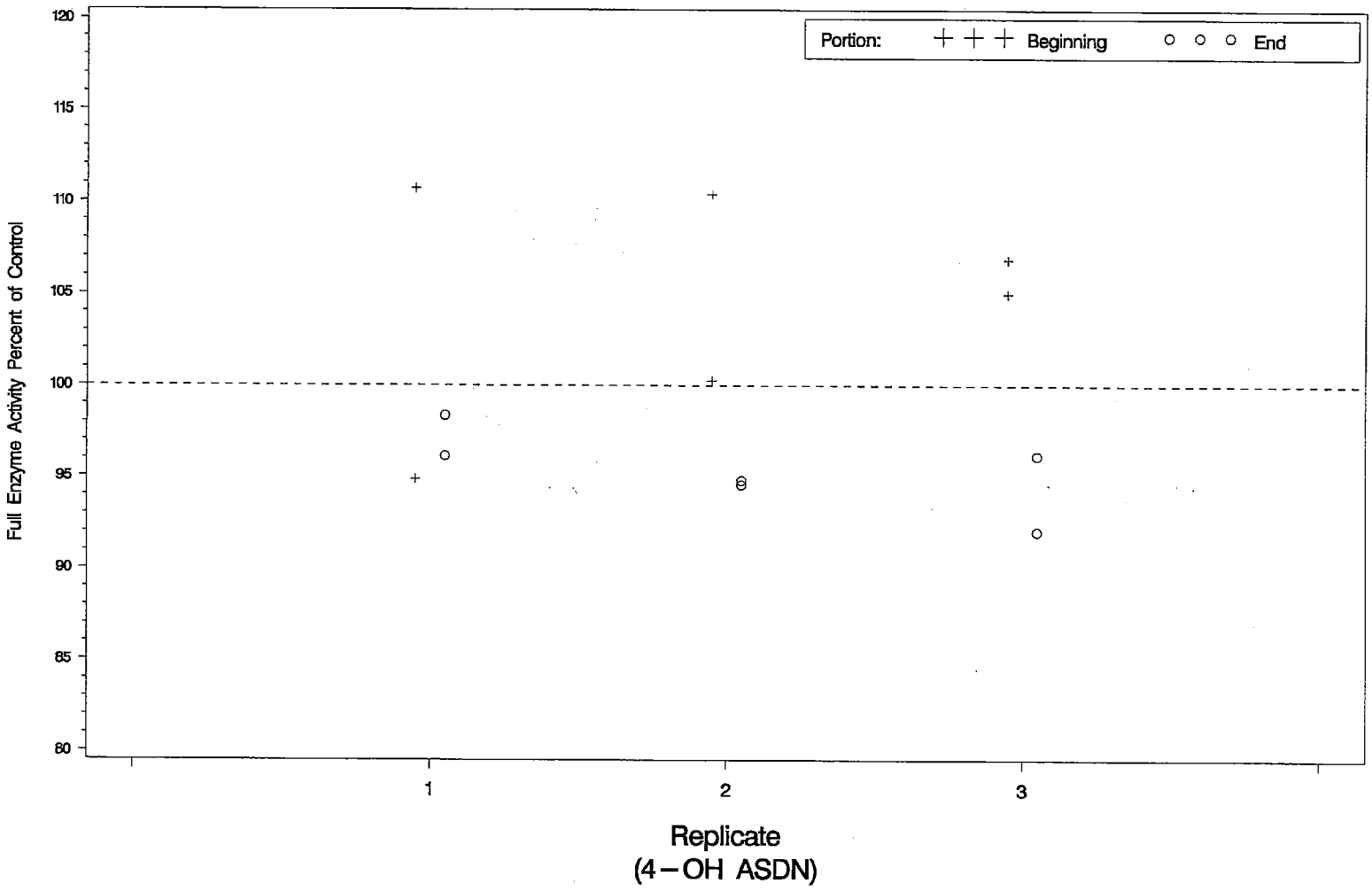



**Figure 6. Full Enzyme Controls. Percent of Control by Replicate and Portion of Replicate (Beginning or End). Recombinant Aromatase Assay. Reference Line at 100%**

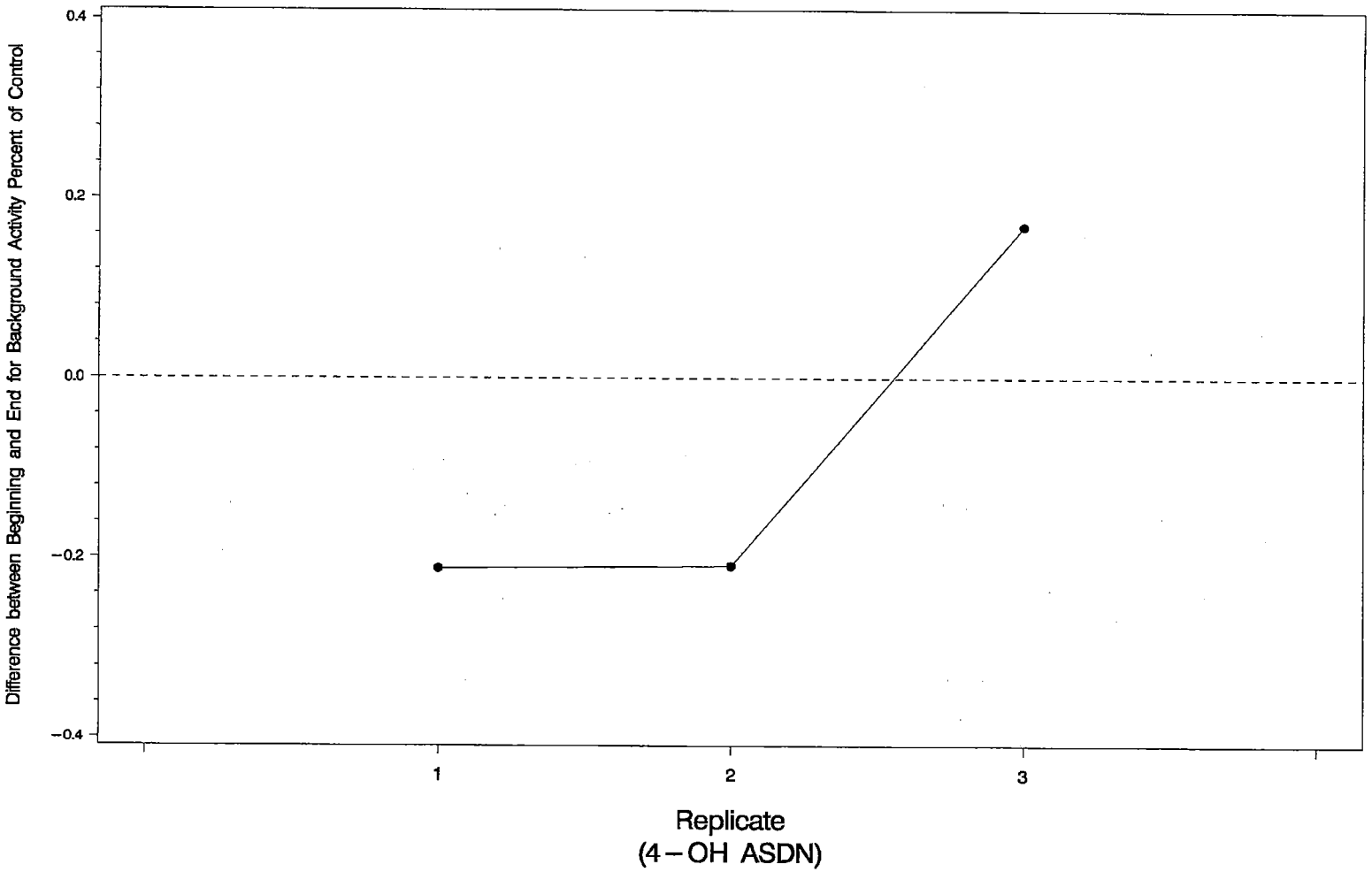



**Figure 7. Background Activity Controls. Difference Between the Averages of the Two End Percent of Control Responses and the Average of the Two Beginning Responses by Replicate (End Minus Beginning). Recombinant Aromatase Assay. Reference Line at 0%**

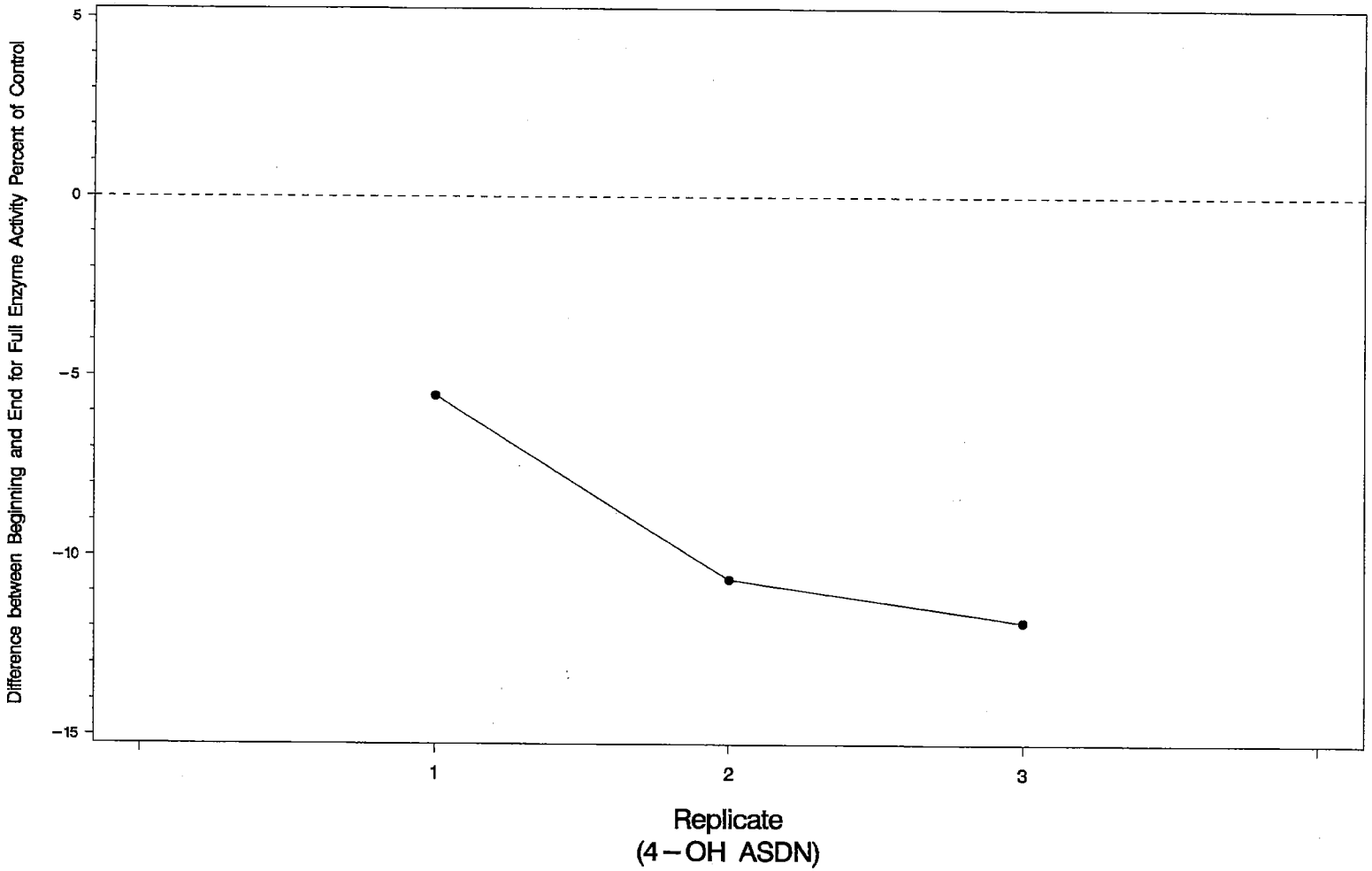



**Figure 8. Full Enzyme Activity Controls. Difference Between the Averages of the Two End Percent of Control Responses and the Average of the Two Beginning Responses by Replicate (End Minus Beginning). Recombinant Aromatase Assay. Reference Line at 0%**

**Table A-1. Percent of Control Activity in Recombinant Assay by Replicate, 4-OH ASDN Concentration Within Replicate, and Repetition Within Concentration.**

| Replicate | Log [4-OH ASDN] | Percent of Control | | |
|---|---|---|---|---|
| | | Repetition 1 | Repetition 2 | Repetition 3 |
| 1 | -6.00 | 10.01 | 9.64 | 10.28 |
| | -7.00 | 49.15 | 43.37 | 43.00 |
| | -7.30 | 62.41 | 58.89 | 60.76 |
| | -7.60 | 72.78 | 73.45 | 71.33 |
| | -8.00 | 87.54 | 85.00 | 79.86 |
| | -9.00 | 99.32 | 89.32 | 96.12 |
| 2 | -6.00 | 9.29 | 9.56 | 7.93 |
| | -7.00 | 47.83 | 45.80 | 43.98 |
| | -7.30 | 58.90 | 56.17 | 55.44 |
| | -7.60 | 74.03 | 71.86 | 71.14 |
| | -8.00 | 84.36 | 82.04 | 78.26 |
| | -9.00 | 93.35 | 87.18 | 90.58 |
| 3 | -6.00 | 10.01 | 9.07 | 8.38 |
| | -7.00 | 46.65 | 43.55 | 43.76 |
| | -7.30 | 63.64 | 62.52 | 60.09 |
| | -7.60 | 76.92 | 72.60 | 74.16 |
| | -8.00 | 84.67 | 83.95 | 92.36 |
| | -9.00 | 94.33 | 94.38 | 97.13 |

**Table A-2. Background Activity Control and Full Enzyme Activity Control Corrected Aromatase Activity by Replicate and Portion (Beginning or End). Recombinant Aromatase Assay.**

| Aromatase Activity | Replicate | Portion | Corrected Activity | % of Control[a] |
|---|---|---|---|---|
| Background Activity Control | 1 | Beginning | 0.00057 | 0.32177 |
| | | Beginning | -0.00020 | -0.11004 |
| | | End | 0.00007 | 0.04168 |
| | | End | -0.00045 | -0.25341 |
| | 2 | Beginning | 0.00031 | 0.17303 |
| | | Beginning | 0.00006 | 0.03572 |
| | | End | -0.00028 | -0.15458 |
| | | End | -0.00010 | -0.05417 |
| | 3 | Beginning | 0.00029 | 0.09833 |
| | | Beginning | -0.00078 | -0.26759 |
| | | End | -0.00041 | -0.14165 |
| | | End | 0.00090 | 0.31091 |
| Full Activity Control | 1 | Beginning | 0.19644 | 110.70880 |
| | | Beginning | 0.16829 | 94.84376 |
| | | End | 0.17449 | 98.34151 |
| | | End | 0.17053 | 96.10593 |
| | 2 | Beginning | 0.19815 | 110.39717 |
| | | Beginning | 0.17996 | 100.26338 |
| | | End | 0.17013 | 94.78698 |
| | | End | 0.16971 | 94.55246 |
| | 3 | Beginning | 0.30542 | 104.98985 |
| | | Beginning | 0.31086 | 106.86002 |
| | | End | 0.27966 | 96.13552 |
| | | End | 0.26767 | 92.01460 |

a. The corrected aromatase activity values were divided by the average of the four FEAC control activity values within each replicate, and expressed as percent of control.

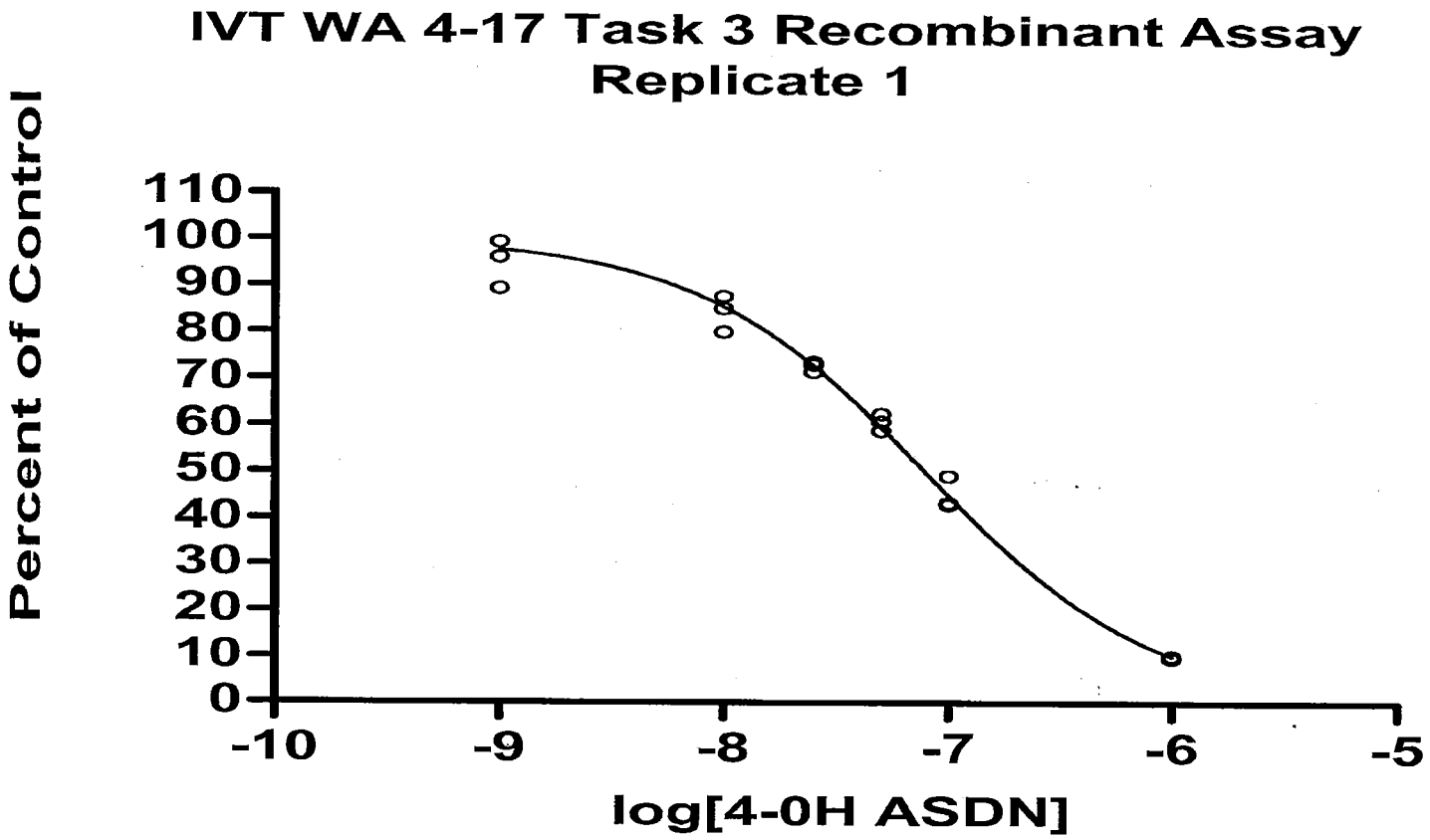



**Figure A-1.** **Replicate 1. Individual Percent of Control Values Vs. (Base 10) Logarithm of 4-OH ASDN Inhibitor Concentration. Concentration Response Curve Fitted to Average Responses Within Concentrations. Recombinant Aromatase Assay**

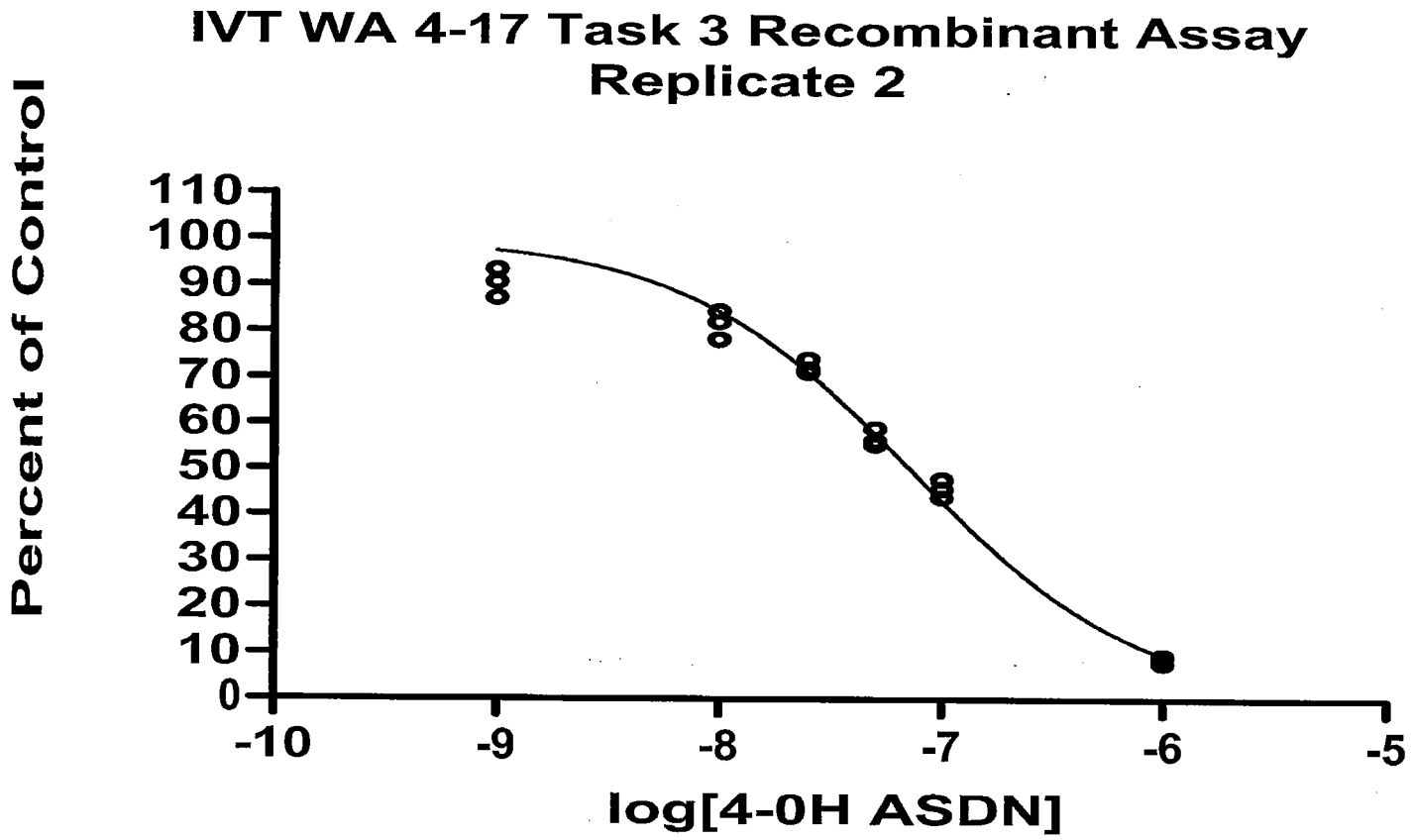



**Figure A-2.** **Replicate 2. Individual Percent of Control Values Vs. (Base 10) Logarithm of 4-OH ASDN Inhibitor Concentration. Concentration Response Curve Fitted to Average Responses Within Concentrations. Recombinant Aromatase Assay**

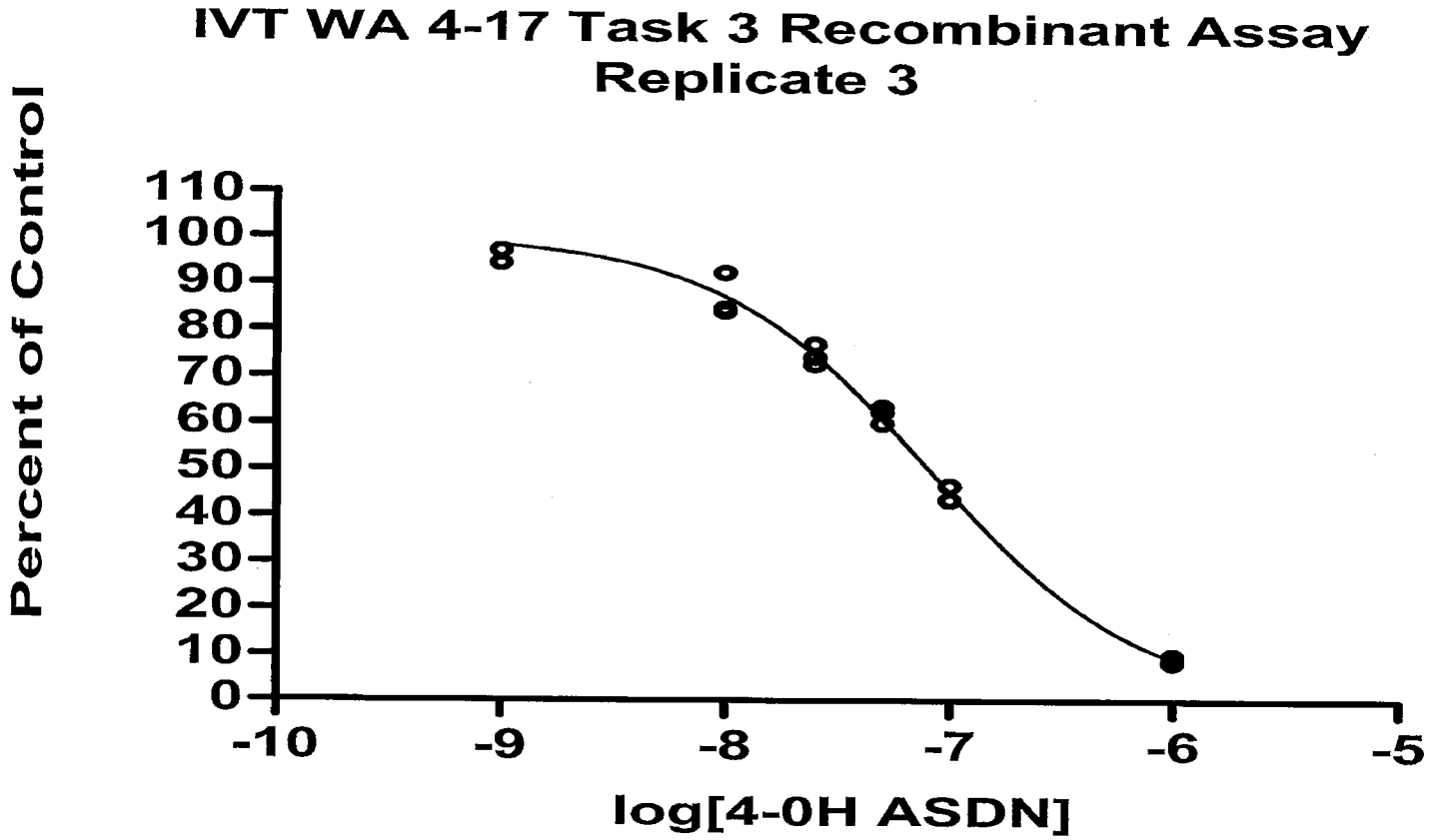



**Figure A-3.** **Replicate 3. Individual Percent of Control Values Vs. (Base 10) Logarithm of 4-OH ASDN Inhibitor Concentration. Concentration Response Curve Fitted to Average Responses Within Concentrations. Recombinant Aromatase Assay**

# APPENDIX C

## WIL Research Laboratories Draft Final Report

# DRAFT TASK REPORT

# VALIDATION OF THE RECOMBINANT MICROSOMAL AROMATASE ASSAY: POSITIVE CONTROL STUDY (WA 4-17, TASK 3)

## WA 4-17 Task 3: Conduct Positive Control Studies in the Participating Laboratories

**EPA Contract Number 68-W-01-023**
**Work Assignment 4-17**

**WIL-431010**

**Sponsor:**

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

**Performing Laboratory:**

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

DRAFT REPORT

| | |
|---|---|
| Title: | VALIDATION OF THE RECOMBINANT MICROSOMAL AROMATASE ASSAY: POSITIVE CONTROL STUDY (WA 4-17, TASK 3)<br><br>Task 3: Conduct Positive Control Studies in the Participating Laboratories |
| Authors: | Christopher J. Bowman, Ph.D. (Study Director)<br>Jennifer A. Thomas-Wohlever, Ph.D. |
| Performing Laboratory: | WIL Research Laboratories, LLC<br>1407 George Road<br>Ashland, OH 44805-9281 |
| Sponsor: | Battelle Memorial Institute<br>505 King Avenue<br>Columbus, OH 43201-2693 |
| Sponsors Representatives: | David P. Houchens, Ph.D.<br>EDSP Program Manager<br>Battelle<br><br>Jerry D. Johnson, Ph.D.<br>Diplomate, A.B.T.<br>Work Assignment Leader<br>Battelle |
| Study Initiation Date: | December 8, 2004 |
| Study Completion Date: | |
| Experimental Start Date: | December 28, 2004 |
| Experimental Start Date: | April 8, 2005 |
| Draft Task Report Date | May 20, 2005 |

# Quality Assurance Statements

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

Intralaboratory data requiring statistical analysis were analyzed by BioSTAT Consultants, Inc., following the current procedural guidelines of BioSTAT Consultants, Inc. BioSTAT Consultants, Inc. provided a statistical analysis report, which is included as Appendix 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 of Inspection | Phases Inspected | Date(s) Findings Reported to Study Director | Date(s) Findings Reported to Management | Auditors |
|---|---|---|---|---|
| 11/29/2004 | Protocol Review | 11/29/2004 | 12/20/2004 | L. Goodrich |
| 12/29/2004 | Microsomal Preparation | 12/29/2004 | 1/27/2005 | AD Deppe |
| 12/29/2004 | Test Article Dilution | 12/29/2004 | 1/27/2005 | AD Deppe |
| 12/29/2004 | Aromatase Assay Preparation | 12/29/2004 | 1/27/2005 | AD Deppe |
| 1/13,14/2005 | Study Records (A-1) (12/28-30/2004) | 1/14/2005 | 2/16/2005 | J. House |
| 1/13,14/2005 | Study Records (B-1) (12/28-30/2004) | 1/14/2005 | 2/16/2005 | J. House |
| 1/18/2005 | Protocol Amendment I Review | 1/18/2005 | 2/16/2005 | T. Booth |
| 1/31/2005 2/1-3/2005 | Aromatase Assay Data Spreadsheets | 2/3/2005 | 3/25/2005 | J. House |
| 3/18/2005 | Protocol Amendment II Review | 6/9/2005 | 7/25/2005 | P. Brant |
| 4/12-13/2005 | Supplemental Audit (Replicates 4 to 6) | 4/13/2005 | 5/28/2005 | ES Crawford |
| 5/15,16/2005 | Draft Report | 5/16/2005 | 6/29/2005 | ES Crawford |

### *Approval*

This study was inspected according to the criteria discussed above.

Report Audited By:

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

Report Released By:

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

# COMPLIANCE STATEMENT

This study, designated WIL-431010, was conducted in compliance with the United States Environmental Protection Agency (EPA) Good Laboratory Practice Standards (40 CFR Part 160), October 16, 1989; the United States Environmental Protection Agency (EPA) Good Laboratory Practice Standards (40 CFR Part 792), September 18, 1989; the standard operating procedures of WIL Research Laboratories, LLC, and the protocol as approved by the sponsor.

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

Date

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# 1.0 Executive Summary

The recombinant 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 amount 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 recombinant 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 (Work Assignment (WA) 4-17, Task 3).

The results of this positive control study using recombinant microsomes and the known aromatase inhibitor 4-OH ASDN were as expected, based on the prevalidation work (WA 2-24 and 4-10), the training data (WA 4-16, Task 3) and WA 4-16, Task 4 (WIL-431006). 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 (enzyme retained 99.83 percent of control activity) to almost full inhibition (6.77 percent of control activity), respectively. The overall $IC_{50}$ in this study for 4-OH ASDN was 65.5 nM (arithmetic mean). 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 recombinant 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 recombinant microsome assay using ten potential aromatase inhibitors will be evaluated under WA 4-17, Task 4 (WIL-431011).

# 2.0 Introduction

## *2.1 Background*

The Food Quality Protection Act of 1996 was enacted by Congress to authorize the EPA to implement a screening program on pesticides and other chemicals found in food or water sources for endocrine effects in humans. Thus, the U.S. EPA is implementing an Endocrine Disruptor Screening Program (EDSP). In this program, comprehensive toxicological and ecotoxicological screens and tests are being developed for identifying and characterizing the endocrine effects of various environmental contaminants, industrial chemicals and pesticides. The program's aim is to develop a two-tiered approach, e.g., a combination of *in vitro* and *in vivo* mammalian and ecotoxicological screens (Tier 1) and a set of *in vivo* tests (Tier 2) for identifying and characterizing endocrine effects of pesticides, industrial chemicals and environmental contaminants. Validation of the individual screens and tests is required, and the Endocrine Disruptor Methods Validation Advisory Committee 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 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 pharmaceuticals and environmental chemicals to inhibit aromatase activity. In addition, human JEG-3 and JAR choriocarcinoma cell culture lines, originally isolated from cytotrophoblasts of malignant recombinant tissues, have been used as *in vitro* systems for measuring the effects of compounds on aromatase activity. These cell lines are also used for investigations on the effects of agents in recombinant toxicology.

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

The human recombinant microsomal aromatase assay was recommended as an *in vitro* aromatase screening assay to be included in the Tier 1 Screening Battery. This assay should 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 recombinant microsomes, demonstrate the utility of the microsomal assay to detect known aromatase inhibitors, and compare the performance of a recombinant assay system and the placental microsomal assays. Concerns with this initial work involving high variability in some assay replicates and partial inhibition curves were addressed in a supplemental prevalidation study (WA 4-10). The objective of the current work assignment is to use the now optimized assay to obtain intra- and inter-laboratory assay variability estimates to complete the validation of the human recombinant 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 recombinant 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 recombinant microsome aromatase assay at WIL Research Laboratories, LLC using the known aromatase inhibitor 4-OH ADSN 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; reported purity of 100%) was manufactured by Sigma-Aldrich (St. Louis, MO) and received from Battelle Marine Science Laboratory (Sequim, WA). 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 assessed by the lead laboratory using high performance liquid chromatography (HPLC). The results of this analysis are presented in the report contained in Appendix D.

A mixture of ASDN and [$^{3}$H]ASDN was made such that the final concentration of ASDN in the assay was 100 nM, and each assay 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 the stock were made in buffer to a final concentration of 1 µg/mL. To make 8 mL of substrate solution (enough for 80 tubes), 4.5 mL of the 1 µg/mL solution of ASDN, 800 µL of the [$^{3}$H]ASDN dilution and 2.7 mL of buffer were combined. For accuracy, the weight of each component added to the substrate solution was recorded. To determine the specific activity of the ASDN substrate, aliquots of substrate solution (approximately 20 µL, weighed) were combined with scintillation cocktail for radiochemical content analysis.

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

| Test Chemical Information | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Chemical name | Chemical code | Mfr. Purity | CAS No. | Molecular formula | Molecular weight | Stock Solution ID | Target Stock Formulation Concentration (mg/mL) | Vehicle | Storage Conditions |
| 4-Androsten-4-ol-3,17 dione | 4-OH ASDN | 99%* | 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 95% 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 | $1 \times 10^{-4}$ | 0 | $1 \times 10^{-4}$ | $1 \times 10^{-6}$ |
| 4-OH ASDN Concentration 2 | 100 | $1 \times 10^{-4}$ | 900 | $1 \times 10^{-5}$ | $1 \times 10^{-7}$ |
| 4-OH ASDN Concentration 3 | 50 | $1 \times 10^{-4}$ | 950 | $5 \times 10^{-6}$ | $5 \times 10^{-8}$ |
| 4-OH ASDN Concentration 4 | 25 | $1 \times 10^{-4}$ | 975 | $2.5 \times 10^{-6}$ | $2.5 \times 10^{-8}$ |
| 4-OH ASDN Concentration 5 | 100 | $1 \times 10^{-5}$ | 900 | $1 \times 10^{-6}$ | $1 \times 10^{-8}$ |
| 4-OH ASDN Concentration 6 | 100 | $1 \times 10^{-6}$ | 900 | $1 \times 10^{-7}$ | $1 \times 10^{-9}$ |

## 3.3 Microsomes

Human recombinant microsomes from baculovirus were received as multiple frozen aliquots from RTI International, who acquired the microsomes from Gentest™ (Human CYP19 + P450 Reductase SUPERSOMES, Woburn, MA). Upon receipt, the sample code number MET-0254A was assigned and the microsomes were stored between approximately -70 to -80 °C. On the day of the assay, microsomes were thawed rapidly in a 37 ± 1°C water bath, re-homogenized using a Potter Elvejhem homogenizer and then kept on ice until used. Fresh aliquots of microsomes were used for each assay. The reported protein concentration was 4.9 mg/mL. Using this figure, a 20-fold dilution was made to a concentration of approximately 0.25 mg/mL. The actual protein concentration of this dilution was determined using the protein assay described in section 3.5. A second dilution was made to achieve the final 0.008 mg/mL 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 | A28H21 |
| 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 was included in excess in the aromatase assay. First, 0.025 g NADPH was weighed and transferred to a 5-mL volumetric flask to make a 5 mg/mL solution in phosphate buffer (see Section 3.4.2.). Adding 100 µL of this NADPH solution to the reaction mixture resulted in a final assay concentration of 0.3 mM. NADPH was prepared fresh every assay day and was stored on ice until 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 final 0.1 M pH 7.4 solution. The assay buffer was stored refrigerated for up to one month.

## 3.5 Protein Determination

The protein concentration of the microsome preparation was determined on each day of use of the microsomes in the aromatase assay. A 6-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). 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 BioRad DC Protein Kit Reagent A was added and mixed. Next, 1 mL of BioRad DC Protein Kit Reagent B was added to each standard or unknown and the samples were vortex mixed. The samples were allowed to sit at room temperature for at least 15 minutes to allow for color development. The absorbances were stable for about 1 hour. Each sample (unknown and standards) was transferred to appropriate cuvettes and the absorbance (750 nm) was measured using a spectrophotometer. The protein concentration of the microsomal sample was determined by extrapolation of the absorbance value using the curve developed using the protein standards.

## 3.6 Cytochrome P450 Aromatase (CYP19) Activity

Aromatase activity was determined via an *in vitro* screening assay using human recombinant microsomes provided by RTI International (purchased from Gentest™) as summarized in Appendix B. The assays were performed in 13x100 mm glass test tubes. Each test tube was uniquely identified with replicate, repetition, and group information summarized in the following table as necessary to differentiate the tubes. In addition to tubes containing test chemical, Full Enzyme Activity Controls (includes vehicle but no test chemical) and Background

Activity Controls (tubes contain no test chemical or NADPH cofactor) were used to determine 100% and 0% activity.

| 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 reference chemical vehicle control | N/A |
| Background Activity Control-Beginning | 2 | Complete assay with reference chemical vehicle control omitting NADPH | N/A |
| 4-OH ASDN Concentration 1 | 3 | Complete assay with 4-OH ASDN added | $1 \times 10^{-6}$ |
| 4-OH ASDN Concentration 2 | 3 | Complete assay with 4-OH ASDN added | $1 \times 10^{-7}$ |
| 4-OH ASDN Concentration 3 | 3 | Complete assay with 4-OH ASDN added | $5 \times 10^{-8}$ |
| 4-OH ASDN Concentration 4 | 3 | Complete assay with 4-OH ASDN added | $2.5 \times 10^{-8}$ |
| 4-OH ASDN Concentration 5 | 3 | Complete assay with 4-OH ASDN added | $1 \times 10^{-8}$ |
| 4-OH ASDN Concentration 6 | 3 | Complete assay with 4-OH ASDN added | $1 \times 10^{-9}$ |
| Full Enzyme Activity Control-End | 2 | Complete assay with reference chemical vehicle control | N/A |
| Background Activity Control-End | 2 | Complete assay with reference chemical vehicle control omitting NADPH | N/A |

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

Propylene glycol (100 µL), ASDN substrate solution (100 µL), NADPH (100 µL, excluded from background control), and vehicle or test chemical (20 µL) were added to the appropriate test tube with buffer to make 1 mL total volume. Microsomes were diluted to the appropriate concentration as detailed in section 3.3 and used within 2 hours of thawing. Both the reaction mixture and the microsomes were incubated at 37 ± 1 °C independently for at least 5 minutes. The reactions were initiated by the addition of diluted microsomes (1 mL) to each reaction mixture, with each assay tube initiated at 15 second intervals. Each assay tube was incubated at 37 ± 1 °C for 15 minutes. At the conclusion of the reaction time, tubes were quenched with 2.0 mL of methylene chloride in the order in which microsomes had been added, one every 15 seconds. The tubes were vortex-mixed for approximately 5 seconds and placed on ice until all tubes were quenched. The tubes were then vortex-mixed for an additional 20 to 25 seconds. The tubes were centrifuged for 10 minutes at approximately 162 $x\ \hat{g}$ to facilitate separation of the organic and aqueous layers. The methylene chloride layers were removed and discarded; the aqueous layers were extracted again with methylene chloride (2 mL). This extraction procedure was performed one additional time. The aqueous layers were transferred to vials and duplicate aliquots (0.5 mL) were transferred to 20-mL liquid scintillation counting vials. Liquid scintillation cocktail (Ultima Gold, Packard Bio Sciences, approximately 10 mL) was added to each counting vial and shaken to mix the solution.

Analysis of the samples was performed using a liquid scintillation counter (LSC). Radiolabel found in the aqueous fractions represents [$^3$H] OH formed from the hydrolysis of [$^3$H] ASDN. One [$^3$H] OH molecule is released per molecule of radiolabeled ASDN converted to estrogen in a stereospecific reaction. Thus, the amount of estrogen product formed is determined by dividing the total amount of [$^3$H] OH 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 is expressed in nmol(mg protein)$^{-1}$min$^{-1}$ and was calculated by

dividing the amount of estrogen formed by the product of milligram microsomal protein used and incubation time, *i.e.*. 15 minutes. (See Section 3.7)

Each assay replicate was performed on the day shown in the following table. The same technician performed each replicate. Three replicates were completed on December 28, 29 and 30, 2004. The results of those three replicates are documented in the study records and are summarized in Appendix G. These first three replicates were deemed unacceptable because the concentration of microsomes analyzed in the protein assay was below the range of the standard curve. The three additional replicates conducted in April were authorized by the EPA and are discussed in this report as Replicates 1, 2, and 3 (Appendix F).

| Assay Dates by Technician | | |
|---|---|---|
| Replicate | Date | Technician |
| 1 | 4/6/2005 | JG |
| 2 | 4/7/2005 | JG |
| 3 | 4/8/2005 | 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 run number, technician, chemical and log chemical concentration, total DPM minus background DPM, and percent activity. For each aqueous sample after extraction the spreadsheet calculated DPM/mL for each aliquot counted, average DPM/mL per aliquot, and total sample DPM. Total assay DPM was calculated by the multiplication of the volume of substrate solution added to the incubation by the substrate solution radiochemical content (DPM 1g, assume 1g/mL) and yields the total DPM present in the assay tube at initiation. Background DPM was calculated as the average of the DPM present in the aqueous portion for the background tubes, and was subtracted from total sample DPM for all samples to provide DPM for calculating aromatase activity. This corrected DPM 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 milligram microsomal protein used and the incubation time.

The average activity in the Full Enzyme Activity Control samples for a given replicate was calculated. Percent of control activity remaining in the presence of various inhibitor concentrations was calculated by dividing the aromatase activity at a given concentration by the average Full Enzyme Activity Control and multiplying by 100. Thus, the average percent activity across the four Background Activity repeat tubes must necessarily equal 0 within each replicate and the average percent activity across the four Full Enzyme Activity Control 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 sometimes extended 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 (GraphPad, San Diego) for curve fitting. $IC_{50}$ was calculated using Prism (v. 4.02) software to fit the percent of control activity versus log concentration data to a curve using the following equation:

$$Y = 100/(1+10^{((\text{Log}IC_{50}-X)*\text{Hill Slope})})$$

where X is the logarithm of inhibitor concentration and Y is the percent of activity. 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 was 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 permitted estimation of the $IC_{50}$ value on inhibition

curves that did not span the entire inhibition range from 100% to 0%. Each response curve was classified in the following manner: complete curves showed inhibition from 0 to 100% for any particular chemical, incomplete curves showed at least 50% inhibition but did not span the entire range, and chemicals that resulted in less than 80% inhibition across the inhibitor concentration range were not inhibitors. The addition of 4-OH ASDN resulted in a complete inhibition curve, and the concentration response fits were performed for each replicate. The resultant $\mu$ (log $IC_{50}$) and $\beta$ (Hill slope) summarize the extent of aromatase inhibition. The average value and standard error of log $IC_{50}$ and $\beta$ were calculated based on a one-way random effects analysis of variance model fit. For each replicate the estimated log $IC_{50}$ ($\mu$), the within replicate standard error of $\mu$, the $IC_{50}$, and the slope ($\beta$) are displayed in a table (Section 4.6).

Slope and log $IC_{50}$ were also compared across replicates based on random effects analysis of variance, treating the replicates as random effects with mean $\beta_{avg}$ and $\mu_{avg}$. $\beta$ and $\mu$ were estimated, separately within each replicate, and plotted along with the average across replicates, where

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

The associated 95% confidence interval from the analysis of variance across replicates is also displayed on separate plots for $\beta$ and $\mu$.

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

Within each replicate, quadruplicate repetitions were made of the Background Activity Control tubes and the Full Enzyme Activity Control tubes. Half the repetitions were carried out at the beginning of the replicate and half at the end. The control tubes at the beginning were equivalent to those at the end. The average of the four Background Activity Controls within a replicate must necessarily be 0 and the average of the four Full Enzyme Activity Controls within a replicate must necessarily be 100 when reported as a percent of control. The two beginning controls and the two end controls were 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 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 Control 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 were in control, the portion main effect and portion by replicate interaction were 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*

Purity Report: Appendix D

The radiochemical purity 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 0.585, 0.575, and 0.580 µg ASDN per gram 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.

| Assay Substrate Analysis Results | | | | |
|---|---|---|---|---|
| Radiochemical Code | Radiochemical ID | Radiochemical Stock Concentration (µCi/g) | Assay Substrate Final Concentration (µg/g) (ASDN + [$^3$H]ASDN) | Substrate soln Specific Activity (dpm/nmol) |
| [$^3$H]ASDN | MET-0251A | 0.632 | 0.585 | 687417 |
| [$^3$H]ASDN | MET-0251A | 0.684 | 0.575 | 757235 |
| [$^3$H]ASDN | MET-0251A | 0.750 | 0.580 | 821771 |

## *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 replicates. 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 | 05/24/2005 |

## *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 concentration of microsomes used was 6.76, 7.28, and 5.88 mg/mL for replicates 1, 2 and 3 respectively.

| Protein Analysis Results | | |
|---|---|---|
| Replicate | Assay Date | Protein stock concentration (mg/mL, measured) |
| 1 | 4/6/2005 | 6.76 |
| 2 | 4/7/2005 | 7.28 |
| 3 | 4/8/2005 | 5.88 |

## 4.4 *Control Aromatase Activity*

Figure 1 – Summary of Full Enzyme Activity Results
Figure 2 – Summary of Background Enzyme Activity Results
Appendix F – Individual Replicate Spreadsheets

Full Enzyme Activity Controls were conducted in duplicate at the beginning and end of each assay. The following table presents the mean beginning, end and overall activities (nmol(mg protein)$^{-1}$ min$^{-1}$) within and among replicates. Figure 1 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 Replicates 1, 2, and 3, the mean percent of control (beginning, end) values were (99.5, 100.5), (100.6, 99.4), and (100.7, 99.3), respectively, indicating that the conditions were constant within each replicate. The overall percent of control across replicates for the beginning portion was 100.3 and the end portion was 99.7, indicating that the conditions were constant across each replicate test.

| Full Enzyme Activity Control Aromatase Activity | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Replicate | Beginning (nmol(mg protein)$^{-1}$ min$^{-1}$) | End (nmol(mg protein)$^{-1}$ min$^{-1}$) | Within Replicate Mean | | | | Overall Mean | | | |
| | | | Mean | SD | Sem | % CV | Mean | SD | Sem | %CV |
| 1 | 0.271 | 0.274 | 0.272 | 0.005 | 0.002 | 1.77 | 0.284 | 0.032 | 0.009 | 11.4% |
| 2 | 0.256 | 0.253 | 0.254 | 0.011 | 0.006 | 4.36 | | | | |
| 3 | 0.328 | 0.323 | 0.326 | 0.005 | 0.003 | 1.62 | | | | |

In comparison with the Full Enzyme Activity for Replicates 1 through 3 shown above (average of 0.284 nmol(mg protein)$^{-1}$ min$^{-1}$), the Full Enzyme Activity for the 12/28/04, 12/29/04 and 12/30/04 replicates were 0.345, 0.396, and 0.595 nmol(mg protein)$^{-1}$ min$^{-1}$, respectively, with an average of 0.445 ± 0.132. The activity of these replicates was higher than expected. Inspection of the method revealed that the high activity was associated with the incorrect identification of the protein concentration of the microsomal stock (the solution being analyzed fell below the range of the standard curve of the protein assay). Therefore, the enzyme concentration was underestimated and resulted in an overestimation of the final aromatase activity (enzyme concentration is in the denominator of the equation used to determine activity). Changing the dilution scheme of the microsomes for Replicates 1 through 3 ensured that the concentration of the first dilution of microsomes was within the range of the standard curve resulting in the activity values shown in the preceding table.

Background Activity Controls were conducted in duplicate at the beginning and end of each assay. Figure 2 presents the individual Background Activity values and the percent of control values from 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 (potentially 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 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

Figure 3 – Plot of 4-OH ASDN Inhibition Replicate 1
Figure 4 – Plot of 4-OH ASDN Inhibition Replicate 2
Figure 5 – Plot of 4-OH ASDN Inhibition Replicate 3
Figure 6 – Plot of Average 4-OH ASDN Inhibition Per Replicate

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 8% across triplicate samples (tubes) at all concentrations and replicates.

Test Chemical Aromatase Activity – Percent of Control

| Test chemical | Repl | Log [test chemical] | Percent of Control | | | Overall | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Tube 1 | Tube 2 | Tube 3 | Mean | s.d. | s.e.m. | %CV |
| 4-OH ASDN | 1 | -6.00 | 7.13 | 7.38 | 7.54 | 7.35 | 0.21 | 0.12 | 2.81 |
| 4-OH ASDN | 1 | -7.00 | 42.63 | 40.54 | 40.76 | 41.31 | 1.15 | 0.66 | 2.78 |
| 4-OH ASDN | 1 | -7.30 | 58.03 | 58.90 | 17.43[a] | 58.46 | 0.62 | 0.36 | 1.05 |
| 4-OH ASDN | 1 | -7.60 | 76.81 | 72.53 | 73.52 | 74.29 | 2.24 | 1.29 | 3.01 |
| 4-OH ASDN | 1 | -8.00 | 89.60 | 92.60 | 91.77 | 91.32 | 1.55 | 0.89 | 1.70 |
| 4-OH ASDN | 1 | -9.00 | 104.39 | 99.62 | 102.51 | 102.17 | 2.41 | 1.39 | 2.35 |
| 4-OH ASDN | 2 | -6.00 | 6.32 | 6.45 | 6.76 | 6.51 | 0.22 | 0.13 | 3.44 |
| 4-OH ASDN | 2 | -7.00 | 37.74 | 37.31 | 37.47 | 37.51 | 0.21 | 0.12 | 0.57 |
| 4-OH ASDN | 2 | -7.30 | 56.26 | 56.47 | 53.38 | 55.37 | 1.73 | 1.00 | 3.13 |
| 4-OH ASDN | 2 | -7.60 | 71.20 | 69.05 | 69.26 | 69.84 | 1.19 | 0.68 | 1.70 |
| 4-OH ASDN | 2 | -8.00 | 85.72 | 88.46 | 86.66 | 86.95 | 1.40 | 0.81 | 1.60 |
| 4-OH ASDN | 2 | -9.00 | 99.44 | 97.60 | 98.16 | 98.40 | 0.94 | 0.54 | 0.96 |
| 4-OH ASDN | 3 | -6.00 | 5.89 | 6.64 | 6.81 | 6.45 | 0.49 | 0.28 | 7.59 |
| 4-OH ASDN | 3 | -7.00 | 36.57 | 38.69 | 38.90 | 38.05 | 1.29 | 0.74 | 3.39 |
| 4-OH ASDN | 3 | -7.30 | 52.48 | 53.86 | 54.76 | 53.70 | 1.15 | 0.66 | 2.13 |
| 4-OH ASDN | 3 | -7.60 | 67.87 | 76.43 | 71.26 | 71.85 | 4.31 | 2.49 | 6.00 |
| 4-OH ASDN | 3 | -8.00 | 86.67 | 96.28 | 84.92 | 89.29 | 6.11 | 3.53 | 6.85 |
| 4-OH ASDN | 3 | -9.00 | 97.90 | 103.29 | 95.56 | 98.92 | 3.96 | 2.29 | 4.01 |

[a] = Indicates value was not included in analysis

As shown in the following table, the inter-assay coefficient of variation in percent of control across replicates was also less than 8%. The highest concentration of 4-OH ASDN ($1 \times 10^{-6}$ M) inhibited aromatase activity to a mean of 6.77% of the Full Enzyme Activity 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 characterized by a sigmoidal dose response (See Figures 3, 4, 5 and 6). The lowest concentration of 4-OH ASDN ($1 \times 10^{-9}$ M) did not inhibit aromatase activity (98.40-102.17% of control; see following table, and 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 | | | |
|---|---|---|---|---|---|---|---|---|
| | | Repl 1 | Repl 2 | Repl 3 | Mean | s.d. | s.e.m. | % CV |
| 4-OH ASDN | -6.00 | 7.35 | 6.51 | 6.45 | 6.77 | 0.50 | 0.29 | 7.45 |
| 4-OH ASDN | -7.00 | 41.31 | 37.51 | 38.05 | 38.96 | 2.06 | 1.19 | 5.28 |
| 4-OH ASDN | -7.30 | 58.46 | 55.37 | 53.70 | 55.85 | 2.42 | 1.40 | 4.33 |
| 4-OH ASDN | -7.60 | 74.29 | 69.84 | 71.85 | 71.99 | 2.23 | 1.29 | 3.10 |
| 4-OH ASDN | -8.00 | 91.32 | 86.95 | 89.29 | 89.19 | 2.19 | 1.26 | 2.46 |
| 4-OH ASDN | -9.00 | 102.17 | 98.40 | 98.92 | 99.83 | 2.05 | 1.18 | 2.05 |

## 4.6 $IC_{50}$ and Slope Determination

Figure 7 – Overall 4-OH ASDN Inhibition Response Curve
Figure 8 – Response Curve Summary Table - Log $IC_{50}$ (μ) Results
Figure 9 – Response Curve Summary Table - Hill Slope (β) Results

Based on the curve fit to the percent of control aromatase activity across six concentrations of 4-OH ASDN, the calculated $IC_{50}$ values were 73.1, 61.2, and 62.1 nM for Replicates 1, 2, and 3, respectively. The overall curve fit to the data from the three replicates resulted in an $IC_{50}$ of 65.5 nM based on arithmetic calculation of the mean. The inter-assay variability (%CV) of the arithmetic $IC_{50}$ determination was 10.1%. Using the statistical method described in Section 3.7, the overall $IC_{50}$ based on $\mu_{AVG}$ and $\beta_{AVG}$ was 65.3 nM (See Figure 7). The Log $IC_{50}$ (μ) and 95% confidence interval is plotted per replicate and across replicates in Figure 8. The β (slope) and associated 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 2%.

| $IC_{50}$ and Slope Results | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Test chemical | Repli cate | Log [$IC_{50}$] | s.e. log[$IC_{50}$] | $IC_{50}$ (nM) | Slope (β) (se) | Status | Overall | | | |
| | | | | | | | $IC_{50}$ | SD | sem | %CV |
| 4-OH ASDN | 1 | -7.136 | 0.011 | 73.11 | -0.9929 (0.0196) | Complete | 65.48 | 6.63 | 3.83 | 10.1 |
| | 2 | -7.213 | 0.007 | 61.24 | -0.9689 (0.0130) | Complete | | | | |
| | 3 | -7.207 | 0.016 | 62.09 | -0.9866 (0.0301) | Complete | | | | |

Status = codes as described in the statistical analysis section that describe the curve fit that led to the $IC_{50}$ calculation.

## 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 $\mu_{AVG}$ and $\beta_{AVG}$. The overall Log $IC_{50}$ value was -7.185 (95% confidence interval of -7.293 to -7.078) resulting in the final $IC_{50}$ value of 65.3 nM. The overall slope was -0.977 (95% confidence interval of -1.021 to -0.934).

# 5.0 Discussion

The results of this positive control study using commercially available human recombinant microsomes from baculovirus and the known aromatase inhibitor 4-OH ASDN were as expected based on the prevalidation work (WA 2-24 and 4-10), the training data (WA 4-16, Task 3) and the results of WA 4-16 Task 4 (WIL-431006). Generally, treating the microsomes with $1x10^{-9}$ to $1x10^{-6}$ M 4-OH ASDN resulted in a sigmoidal dose response curve ranging from no inhibition (99.83% of control) to almost full inhibition (6.77%). Therefore, the response curves were considered complete curves. The overall $IC_{50}$ in this study for 4-OH ASDN was 65.5 nM

(arithmetic mean). 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.

A potential problem arose, which was identified by unusually high Full Enzyme Activity Controls in replicates conducted on 12/28/04, 12/29/04 and 12/30/04. Inspection of the data worksheets revealed that the concentration of the microsomes in the protein assay were below the range of the standard curve. Protein concentrations outside the range of the standard curve may not be correctly calculated. If the protein concentration was underestimated by the protein assay, the aromatase activity would be high, as activity is calculated per milligram of protein. The replicates were repeated (Replicates 1, 2, and 3) with an adjusted microsome dilution scheme to ensure that the solution being assayed for protein concentration was within the range of the standard curve. The average activity for the December replicates was $0.445 \pm 0.132$ nmol(mg protein)$^{-1}$ min$^{-1}$, calculated using $4.6 \pm 0.8$ mg/mL protein. The average activity for Replicates 1 through 3 was $0.28 \pm 0.03$ nmol(mg protein)$^{-1}$ min$^{-1}$, calculated using $6.6 \pm 0.7$ mg/mL protein.

# 6.0 Conclusion

The response of the recombinant human aromatase in baculovirus microsomes using the known aromatase inhibitor 4-hydroxyandrostenedione as a positive control was successfully validated in this laboratory. Additional testing of this microsomal system using nine additional test chemicals is planned under WA 4-17, Task 4 (WIL-431011).

# 7.0 References

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

US EPA, Quality Assurance Project Plan (QAPP) for Work Assignment 4-17 Recombinant Aromatase Validation Study, (WA 4-17, Task 3), WIL Research Laboratories, LLC, Ashland, OH, 2004

# 8.0 Key Personnel and Report Submission

Report Submitted By:

| | |
|---|---|
| Christopher J. Bowman, Ph.D.<br>Staff Toxicologist, Developmental<br>and Reproductive Toxicology<br>Study Director | Date |

Report Reviewed By:

| | |
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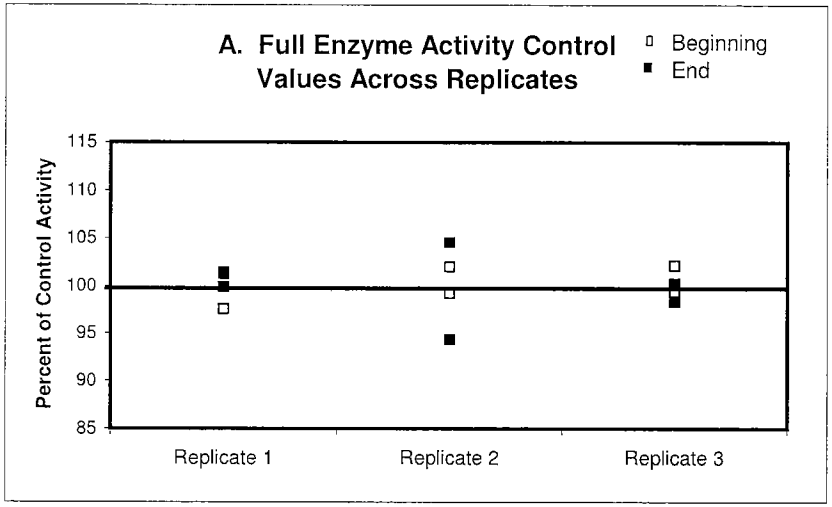
| Terry L. Johnson, Ph.D.<br>Associate Director, Metabolism | Date |
| Mark D. Nemec, B.S., D.A.B.T.<br>Director, Developmental and<br>Reproductive Toxicology | Date |
| Daniel W. Sved, Ph.D.<br>Director, Metabolism and Analytical Chemistry | Date |
| Jennifer A. Thomas-Wohlever, Ph.D.<br>Research Scientist, Metabolism | Date |
| Robert A. Wally, B.S., RAC<br>Acting Manager, Reporting and Regulatory<br>Technical 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 | 0.2656 | 97.6 | | | 0.2521 | 99.2 | | | 0.3326 | 102.1 | | | | |
| | 0.2760 | 101.4 | 0.2708 | 99.5 | 0.2593 | 102.0 | 0.2557 | 100.6 | 0.3234 | 99.3 | 0.3280 | 100.7 | 0.2848 | 100.3 |
| End | 0.2718 | 99.8 | | | 0.2657 | 104.5 | | | 0.3263 | 100.2 | | | | |
| | 0.2755 | 101.2 | 0.2737 | 100.5 | 0.2399 | 94.3 | 0.2528 | 99.4 | 0.3202 | 98.3 | 0.3233 | 99.3 | 0.2832 | 99.7 |
| | Average % Activity: | 100 | | | | 100 | | | | 100 | | | | |



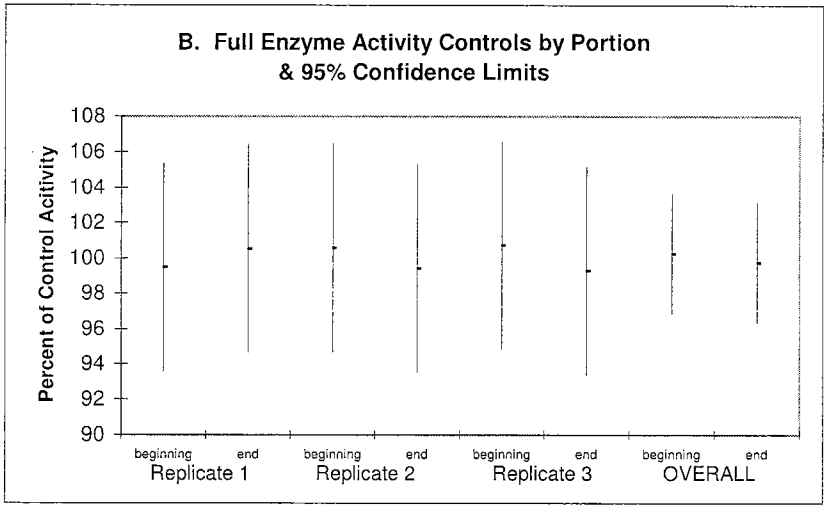
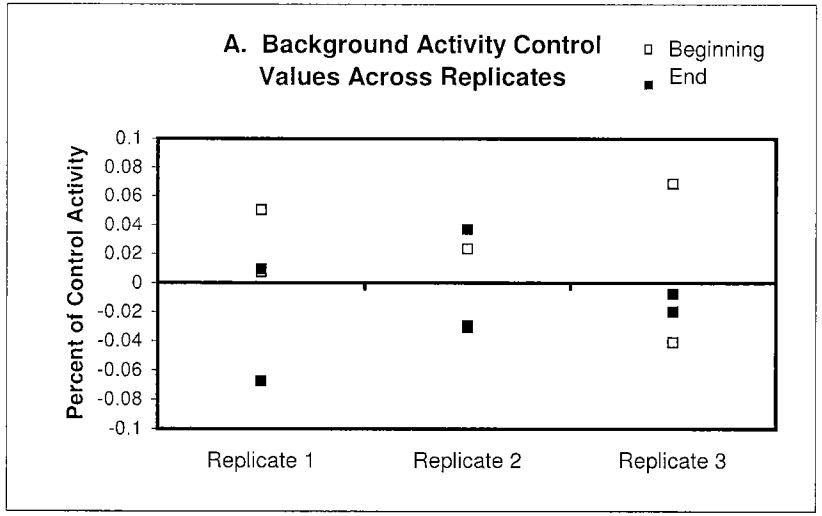
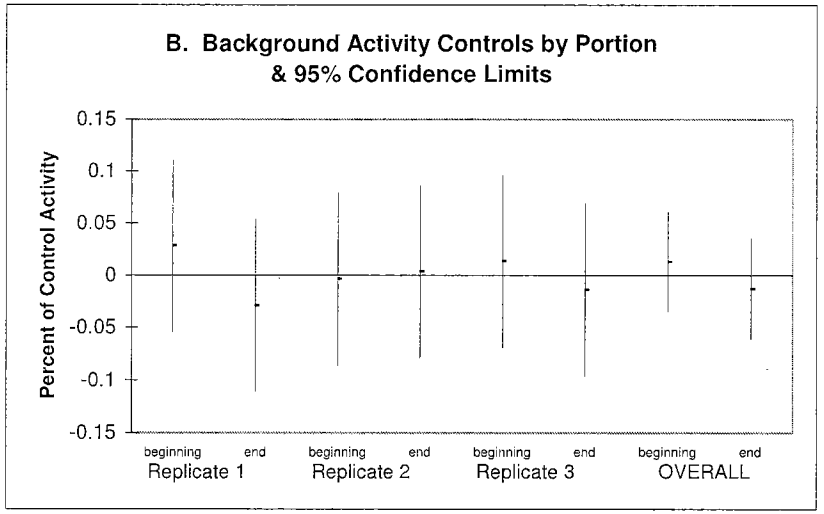



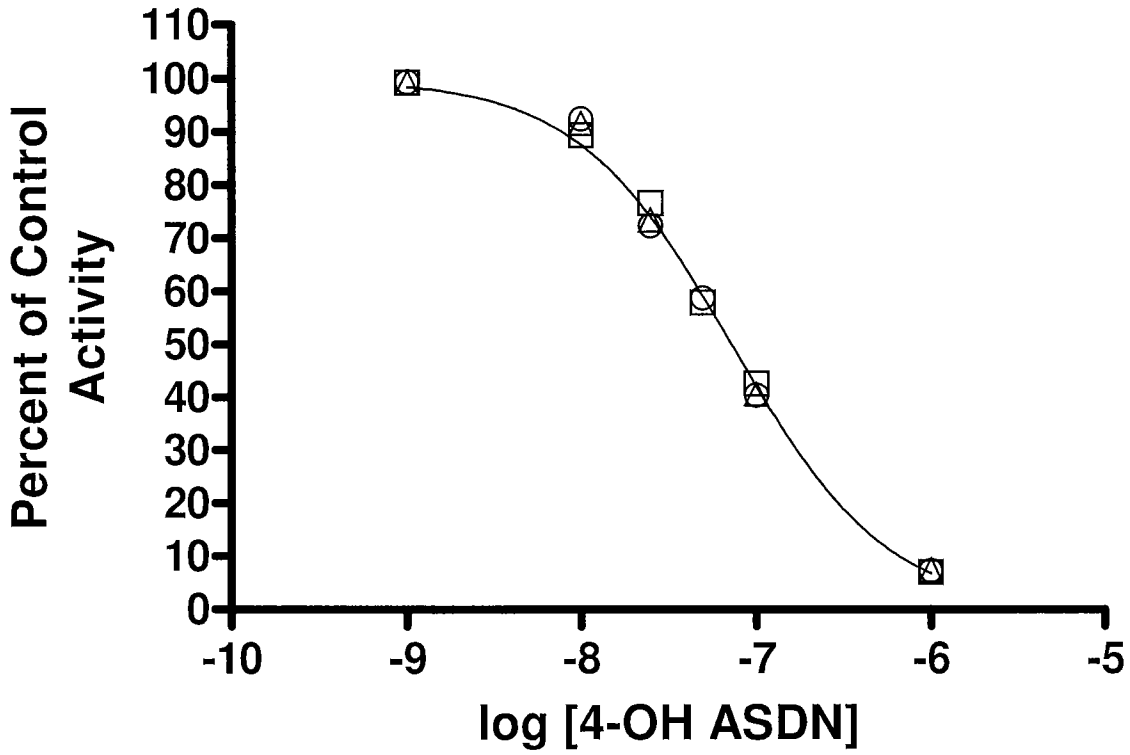
Figure 2. Summary of Background Enzyme Activity Results

| | 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 | 1.37E-04 | 0.050 | | | 5.94E-05 | 0.0234 | | | 2.23E-04 | 0.0686 | | | | |
| | 2.00E-05 | 0.007 | 7.86E-05 | 0.029 | -7.77E-05 | -0.0306 | -9.12E-06 | -0.004 | -1.34E-04 | -0.0411 | 4.48E-05 | 0.014 | 3.81E-05 | 0.013 |
| End | 2.60E-05 | 0.010 | | | -7.60E-05 | -0.0299 | | | -6.50E-05 | -0.0200 | | | | |
| | -1.83E-04 | -0.067 | -7.86E-05 | -0.029 | 9.42E-05 | 0.0371 | 9.12E-06 | 0.004 | -2.46E-05 | -0.0076 | -4.48E-05 | -0.014 | -3.81E-05 | -0.013 |
| | Sum of Background Activity: | 0 | | | | -1.2E-16 | | | | -4.9E-17 | | | | |



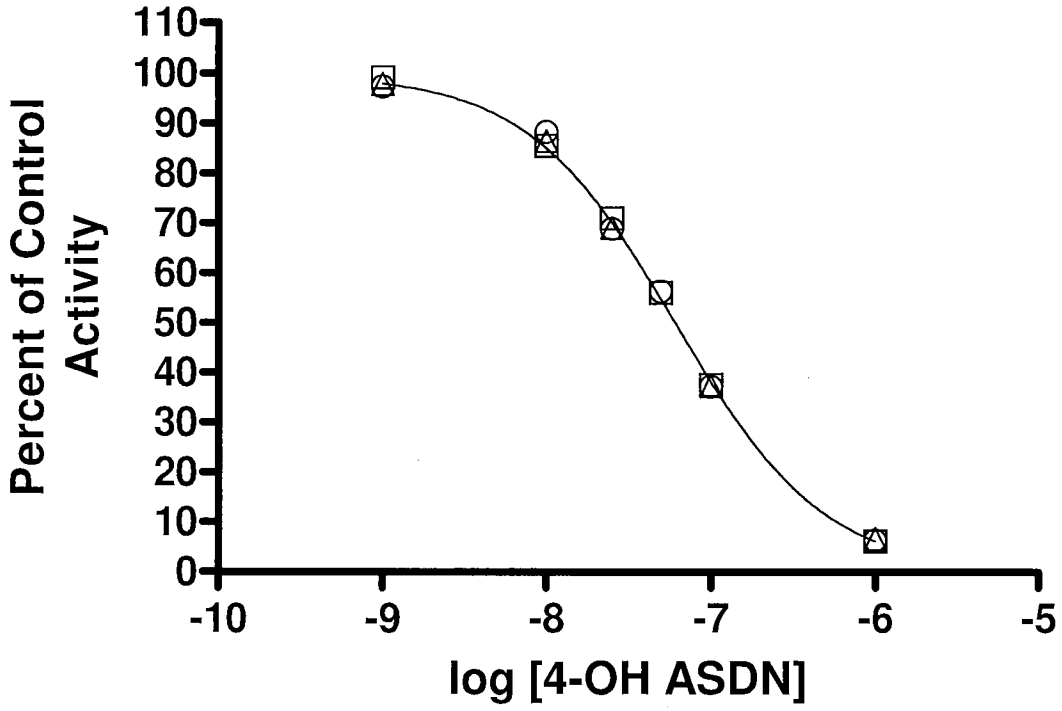
# Figure 3. Plot of 4-OH ASDN Inhibition Replicate 1




| | Replicate 1 |
|---|---|
| aromatase eqn | |
| Best-fit values | |
| LOGEC50 | -7.136 |
| HILLSLOPE | -0.9929 |
| Std. Error | |
| LOGEC50 | 0.01078 |
| HILLSLOPE | 0.01961 |
| 95% Confidence Intervals | |
| LOGEC50 | -7.159 to -7.113 |
| HILLSLOPE | -1.035 to -0.9511 |
| Goodness of Fit | |
| Degrees of Freedom | 15 |
| $R^2$ (unweighted) | 0.9965 |
| Weighted Sum of Squares (1/Y) | 0.8578 |
| Absolute Sum of Squares | 62.16 |
| Sy.x | 2.036 |
| Data | |
| Number of X values | 6 |
| Number of Y replicates | 3 |
| Total number of values | 17 |
| Number of missing values | 1 |

| log [4-OH ASDN] | Percent of Control Activity | | |
|---|---|---|---|
| | Y1 | Y2 | Y3 |
| -6.00 | 7.13 | 7.38 | 7.54 |
| -7.00 | 42.63 | 40.54 | 40.76 |
| -7.30 | 58.03 | 58.90 | ~~17.43~~ |
| -7.60 | 76.81 | 72.53 | 73.52 |
| -8.00 | 89.60 | 92.60 | 91.77 |
| -9.00 | 99.50 | 99.62 | 99.50 |

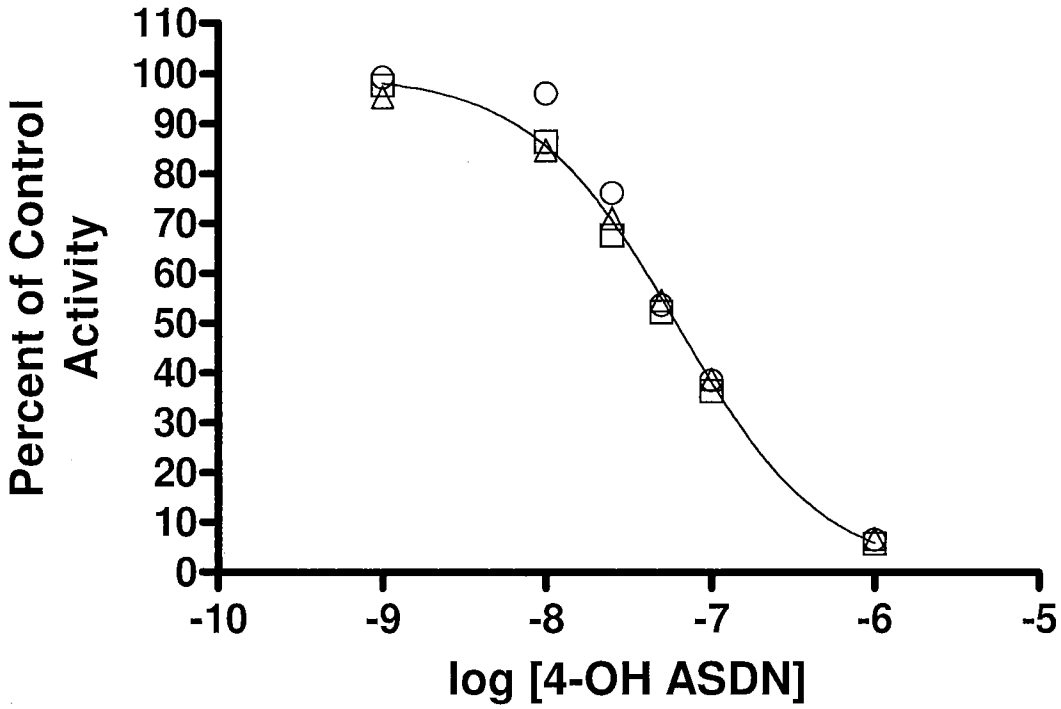
# Figure 4. Plot of 4-OH ASDN Inhibition Replicate 2




| | Replicate 2 |
|---|---|
| aromatase eqn | |
| Best-fit values | |
| LOGEC50 | -7.213 |
| HILLSLOPE | -0.9689 |
| Std. Error | |
| LOGEC50 | 0.006991 |
| HILLSLOPE | 0.01298 |
| 95% Confidence Intervals | |
| LOGEC50 | -7.228 to -7.198 |
| HILLSLOPE | -0.9965 to -0.9414 |
| Goodness of Fit | |
| Degrees of Freedom | 16 |
| $R^2$ (unweighted) | 0.9984 |
| Weighted Sum of Squares (1/Y) | 0.4352 |
| Absolute Sum of Squares | 27.12 |
| Sy.x | 1.302 |
| Data | |
| Number of X values | 6 |
| Number of Y replicates | 3 |
| Total number of values | 18 |
| Number of missing values | 0 |

| log [4-OH ASDN] | Percent of Control Activity | | |
|---|---|---|---|
| | Y1 | Y2 | Y3 |
| -6.00 | 6.32 | 6.45 | 6.76 |
| -7.00 | 37.74 | 37.31 | 37.47 |
| -7.30 | 56.26 | 56.47 | 53.38 |
| -7.60 | 71.20 | 69.05 | 69.26 |
| -8.00 | 85.72 | 88.46 | 86.66 |
| -9.00 | 99.44 | 97.60 | 98.16 |

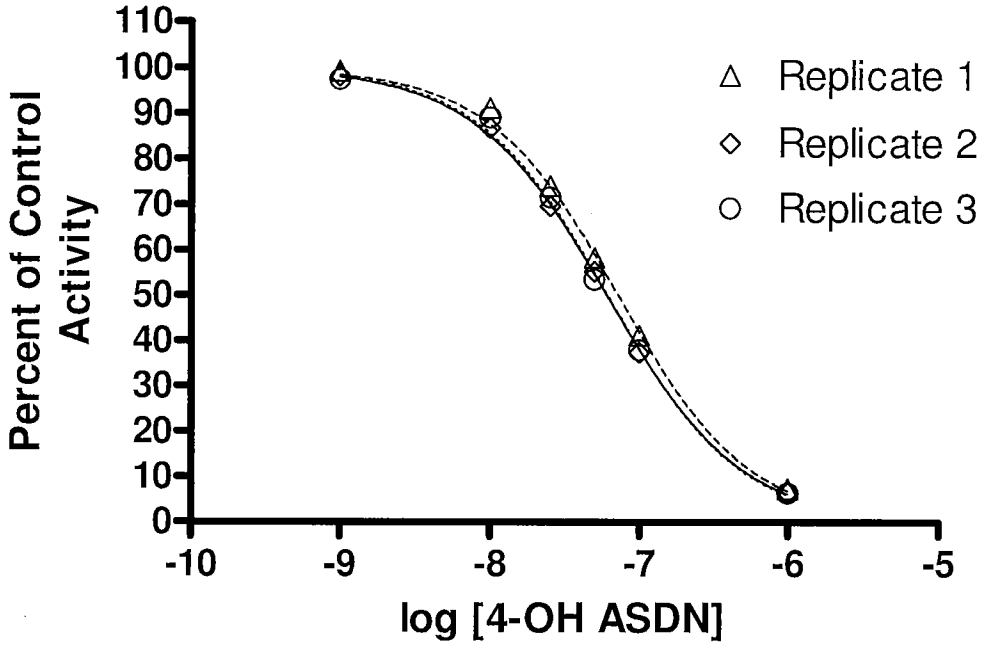
# Figure 5. Plot of 4-OH ASDN Inhibition Replicate 3




| | Replicate 3 |
|---|---|
| aromatase eqn | |
| Best-fit values | |
| LOGEC50 | -7.207 |
| HILLSLOPE | -0.9866 |
| Std. Error | |
| LOGEC50 | 0.01557 |
| HILLSLOPE | 0.03007 |
| 95% Confidence Intervals | |
| LOGEC50 | -7.240 to -7.174 |
| HILLSLOPE | -1.050 to -0.9229 |
| Goodness of Fit | |
| Degrees of Freedom | 16 |
| $R^2$ (unweighted) | 0.9901 |
| Weighted Sum of Squares (1/Y) | 2.213 |
| Absolute Sum of Squares | 174.3 |
| Sy.x | 3.300 |
| Data | |
| Number of X values | 6 |
| Number of Y replicates | 3 |
| Total number of values | 18 |
| Number of missing values | 0 |

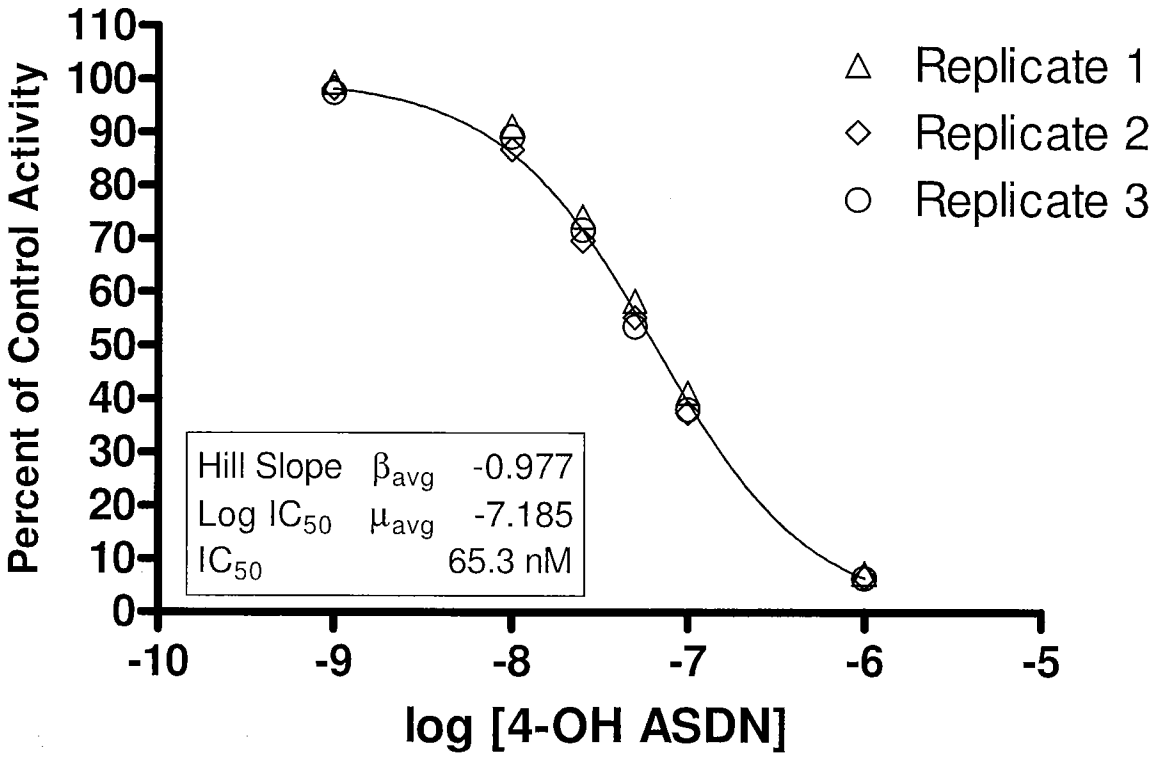
| log [4-OH ASDN] | Percent of Control Activity | | |
|---|---|---|---|
| | Y1 | Y2 | Y3 |
| -6.00 | 5.89 | 6.64 | 6.81 |
| -7.00 | 36.57 | 38.69 | 38.90 |
| -7.30 | 52.48 | 53.86 | 54.76 |
| -7.60 | 67.87 | 76.43 | 71.26 |
| -8.00 | 86.67 | 96.28 | 84.92 |
| -9.00 | 97.90 | 99.50 | 95.56 |

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



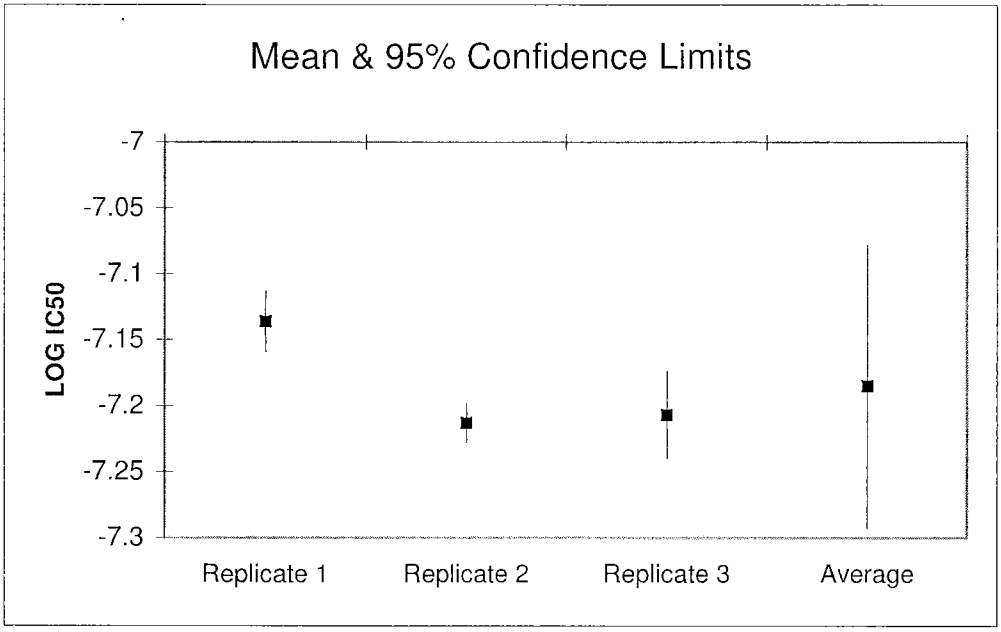

| | Replicate 1 | Replicate 2 | Replicate 3 |
|---|---|---|---|
| aromatase eqn | | | |
| Best-fit values | | | |
| LOGEC50 | -7.136 | -7.213 | -7.207 |
| HILLSLOPE | -0.9929 | -0.9689 | -0.9866 |
| Std. Error | | | |
| LOGEC50 | 0.01078 | 0.006991 | 0.01557 |
| HILLSLOPE | 0.01961 | 0.01298 | 0.03007 |
| 95% Confidence Intervals | | | |
| LOGEC50 | -7.159 to -7.113 | -7.228 to -7.198 | -7.240 to -7.174 |
| HILLSLOPE | -1.035 to -0.9511 | -0.9965 to -0.9414 | -1.050 to -0.9229 |
| Goodness of Fit | | | |
| Degrees of Freedom | 15 | 16 | 16 |
| $R^2$ (unweighted) | 0.9965 | 0.9984 | 0.9901 |
| Weighted Sum of Squares (1/Y) | 0.8578 | 0.4352 | 2.213 |
| Absolute Sum of Squares | 62.16 | 27.12 | 174.3 |
| Sy.x | 2.036 | 1.302 | 3.300 |
| Data | | | |
| Number of X values | 6 | 6 | 6 |
| Number of Y replicates | 3 | 3 | 3 |
| Total number of values | 17 | 18 | 18 |
| Number of missing values | 1 | 0 | 0 |

# Figure 7. Overall 4-OH ASDN Inhibition Response Curve



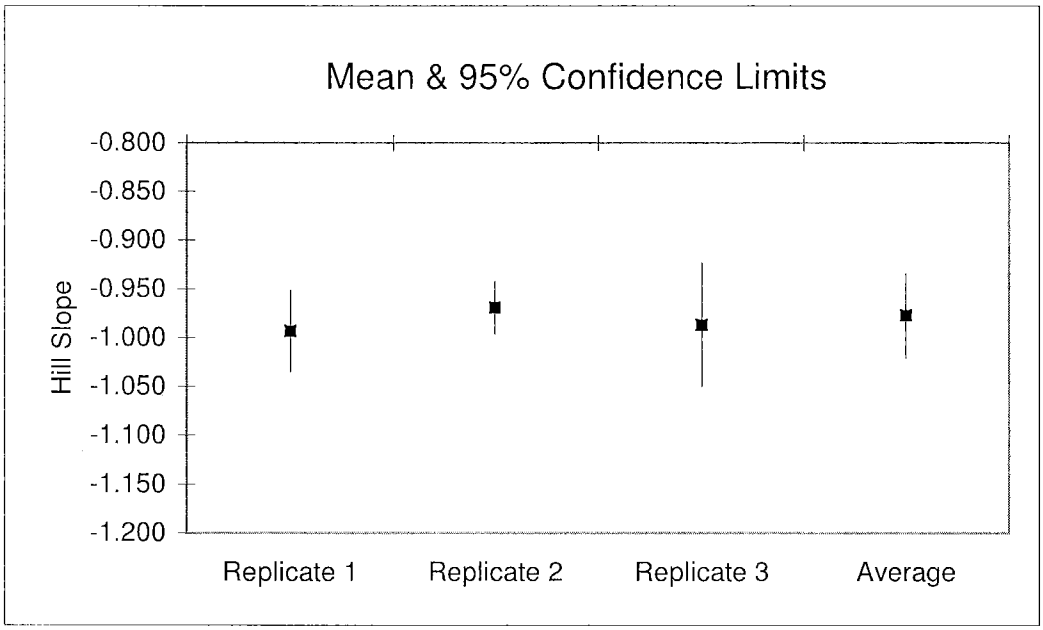
# Figure 8. Response Curve Summary Table - LOG $IC_{50}$

| | | Replicate | Status | Values | Std. Error | 95% Confidence Level | |
|---|---|---|---|---|---|---|---|
| μ | LOG IC50 | 1 | C | -7.136 | 0.011 | -7.159 | -7.113 |
| μ | LOG IC50 | 2 | C | -7.213 | 0.007 | -7.228 | -7.198 |
| μ | LOG IC50 | 3 | C | -7.207 | 0.016 | -7.240 | -7.174 |
| μ (avg) | AVG LOG IC50 | | | -7.185 | 0.025 | -7.293 | -7.078 |



# Figure 9. Response Curve Summary Table - Hill Slope

| | | Replicate | Status | Values | Std. Error | 95% Confidence Level | |
|---|---|---|---|---|---|---|---|
| β | HILLSLOPE | 1 | C | -0.9929 | 0.020 | -1.035 | -0.951 |
| β | HILLSLOPE | 2 | C | -0.9689 | 0.013 | -0.997 | -0.941 |
| β | HILLSLOPE | 3 | C | -0.9866 | 0.030 | -1.050 | -0.923 |
| β (avg) | AVG HILLSLOPE | | | -0.977 | 0.01 | -1.021 | -0.934 |



# Appendix A

## Study Protocol

Study Number: WIL-431010

**PROTOCOL AMENDMENT I**

Sponsor: Battelle Memorial Institute

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

A. Title of Study:

Validation of the Recombinant Microsomal Aromatase Assay: Positive Control Study (WA 4-17, Task 3)

B. Protocol Additions/Modifications:

1) **4.2.3 Lot Number:**

This section is changed to the following:

SW0045

2) **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.

3) **10 STATISTICAL ANALYSES:**

This entire section is changed to the following:

**10.1 Concentration Response Fits for the Test Substance**

For the test substance multiple independent replicates of the concentration response curve fit were carried out. The number of replicates conducted were three. 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
$\beta$ ≡ slope of the concentration response curve ($\beta$ 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 $\varepsilon$ 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 $\mu$) and slope ($\beta$). 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 $\log_{10}IC_{50}$ or $\beta$ can be calculated based on a one-way random effects analysis of variance model fit.

[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%.

For each test substance and replicate the estimated $\log_{10}IC_{50}$ ($\mu$), the within replicate standard error of $\mu$, the $IC_{50}$, the slope ($\beta$), the within replicate standard error of $\beta$, 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 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 ($\beta$, $\mu$) as a random variable with mean ($\beta_{avg}$, $\mu_{avg}$). 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 ($\beta$) and $\log_{10}IC_{50}$ ($\mu$) will also be compared across replicates based on random effects analysis of variance, treating the replicates as random effects. $\beta$ and $\mu$ 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.

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

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

4) 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

Study Number: WIL-431010

**PROTOCOL AMENDMENT II**

Sponsor: Battelle Memorial Institute

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

A. Title of Study:

Validation of the Recombinant Microsomal Aromatase Assay: Positive Control Study (WA 4-17, Task 3)

B. Protocol Additions/Modifications:

1) **3** **STUDY SCHEDULE:**

This section is changed to the following:

| | |
|---|---|
| Proposed First Replicate Assay Date: | December 28, 2004 |
| Proposed Last Replicate Assay Date: | March 31, 2005 |
| Proposed Unaudited Data Submission Date: | April 8, 2005 |
| Proposed Audited Report Date: | April 27, 2005 |

2) **8** **$IC_{50}$ DETERMINATION OF AROMATASE ASSAY RESULTS:**

The following is added to this section:

Three additional independent experimental replicates ($4^{th}$, $5^{th}$ and $6^{th}$) will be conducted under the same conditions as the first three replicates except that adjustments will made to ensure that the protein concentration of the microsomes fall within the range of the standard curve in the protein assay described in section 6.

3) **9** **DATA ANALYSIS:**

The following is added to this section:

Data from the first 3 replicates dated December 28, 29 and 30, 2004 will be presented as an appendix to the report and referred to by date (spreadsheets and curve fits only).

4) **10.1** **Concentration Response Fits for the Test Substance:**

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

For the test substance, multiple independent replicates of the concentration response curve fit will be carried out. The number of replicates conducted will be six. Only the data from the 4th, 5th and 6th replicates will be analyzed as described in section 10. The first 3 replicates will not be analyzed as described in section 10 since the microsomal protein determination values were outside the range of the protein assay standard curve. For reporting purposes, replicates 4, 5 and 6 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.

C. Reasons for Protocol Additions/Modifications:

1) The study schedule has been revised based on the Sponsor request to repeat the first three replicates (see March 14, 2005 e-mail).

2) The Sponsor has requested that the first three replicates be repeated so that the protein concentrations of the microsomes fall within the range of the standard curve in the protein assay.

3) This statement was added to clarify that the data from the first three replicates will only be presented as an appendix to the report.

4) This section was changed to clarify that only data from the last three replicates would be presented and analyzed according to section 10. Also, clarification is provided as to how the replicates will be referred to in the final report.

Approved By:

**Battelle Memorial Institute**

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

3-22-05
Date

Prepared By:

**WIL Research Laboratories, LLC**

Christopher J. Bowman, Ph.D.
Study Director

3/21/05
Date

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

3/25/2005
Date

Study Number: WIL-431010

**PROTOCOL AMENDMENT III**

Sponsor: Battelle Memorial Institute
EPA Contract No.: 68-W-01-023

A. Title of Study:

Validation of the Recombinant Microsomal Aromatase Assay: Positive Control Study (WA 4-17, Task 3)

B. Protocol Modification:

1) **10.3** **Full Enzyme Activity and Background Activity Control Values Across Replicates:**

The seventh sentence of the second paragraph is changed to the following (change in bold):

For the purposes of evaluation, replicate will be treated as a **random** effect.

C. Reason for Protocol Modification:

1) The replicate was changed from a "fixed effect" to a "random effect" to have this effect treated consistently throughout the statistical analysis plan.

| COPIES TO | |
|---|---|
| ACCOUNTING | Y |
| ANALYTICAL | Y |
| AQUATICS | |
| CENTRAL FILE | Y |
| CLIN. PATH | Y |
| DART LAB | Y |
| HISTOLOGY | Y |
| INHALATION | |
| IN-LIFE MANAGER | 2 |
| METABOLISM | |
| NECROPSY | Y |
| PHARMACY | Y |
| PATHOLOGIST | |
| QA | Y |
| RPT. WRITING | 2 |
| STUDY BOOK | B |
| STUDY DIRECTOR | CJB |

AH JHW
CLP JLH
OAS RP
WLP MS2

Approved By:
**Battelle Memorial Institute**

Jerry D. Johnson

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

5-31-05
Date

Prepared By:
**WIL Research Laboratories, LLC**

Christopher J. Bowman, Ph.D.
Study Director

5/27/05
Date

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

6/2/2005
Date

# PROTOCOL

## VALIDATION OF THE RECOMBINANT MICROSOMAL AROMATASE ASSAY: POSITIVE CONTROL STUDY (WA 4-17, TASK 3)

**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 3: 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 recombinant 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 recombinant 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 a human recombinant microsome 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 recombinant 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.
Work Assignment Leader/Study Monitor
Endocrine Disruptor Screening Program
Battelle Memorial Institute
Tel: (614) 424-4499
Fax: (614) 424-5221
Email: johnsojd@battelle.org

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

### 2.2 U.S. EPA Representatives:

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

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

**2.3 WIL Study Director:**

Christopher J. Bowman, Ph.D.
Staff Toxicologist, Developmental
and Reproductive Toxicology
Tel: (419) 289-8700
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Email: cbowman@wilresearch.com

**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 $\mu$L 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 $\mu$L 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 recombinant 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 ([1$\beta$-$^3$H]-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

Recombinant microsomes will be supplied to each laboratory by the lead laboratory (RTI International). Human recombinant microsomes will be obtained from Gentest™ (Woburn, MA). The product name is Human CYP19 (Aromatase) Supersomes™ and the catalog number is 456260. The Supersomes™ package size is 0.5 nmoles cytochrome P450 in 0.5 mL. Supplier-provided values for protein concentration, cytochrome c reductase activity, and aromatase activity will be found on the data sheet accompanying each shipment and will be included in the report. The microsomes must be stored between approximately -70 and -80°C. Microsomes may be thawed and re-frozen up to 4 times if necessary.

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 0.008 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 (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 Recombinant Microsomal Aromatase Assay-Optimized Conditions | |
|---|---|
| Microsomal Protein | 0.004 mg/mL[a] |
| NADPH | 0.3 mM[a] |
| [3H]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 $^3$H2O 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 $^3$H2O 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 $IC_{50}$ 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 | $1 \times 10^{-4}$ | $1 \times 10^{-6}$ |
| 4-OH ASDN Concentration 2 | 3 | 4-OH ASDN added | $1 \times 10^{-5}$ | $1 \times 10^{-7}$ |
| 4-OH ASDN Concentration 3 | 3 | 4-OH ASDN added | $5 \times 10^{-6}$ | $5 \times 10^{-8}$ |
| 4-OH ASDN Concentration 4 | 3 | 4-OH ASDN added | $2.5 \times 10^{-6}$ | $2.5 \times 10^{-8}$ |
| 4-OH ASDN Concentration 5 | 3 | 4-OH ASDN added | $1 \times 10^{-6}$ | $1 \times 10^{-8}$ |
| 4-OH ASDN Concentration 6 | 3 | 4-OH ASDN added | $1 \times 10^{-7}$ | $1 \times 10^{-9}$ |

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.

IC50 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:

$$Y=\text{Bottom} + (\text{Top}-\text{Bottom})/(1+10^{((\text{LogIC}_{50}-X)*\text{HillSlope})})$$

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
$\beta$ = slope of the concentration response curve ($\beta$ 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 $\varepsilon$ 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 $\mu$) and slope ($\beta$). 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 $\beta$ 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 $(\beta, \mu)$ as a random variable with mean $(\beta_{avg}, \mu_{avg})$ and covariance $\Sigma_{(\beta, \mu)}$ 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 $\Sigma_{(\beta, \mu)}$ 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

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

Slope $(\beta)$ and $\log_{10}IC_{50}$ $(\mu)$ will also be compared across replicates based on random effects analysis of variance, treating the replicates as random effects. $\beta$ and $\mu$ 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 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 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."

WIL Research Laboratories will submit one copy of an audited draft report in a timely manner upon completion of data collection prior to issuance of the final report. One revision 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 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 within one month following receipt of comments. If the Sponsor's comments and/or authorization to finalize the report have not been received at 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) of the final report on CD-R will be provided. Requests for additional 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
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Jerry D. Johnson 12-9-04
Jerry D. Johnson, Ph.D., D.A.B.T. Date
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12/9/04
David P. Houchens, Ph.D. Date
Program Manager
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**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
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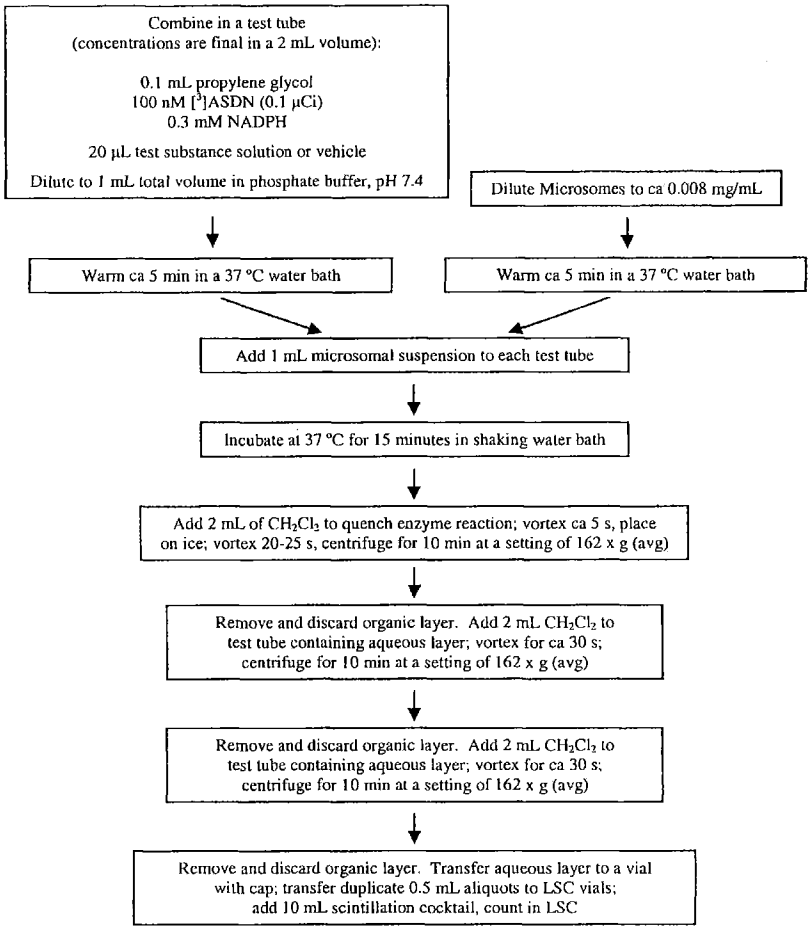
## Appendix A


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 0.008 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.

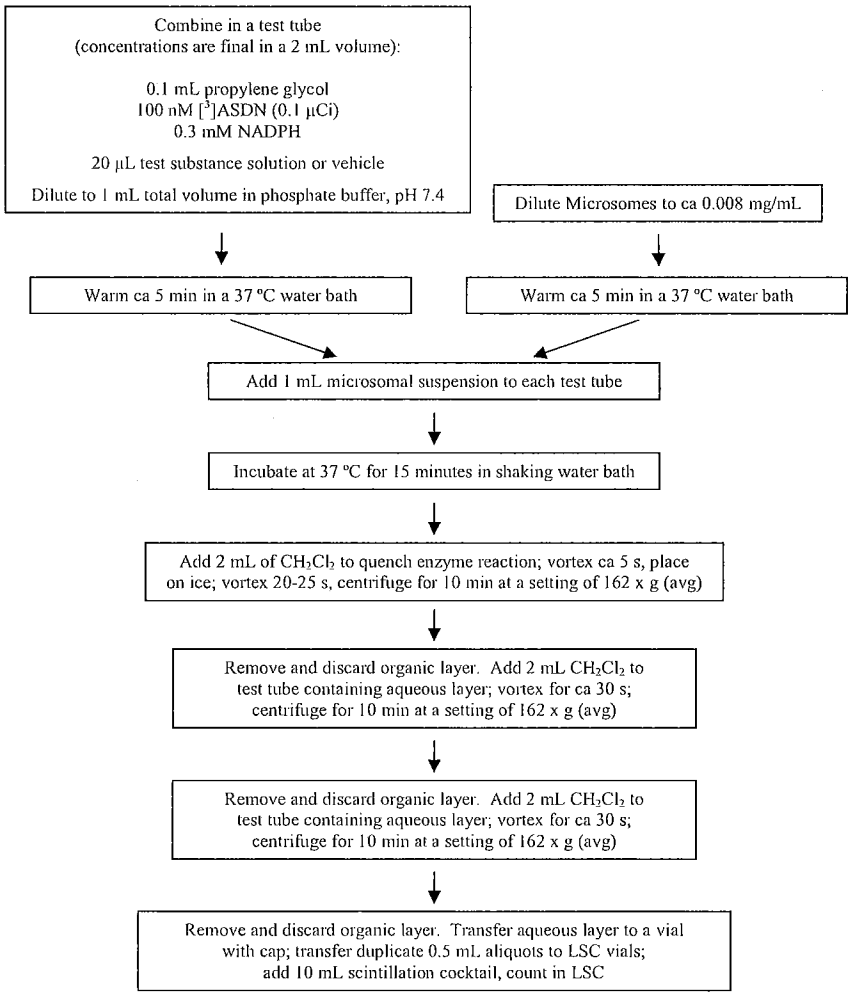
- **Protocol Section 6.** states that for the protein assay, a six-point standard curve would be prepared, ranging from 0.13 to 1.5 mg protein/mL. Due to the bovine serum albumin (BSA) standard (Reference 431006A1-2-1) having a concentration of 2.36 mg/mL and not 2.5 mg/mL, the standard curve had a range of 0.12 to 1.4.

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

# Appendix B

## Assay Procedure

## Appendix B


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 0.008 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

# Appendix C

## Quality Assurance Project Plan

## 1.0 TITLE AND APPROVAL

**Quality Assurance Project Plan (QAPP)**
**For Work Assignment 4-17**
**Recombinant Aromatase Validation Study**

**Task 3 - Conduct Positive Control Studies**
**in Participating Laboratories**

**for**

**EPA CONTRACT NUMBER 68-W-01-023**

**December 7, 2004**

## SIGNATURE PAGE

**Quality Assurance Project Plan for WA 4-17**
**Recombinant Aromatase Validation Study**
**EPA CONTRACT NUMBER 68-W-01-023**

**Concurrences and Approvals**

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

Linda J Phillips 12/7/04
*Signature* *Date*

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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 recombinant microsomes, demonstrate the utility of the microsomal assay to detect known aromatase inhibitors, and compare the performance of the 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, and (2) to test up to 10 reference chemicals with different modes of action in order to test 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 3 and 4 of the work assignment.

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

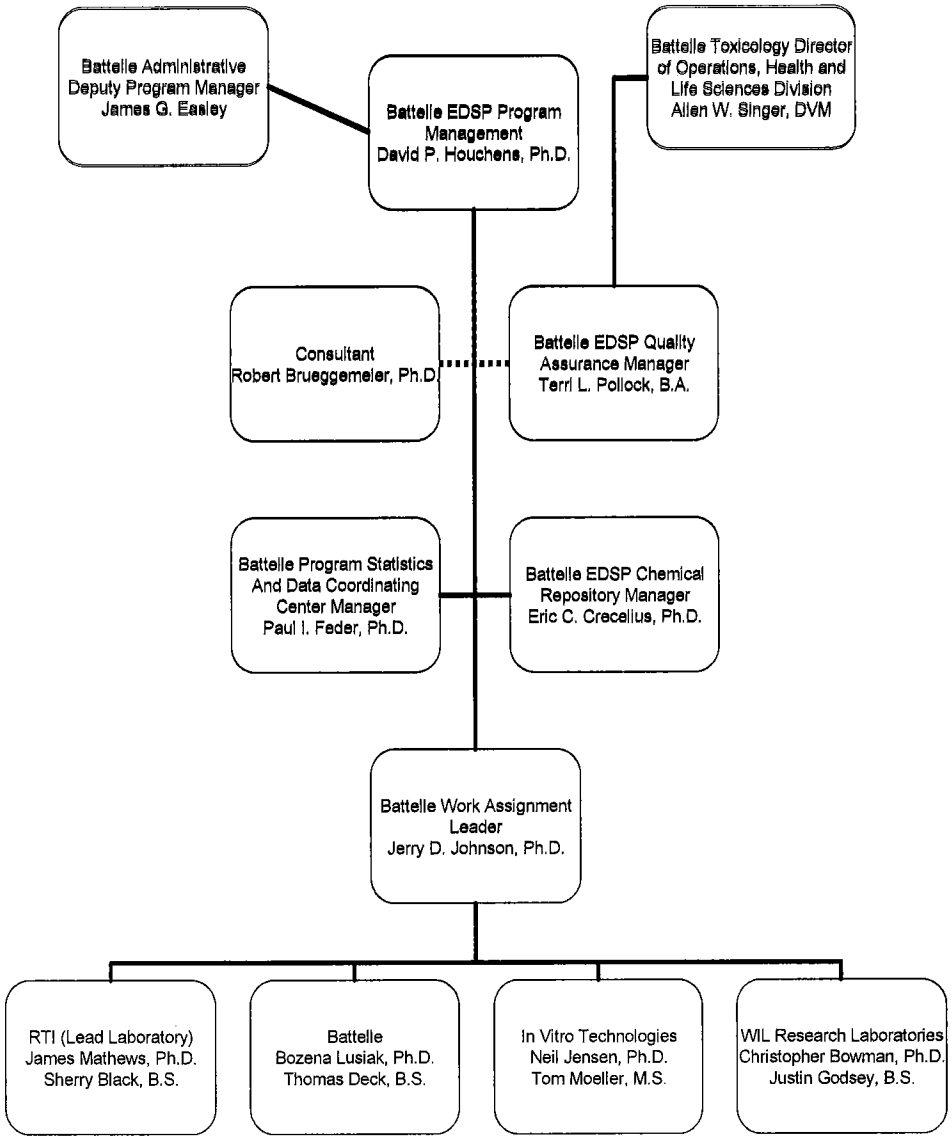


**Figure 4-1. WA 4-17 Organizational Chart**

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 WAL/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 recombinant aromatase assay (WA 2-24) were conducted to optimize the microsomal aromatase assay protocol for recombinant microsomes, demonstrate the utility of the microsomal assay to detect known aromatase inhibitors, and compare the performance of the recombinant assay system and the human placental microsomal assay system.

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 recombinant aromatase assay. A companion work assignment (WA 4-16) has been issued for the conduct of the human placental aromatase assay validation.

The work assignment is comprised of 6 tasks of which two tasks involve experimentation. Only the work in Task 3 is subject to this QAPP. Table 1 summarizes the validation tasks and the laboratory(ies) involved for each experimental task.

**Table 1. WA 4-17 Tasks**

| 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 | Conduct Positive Control Studies in the Participating Laboratories | 3 Participating Laboratories |
| 4 | Conduct Multiple Chemical Studies with Microsomes Supplied by RTI (RTI/Participating Laboratories) | Lead Laboratory + 3 Participating Laboratories |
| 5 | Prepare Study Reports (RTI/Participating Laboratories) | Not an experimental task |
| 6 | Prepare Presentation for EDMVAC | Not an experimental task |

EDMVAC = Endocrine Disruptor Method Validation Committee

## 5.2 Background

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

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

Aromatase is a cytochrome P450 enzyme complex responsible for estrogen biosynthesis and converts androgens, such as testosterone and androstenedione, into the estrogens estradiol and estrone. Aromatase is present in the ovary, placenta, uterus, testis, brain, and extraglandular adipose tissues. Two proteins, cytochrome $P450_{arom}$ 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 recombinant 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, demonstrate the utility of the microsomal assay to detect known aromatase inhibitors, and compare the performance of a recombinant assay system and the human 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 recombinant microsome aromatase assay.

## 6.0 PROJECT/TASK DESCRIPTION

Only Task 3 is under the control by this QAPP. However, this QAPP also addresses the other task in this work assignment and will be reissued prior to the start of the other task together with a finalized task-specific protocol included as an attachment. The Task 3 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 3: Conduct of the 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 recombinant 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) is tested in the aromatase assay at 6 concentrations to construct a dose/response curve from which an $IC_{50}$ may be calculated. Control runs are also 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.

**Task 4: Conduct Studies on Reference Chemicals**

Each participating laboratory will conduct the studies in this task with recombinant microsomes supplied by RTI.

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 must 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 4-17 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 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.

Staff from the participating laboratories will be trained on the performance of the aromatase assay at RTI International as part of Task 3 of WA 4-16. 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 tasks. 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 each task subject to this QAPP will be contained in GLP compliant protocols. A template protocol for Task 3 is attached as an Appendix.

## 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 WA Leader/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 be also 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 is 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 Mamager (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 Recombinant Aromatase Validation Study, EPA Contract Number 68-W-01-023, Work Assignment 4-17. 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 3

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EPA Contract No.:
EPA Work Assignment No.:

TITLE: Template Protocol for WA 4-17 Task 3:
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 D. 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 3: 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 recombinant 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 recombinant 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 a human recombinant microsome 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 recombinant microsomes will be obtained prior to initiation of the first set of experiments to ensure that sufficient quantities are available to conduct the studies.

### 2.1 Substrate

#### 2.1.1 Substrate Name/Supplier

The substrate for the aromatase assay is androstenedione (ASDN). Non-radiolabeled and radiolabeled ASDN will be used. The non-radiolabeled ASDN and the radiolabeled androstenedione ([1β-$^3$H]-androstenedione, [$^3$H]ASDN) will be provided to the laboratories by Battelle's Chemical 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.

### 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

Recombinant 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.

Human recombinant microsomes will be obtained from Gentest™ (Woburn, MA; www.gentest.com). The product name is Human CYP19 (Aromatase) Supersomes™ and the catalog number is 456260. The Supersomes™ package size is 0.5 nmoles cytochrome P450 in 0.5 mL. Supplier-provided values for protein concentration, cytochrome c reductase activity, and aromatase activity will be found on the data sheet accompanying each shipment and will be included in the report.

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 controls 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 | $1 \times 10^{-6}$ |
| 4-OH ASDN Concentration 2 | 3 | Complete assay with 4-OH ASDN added | $1 \times 10^{-7}$ |
| 4-OH ASDN Concentration 3 | 3 | Complete assay with 4-OH ASDN added | $5 \times 10^{-8}$ |
| 4-OH ASDN Concentration 4 | 3 | Complete assay with 4-OH ASDN added | $2.5 \times 10^{-8}$ |
| 4-OH ASDN Concentration 5 | 3 | Complete assay with 4-OH ASDN added | $1 \times 10^{-8}$ |
| 4-OH ASDN Concentration 6 | 3 | Complete assay with 4-OH ASDN added | $1 \times 10^{-9}$ |

[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:

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

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.

## 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
$\beta$ ≡ slope of the concentration response curve ($\beta$ 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 $\epsilon$ 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 $IC_{50}$ ($10^{\mu}$) and slope ($\beta$). 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 $\beta$ 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 $(\beta, \mu)$ as a random variable with mean $(\beta_{avg}, \mu_{avg})$ 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 ($\beta$) and $\log_{10}IC_{50}$ ($\mu$) will also be compared across replicates based on random effects analysis of variance, treating the replicates as random effects. $\beta$ and $\mu$ 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 [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.

### 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.

| PROTOCOL | | Page 14 of 14 |
|---|---|---|

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)

Project No.: WIL-431010
Battelle

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

# 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

Project No.: WIL-431010
Battelle

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

FINAL REPORT

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

Author:

Sherry J. Black 9/28/05

Sherry Black — Date
Research Chemist

Approved:

J. Mathews 9-28-05

James Mathews, Ph. D, DABT — Date
Study Director

# 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).

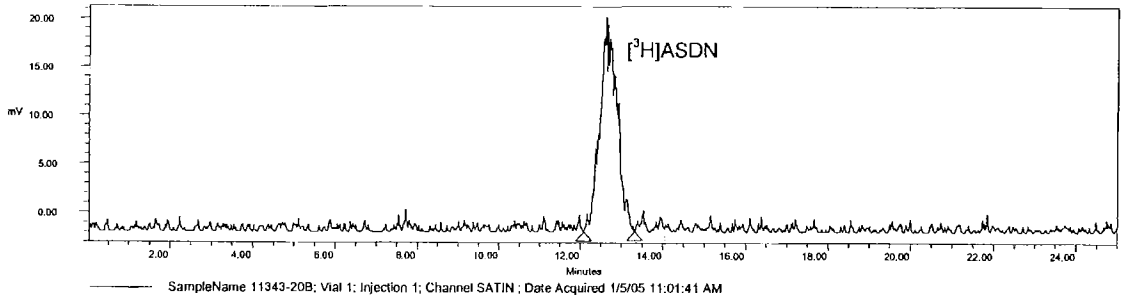
The radiochemical purity of the [$^3$H]ASDN (1:100 dilution in ethanol) was determined using high performance liquid chromatography (HPLC) and liquid scintillation counting. The HPLC system consists of a Waters 2690 Separations Module, a Waters 2487 Dual $\lambda$ Absorbance Detector and a $\beta$-RAM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

# 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.

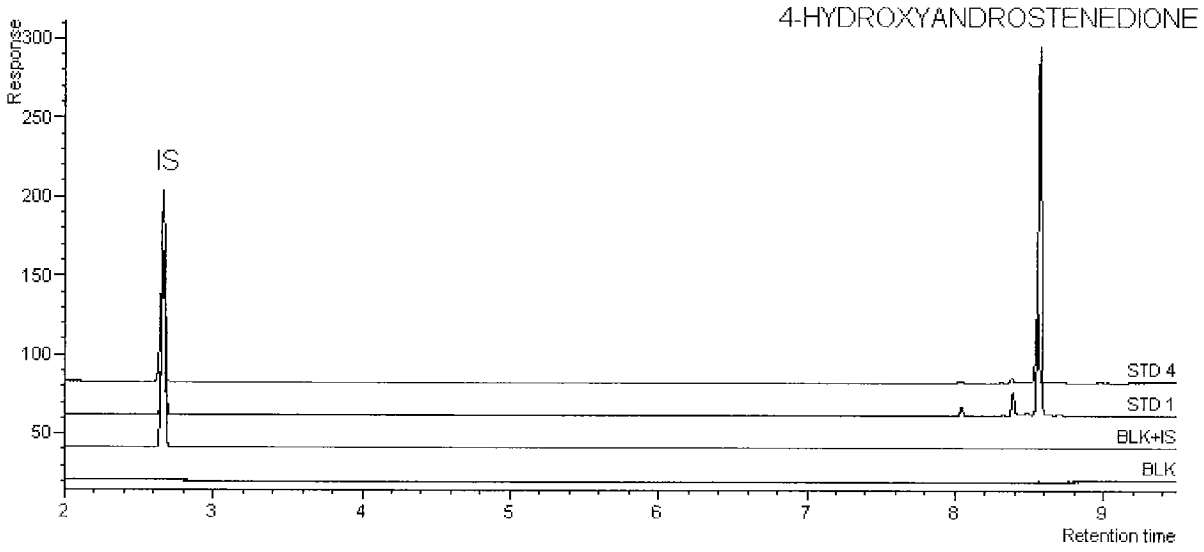



**Figure 2 – Representative Overlaid Chromatograms from a High and Low Vehicle/Calibration Standard, Blank with Internal Standard, and Blank from the Validation (Shown Top to Bottom)**

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 |
|---|---|---|---|---|---|---|
| | 496.8 | | | | -1.9 | |
| 506.4 | 494.5 | 509.6 | 24.2 | 4.7 | -2.3 | 0.6 |
| | 537.5 | | | | 6.1 | |
| 298.1 | 298.4 | NA | NA | NA | -2.9 | NA |
| 202.5 | 198.8 | NA | NA | NA | -1.9 | NA |
| | 100.7 | | | | 1.3 | |
| 99.38 | 99.98 | 100.4 | 0.4 | 0.4 | 0.5 | 1.0 |
| | 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

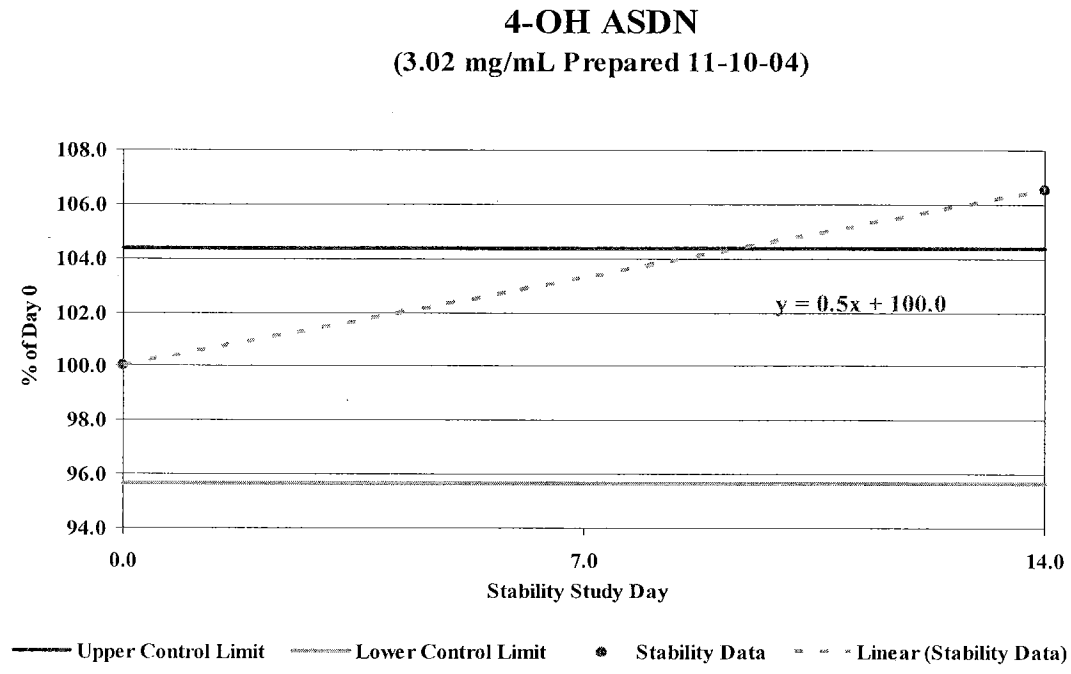
The results from the storage stability study are shown in Table 5 and presented in control chart format in Figure 3.

**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.



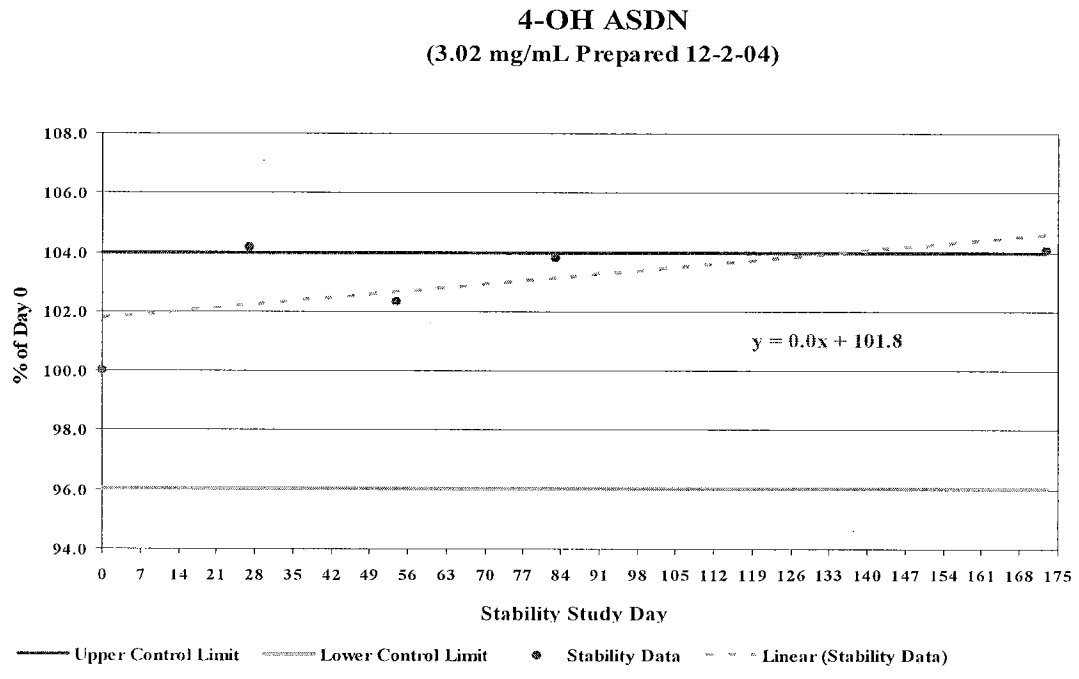




**Figure 3 – Control Charts for the Storage Stability Studies**

### 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.

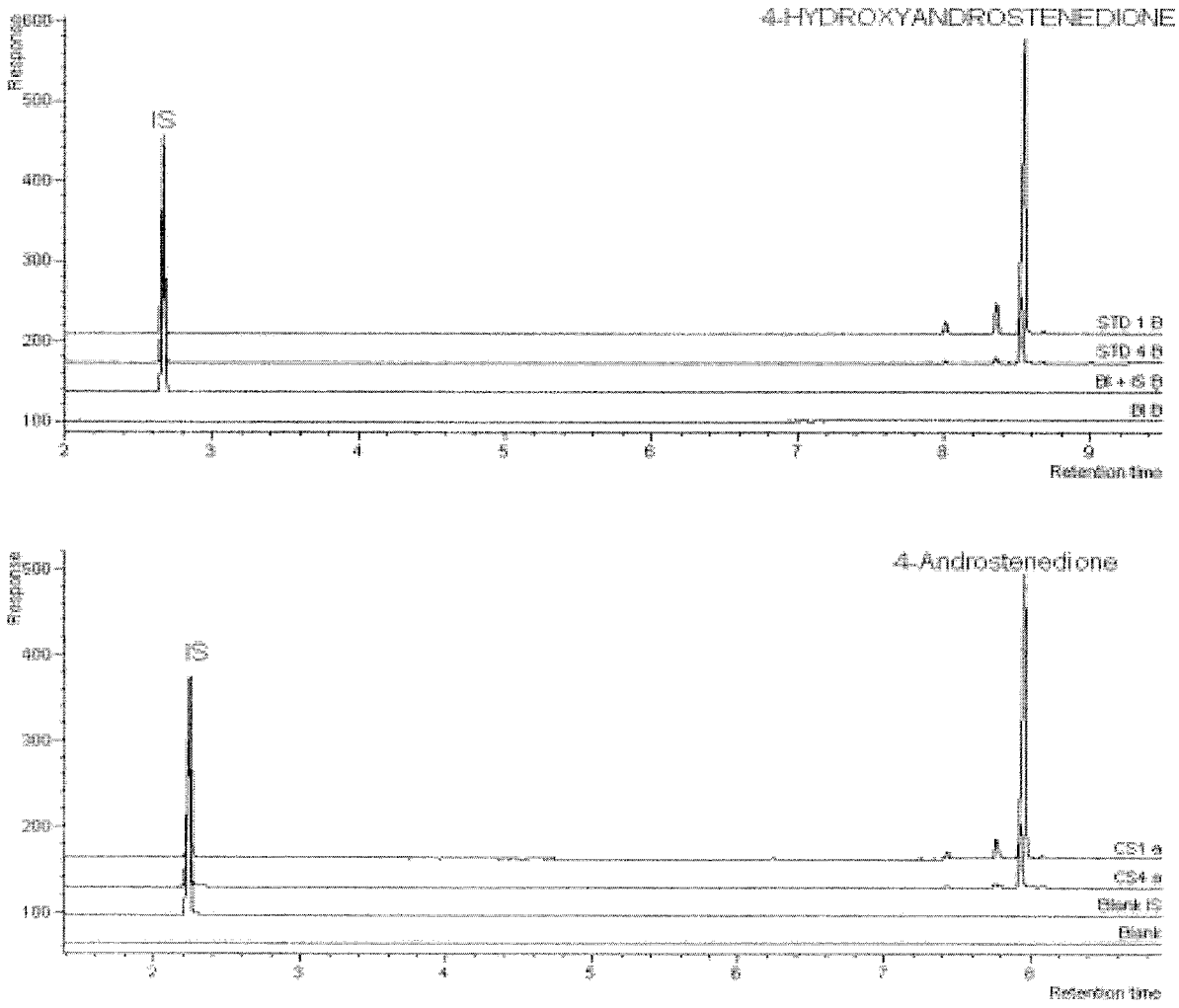



**Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from Formulation Analysis Batch 1-ASDN and Batch 2-ASDN (Shown Top to Bottom)**

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 $\leq$ 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)

| Aliquot # | Weight of aliquot (g) | DPM/Aliq. | DPM/g soln. | |
|---|---|---|---|---|
| 1 | 0.0200 | 26459.69 | 1322985 | |
| 2 | 0.0199 | 28145.74 | 1414359 | |
| 3 | 0.0200 | 28220.33 | 1411017 | |
| 4 | 0.0199 | 28237.6 | 1418975 | |
| 5 | 0.0197 | 28552.89 | 1449385 | |
| | | | Average DPM/g soln | 1403344 |
| | | | SD | 47439 |
| | | | CV | 3.38 |
| | | | µCi/g soln | 0.632 |

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 | 8.1778 g |
| Mass of dilution B used in substrate prep | 4.5853 g |
| Concentration of nonradiolabeled ASDN in substrate soln. | 0.577522 µg/g |

Calculation of Substrate Solution Specific Activity

1) Calculate µg [$^3$H]ASDN/g soln. = 0.00716 µg/g soln.
µg/g soln.

a. µCi/g soln 0.632
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.

= 0.577522 + 0.00716
= 0.584678 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.081 µCi/µg ASDN

687417 dpm/nmol

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Assay Date | 4/6/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | | | | |
| Technician ID | JG | Replicate # | 1 | Microsome type | recombinant | Microsome ID | MET-0254A Vial 4 | | |

| | | | | | | | | Protein stock (mg | Total volume of | |
|---|---|---|---|---|---|---|---|---|---|---|
| Standards: | 1.42 | 0.95 | 0.71 | 0.47 | 0.24 | 0.12 | 0 | BSA) | stock (mL) | Protein stock ID |
| | | | | | | | | | 10 | |

Samples:

| 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 | 0.00000 | 25 | 0.0000 | | | | m, b | |
| 0.95 | 40 | 100 | 0.00000 | 25 | 0.0000 | | | | $se_m$, $se_b$ | |
| 0.71 | 30 | 100 | 0.00000 | 25 | 0.0000 | | | | $r^2$, $se_y$ | |
| 0.47 | 20 | 100 | 0.00000 | 25 | 0.0000 | | | | F, df | |
| 0.24 | 10 | 100 | 0.00000 | 25 | 0.0000 | | | | $ss_{regr}$, $ss_{resid}$ | |
| 0.12 | 5.2 | 100 | 0.00000 | 25 | 0.0000 | | | | | |

Regression results are calculated using the function LINEST

Blank

$r^2$=
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 |
|---|---|---|---|---|---|---|---|---|
| | | | | 1 | 1 | | | |
| | | | | 1 | 1 | | | |
| | | | | 1 | 1 | | | |
| | | | | 65 | 3965 | | | |
| | | | | 65 | 3965 | | | |
| | | | | 65 | 3965 | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Assay Date | 4/6/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | | |
| Technician ID | JG | Replicate # | 1 | Microsome type | recombinant | Microsome ID | MET-0254A Vial 4 |

Protein stock (mg/10 mL): 25 — Protein stock ID:

| Standards: | 1.5 | 1 | 0.75 | 0.5 | 0.25 | 0.13 | Blk |
|---|---|---|---|---|---|---|---|
| | 0.3938 | 0.2958 | 0.2350 | 0.1706 | 0.1060 | 0.0683 | 0.0334 |
| | 0.4193 | 0.3078 | 0.2392 | 0.1741 | 0.1085 | 0.0669 | 0.0366 |
| | 0.4045 | 0.2977 | 0.2359 | 0.1825 | 0.1061 | 0.0714 | 0.0339 |

Samples:

| | |
|---|---|
| | 0.1310 |
| | 0.1244 |
| | 0.1276 |

| mg Protein per µL | µL Standard Used | mg Protein Measured | $A_{raw}$ | $A_{adj}$ | Curve Output |
|---|---|---|---|---|---|
| 0.00150 | 25 | 0.0375 | 0.406 | 0.371 | 0.0365 |
| 0.00100 | 25 | 0.0250 | 0.300 | 0.266 | 0.0258 |
| 0.00075 | 25 | 0.0188 | 0.237 | 0.202 | 0.0193 |
| 0.00050 | 25 | 0.0125 | 0.176 | 0.141 | 0.0132 |
| 0.00025 | 25 | 0.0063 | 0.107 | 0.072 | 0.0062 |
| 0.00013 | 25 | 0.0033 | 0.069 | 0.034 | 0.0023 |

| Variables | Regression results | |
|---|---|---|
| m, b | 0.101 | -0.001 |
| $se_m$, $se_b$ | 0.003 | 0.001 |
| $r^2$, $se_y$ | 0.996 | 0.001 |
| F, df | 979 | 4 |
| $ss_{reg}$, $ss_{resid}$ | 0.001 | 0.000 |

Regression results are calculated using the function LINEST

Blank 0.035

$r^2$= 0.996
m= 0.101
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 |
|---|---|---|---|---|---|---|---|---|
| 0.131 | 0.096 | 0.009 | 25 | 98 | 2000 | 0.007 | 0.007 | 6.763 |
| 0.124 | 0.090 | 0.008 | 25 | 98 | 2000 | 0.006 | | |
| 0.128 | 0.093 | 0.008 | 25 | 98 | 2000 | 0.007 | | |

| Assay Date | 4/6/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | Microsome type | recombinat Microsome ID | MET-0254 | Technician ID | JG | Replicate # | 1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Microsome Dilution Details | | |
|---|---|---|
| Dilution A | 0.098 | mL microsome Stock used |
| | 2 | mL total volume |
| | 20.40816 | dilution factor |
| Dilution B | 0.9 | mL microsome Dilution A used |
| | 36 | mL total volume |
| | 40 | dilution factor |
| Dilution C (if applicable) | | mL microsome Dilution B used |
| | | mL total volume |
| | NA | dilution factor |
| | 816.3265 | total dilution factor |

| Protein Concentration (stock microsomes, mg/mL): | 6.763 |
|---|---|
| Protein Concentration (dilution added to assay, mg/mL): | 0.008285 |

| Test Chemical Concentrations | |
|---|---|
| Level | Final Concentration (M) |
| 1 | 1.00E-06 |
| 2 | 1.00E-07 |
| 3 | 5.00E-08 |
| 4 | 2.50E-08 |
| 5 | 1.00E-08 |
| 6 | 1.00E-09 |
| | |
| | |

| Assay Date | 4/6/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 8 | Microsome type | recombinant | Microsome ID | MET-0254A Vial 4 | 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 | 5796.71 | 11513.42 | 11390.42 | 22780.84 | 0.1 | 140334 | 16.23 | 22656 | 0.0330 | 1 | 0.004 | 15 | 0.2656 |
| | | | 0.5 | 2 | 5633.71 | 11267.42 | | | 0.1 | | | | | 1 | | 15 | |
| | 2 | 2 | 0.5 | 1 | 5865.1 | 11730.2 | 11835.87 | 23671.74 | 0.1 | 140334 | 16.87 | 23577 | 0.0345 | 1 | 0.004 | 15 | 0.2760 |
| | | | 0.5 | 2 | 5970.77 | 11941.54 | | | 0.1 | | | | | 1 | | 15 | |
| | 3 | 2 | 0.5 | 1 | 5878.17 | 11756.34 | 11657.05 | 23314.1 | 0.1 | 140334 | 16.61 | 23219 | 0.0338 | 1 | 0.004 | 15 | 0.2718 |
| | | | 0.5 | 2 | 5778.88 | 11557.76 | | | 0.1 | | | | | 1 | | 15 | |
| | 4 | 2 | 0.5 | 1 | 5929.35 | 11858.7 | 11816.26 | 23632.52 | 0.1 | 140334 | 16.84 | 23538 | 0.0342 | 1 | 0.004 | 15 | 0.2758 |
| | | | 0.5 | 2 | 5886.91 | 11773.82 | | | 0.1 | | | | | 1 | | 15 | |
| Background control | 1 | 2 | 0.5 | 1 | 18.82 | 37.64 | 53.22 | 106.44 | 0.1 | 140334 | 0.08 | 12 | 0.0000 | 1 | 0.004 | 15 | 0.0001 |
| | | | 0.5 | 2 | 34.4 | 68.8 | | | 0.1 | | | | | 1 | | 15 | |
| | 2 | 2 | 0.5 | 1 | 21.37 | 42.74 | 48.21 | 96.42 | 0.1 | 140334 | 0.07 | 2 | 0.0000 | 1 | 0.004 | 15 | 0.0000 |
| | | | 0.5 | 2 | 26.84 | 53.68 | | | 0.1 | | | | | 1 | | 15 | |
| | 3 | 2 | 0.5 | 1 | 24.55 | 49.1 | 48.47 | 96.94 | 0.1 | 140334 | 0.07 | 2 | 0.0000 | 1 | 0.004 | 15 | 0.0000 |
| | | | 0.5 | 2 | 23.82 | 47.64 | | | 0.1 | | | | | 1 | | 15 | |
| | 4 | 2 | 0.5 | 1 | 22.56 | 45.12 | 39.53 | 79.06 | 0.1 | 140334 | 0.06 | -16 | 0.0000 | 1 | 0.004 | 15 | -0.0002 |
| | | | 0.5 | 2 | 16.97 | 33.94 | | | 0.1 | | | | | 1 | | 15 | |
| Positive control | 1 | 2 | 0.5 | 1 | | | | | 0.1 | 140334 | | #VALUE! | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 2 | 2 | 0.5 | 1 | | | | | 0.1 | 140334 | | #VALUE! | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 3 | 2 | 0.5 | 1 | | | | | 0.1 | 140334 | | #VALUE! | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 4 | 2 | 0.5 | 1 | | | | | 0.1 | 140334 | | #VALUE! | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| Negative Control | 1 | 2 | 0.5 | 1 | | | | | 0.1 | 140334 | | #VALUE! | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 2 | 2 | 0.5 | 1 | | | | | 0.1 | 140334 | | #VALUE! | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 3 | 2 | 0.5 | 1 | | | | | 0.1 | 140334 | | #VALUE! | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 4 | 2 | 0.5 | 1 | | | | | 0.1 | 140334 | | #VALUE! | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| 4-OH ASDN | 1-1 | 2 | 0.5 | 1 | 441 | 882 | 876.45 | 1752.9 | 0.1 | 140334 | 1.25 | 1658 | 0.0024 | 1 | 0.004 | 15 | 0.0124 |
| | | | 0.5 | 2 | 435.45 | 870.9 | | | 0.1 | | | | | 1 | | 15 | |
| | 1-2 | 2 | 0.5 | 1 | 445.73 | 891.46 | 905.44 | 1810.88 | 0.1 | 140334 | 1.29 | 1716 | 0.0025 | 1 | 0.004 | 15 | 0.0201 |
| | | | 0.5 | 2 | 459.71 | 919.42 | | | 0.1 | | | | | 1 | | 15 | |
| | 1-3 | 2 | 0.5 | 1 | 458.69 | 918.38 | 924.12 | 1848.24 | 0.1 | 140334 | 1.32 | 1754 | 0.0025 | 1 | 0.004 | 15 | 0.0205 |
| | | | 0.5 | 2 | 464.43 | 928.86 | | | 0.1 | | | | | 1 | | 15 | |
| | 2-1 | 2 | 0.5 | 1 | 2479.05 | 4958.1 | 5004.72 | 10009.44 | 0.1 | 140334 | 7.13 | 9915 | 0.0144 | 1 | 0.004 | 15 | 0.1161 |
| | | | 0.5 | 2 | 2525.67 | 5051.34 | | | 0.1 | | | | | 1 | | 15 | |
| | 2-2 | 2 | 0.5 | 1 | 2362.83 | 4725.66 | 4761.58 | 9523.16 | 0.1 | 140334 | 6.79 | 9428 | 0.0137 | 1 | 0.004 | 15 | 0.1104 |
| | | | 0.5 | 2 | 2398.75 | 4797.5 | | | 0.1 | | | | | 1 | | 15 | |
| | 2-3 | 2 | 0.5 | 1 | 2394.75 | 4789.5 | 4787.14 | 9574.28 | 0.1 | 140334 | 6.82 | 9480 | 0.0138 | 1 | 0.004 | 15 | 0.1110 |
| | | | 0.5 | 2 | 2392.38 | 4784.76 | | | 0.1 | | | | | 1 | | 15 | |
| | 3-1 | 2 | 0.5 | 1 | 3372.2 | 6744.4 | 6794.71 | 13589.42 | 0.1 | 140334 | 9.66 | 13486 | 0.0196 | 1 | 0.004 | 15 | 0.1580 |
| | | | 0.5 | 2 | 3422.91 | 6845.82 | | | 0.1 | | | | | 1 | | 15 | |
| | 3-2 | 2 | 0.5 | 1 | 3452.52 | 6905.04 | 6895.92 | 13791.84 | 0.1 | 140334 | 9.83 | 13697 | 0.0199 | 1 | 0.004 | 15 | 0.1603 |
| | | | 0.5 | 2 | 3443.4 | 6886.8 | | | 0.1 | | | | | 1 | | 15 | |
| | 3-3 | 2 | 0.5 | 1 | 1032.33 | 2064.66 | 2074.1 | 4148.2 | 0.1 | 140334 | 2.96 | 4053 | 0.0059 | 1 | 0.004 | 15 | 0.0475 |
| | | | 0.5 | 2 | 1041.77 | 2083.54 | | | 0.1 | | | | | 1 | | 15 | |
| | 4-1 | 2 | 0.5 | 1 | 4405 | 8810 | 8978.5 | 17957 | 0.1 | 140334 | 12.80 | 17862 | 0.0260 | 1 | 0.004 | 15 | 0.2091 |
| | | | 0.5 | 2 | 4573.5 | 9147 | | | 0.1 | | | | | 1 | | 15 | |
| | 4-2 | 2 | 0.5 | 1 | 4229.28 | 8458.56 | 8481.32 | 16962.64 | 0.1 | 140334 | 12.09 | 16868 | 0.0245 | 1 | 0.004 | 15 | 0.1975 |
| | | | 0.5 | 2 | 4252.04 | 8504.08 | | | 0.1 | | | | | 1 | | 15 | |
| | 4-3 | 2 | 0.5 | 1 | 4313.37 | 8626.74 | 8596.45 | 17192.9 | 0.1 | 140334 | 12.25 | 17098 | 0.0249 | 1 | 0.004 | 15 | 0.2002 |
| | | | 0.5 | 2 | 4283.08 | 8566.16 | | | 0.1 | | | | | 1 | | 15 | |
| | 5-1 | 2 | 0.5 | 1 | 5231.89 | 10463.78 | 10465.79 | 20931.58 | 0.1 | 140334 | 14.92 | 20837 | 0.0303 | 1 | 0.004 | 15 | 0.2439 |
| | | | 0.5 | 2 | 5233.9 | 10467.8 | | | 0.1 | | | | | 1 | | 15 | |
| | 5-2 | 2 | 0.5 | 1 | 5449.13 | 10898.26 | 10814.93 | 21629.86 | 0.1 | 140334 | 15.41 | 21535 | 0.0313 | 1 | 0.004 | 15 | 0.2521 |
| | | | 0.5 | 2 | 5365.8 | 10731.6 | | | 0.1 | | | | | 1 | | 15 | |
| | 5-3 | 2 | 0.5 | 1 | 5347.97 | 10695.94 | 10717.71 | 21435.42 | 0.1 | 140334 | 15.27 | 21341 | 0.0310 | 1 | 0.004 | 15 | 0.2498 |
| | | | 0.5 | 2 | 5369.74 | 10739.48 | | | 0.1 | | | | | 1 | | 15 | |
| | 6-1 | 2 | 0.5 | 1 | 6084.58 | 12169.16 | 12185.86 | 24371.72 | 0.1 | 140334 | 17.37 | 24277 | 0.0353 | 1 | 0.004 | 15 | 0.2842 |
| | | | 0.5 | 2 | 6100.88 | 12201.76 | | | 0.1 | | | | | 1 | | 15 | |
| | 6-2 | 2 | 0.5 | 1 | 5937.13 | 11874.26 | 11630.44 | 23260.88 | 0.1 | 140334 | 16.58 | 23166 | 0.0337 | 1 | 0.004 | 15 | 0.2712 |
| | | | 0.5 | 2 | 5693.31 | 11386.62 | | | 0.1 | | | | | 1 | | 15 | |
| | 6-3 | 2 | 0.5 | 1 | 6033.25 | 12066.5 | 11966.58 | 23933.16 | 0.1 | 140334 | 17.05 | 23839 | 0.0347 | 1 | 0.004 | 15 | 0.2791 |
| | | | 0.5 | 2 | 5933.33 | 11866.66 | | | 0.1 | | | | | | | | |

| Assay Date | 4/6/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | Microsome 6 type | recombinant | Microsome ID MET-0254A Vi | Technician ID JG | Replicate # | 1 |
|---|---|---|---|---|---|---|---|---|---|---|

| Control Type | Portion | Average | SD |
|---|---|---|---|
| Full activity | Beginning | 0.2708 | 0.0074 |
| Full activity | End | 0.2737 | 0.0026 |
| Full activity | Overall | 0.2722 | 0.0048 |
| Background | Beginning | 0.0001 | 8.29403E-05 |
| Background | End | -0.0001 | 0.000148001 |
| Background | Overall | 0.0000 | 0.000133541 |
| 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 | 0.0194 |
| 4-OH ASDN | 1 | 2 | 1.00E-06 | -6.00 | 0.0201 |
| 4-OH ASDN | 1 | 3 | 1.00E-06 | -6.00 | 0.0205 |
| 4-OH ASDN | 2 | 1 | 1.00E-07 | -7.00 | 0.1161 |
| 4-OH ASDN | 2 | 2 | 1.00E-07 | -7.00 | 0.1104 |
| 4-OH ASDN | 2 | 3 | 1.00E-07 | -7.00 | 0.1110 |
| 4-OH ASDN | 3 | 1 | 5.00E-08 | -7.30 | 0.1580 |
| 4-OH ASDN | 3 | 2 | 5.00E-08 | -7.30 | 0.1603 |
| 4-OH ASDN | 3 | 3 | 5.00E-08 | -7.30 | 0.0475 |
| 4-OH ASDN | 4 | 1 | 2.50E-08 | -7.60 | 0.2091 |
| 4-OH ASDN | 4 | 2 | 2.50E-08 | -7.60 | 0.1975 |
| 4-OH ASDN | 4 | 3 | 2.50E-08 | -7.60 | 0.2002 |
| 4-OH ASDN | 5 | 1 | 1.00E-08 | -8.00 | 0.2439 |
| 4-OH ASDN | 5 | 2 | 1.00E-08 | -8.00 | 0.2521 |
| 4-OH ASDN | 5 | 3 | 1.00E-08 | -8.00 | 0.2498 |
| 4-OH ASDN | 6 | 1 | 1.00E-09 | -9.00 | 0.2842 |
| 4-OH ASDN | 6 | 2 | 1.00E-09 | -9.00 | 0.2712 |
| 4-OH ASDN | 6 | 3 | 1.00E-09 | -9.00 | 0.2791 |

| | Percent of control values | | | |
|---|---|---|---|---|
| | Log[test | Replicate | | |
| Level | substance] | 1 | 2 | 3 |
| 1 | -6.00 | 7.13 | 7.38 | 7.54 |
| 2 | -7.00 | 42.63 | 40.54 | 40.76 |
| 3 | -7.30 | 58.03 | 58.90 | 17.43 |
| 4 | -7.60 | 76.81 | 72.53 | 73.52 |
| 5 | -8.00 | 89.60 | 92.60 | 91.77 |
| 6 | -9.00 | 104.39 | 99.62 | 102.51 |

| Aliquot # | Weight of aliquot (g) | DPM/Aliq. | DPM/g soln. | |
|---|---|---|---|---|
| 1 | 0.0197 | 29012.57 | 1472719 | |
| 2 | 0.0200 | 29935.81 | 1496791 | |
| 3 | 0.0200 | 30547.69 | 1527385 | |
| 4 | 0.0200 | 30930.61 | 1546531 | |
| 5 | 0.0199 | 30927.63 | 1554152 | |
| | | | Average DPM/g soln | 1519515 |
| | | | SD | 34270 |
| | | | CV | 2.26 |
| | | | µCi/g soln | 0.684 |

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

| ASDN solution | mg ASDN added | total volume (mL) | dilution factor | [ASDN] in solution (µg/mL) |
|---|---|---|---|---|
| Stock | 10.1 | 10 | | 1010.00 |
| Dilution A | | | 100 | 10.10 |
| Dilution B | | | 10 | 1.01 |

Calculation of concentration nonradiolabeled ASDN in substrate solution

| | |
|---|---|
| Total g substrate solution | 8.1602 g |
| Mass of dilution B used in substrate prep | 4.5807 g |
| Concentration of nonradiolabeled ASDN in substrate soln. | 0.56696 µg/g |

Calculation of Substrate Solution Specific Activity

1) Calculate µg [$^3$H]ASDN/g soln. = 0.00775 µg/g soln.
µg/g soln.

| | |
|---|---|
| a. µCi/g soln | 0.684 |
| 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.

= 0.566960 + 0.00775
= 0.574708 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.191 µCi/µg ASDN

757235 dpm/nmol

| Assay Date | 4/7/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | | |
|---|---|---|---|---|---|---|---|
| Technician ID | JG | Replicate # | 2 | Microsome type | recombinant | Microsome ID | MET-0254A 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 | | 10 | |

Samples:

| 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 | 0.00000 | 25 | 0.0000 | | | | m, b | |
| 0.95 | 40 | 100 | 0.00000 | 25 | 0.0000 | | | | $se_m$, $se_b$ | |
| 0.71 | 30 | 100 | 0.00000 | 25 | 0.0000 | | | | $r^2$, $se_y$ | |
| 0.47 | 20 | 100 | 0.00000 | 25 | 0.0000 | | | | F, df | |
| 0.24 | 10 | 100 | 0.00000 | 25 | 0.0000 | | | | $ss_{reg}$, $ss_{resid}$ | |
| 0.12 | 5.2 | 100 | 0.00000 | 25 | 0.0000 | | | | | |

Regression results are calculated using the function LINEST

Blank

$r^2$=
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 | 4/7/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | | |
|---|---|---|---|---|---|---|---|
| Technician ID | JG | Replicate # | 2 | Microsome type | recombinant | Microsome ID | MET-0254A Vial 5 |

| Standards: | 1.5 | 1 | 0.75 | 0.5 | 0.25 | 0.13 | Blk | Protein stock (mg/10 mL) | Protein stock ID |
|---|---|---|---|---|---|---|---|---|---|
| | 0.3997 | 0.2908 | 0.2176 | 0.1747 | 0.1011 | 0.0638 | 0.0336 | 25 | |
| | 0.4106 | 0.2992 | 0.2368 | 0.1738 | 0.0945 | 0.0630 | 0.0318 | | |
| | 0.4144 | 0.2950 | 0.2381 | 0.1666 | 0.1083 | 0.0548 | 0.0329 | | |

Samples:

| | |
|---|---|
| 0.1286 | |
| 0.1298 | |
| 0.1242 | |

| mg Protein per µL | µL Standard Used | mg Protein Measured | $A_{raw}$ | $A_{adj}$ | Curve Output | Variables | Regression results | |
|---|---|---|---|---|---|---|---|---|
| 0.00150 | 25 | 0.0375 | 0.408 | 0.375 | 0.0367 | m, b | 0.099 | 0.000 |
| 0.00100 | 25 | 0.0250 | 0.295 | 0.262 | 0.0255 | $se_m$, $se_b$ | 0.003 | 0.001 |
| 0.00075 | 25 | 0.0188 | 0.231 | 0.198 | 0.0191 | $r^2$, $se_y$ | 0.997 | 0.001 |
| 0.00050 | 25 | 0.0125 | 0.172 | 0.139 | 0.0133 | F, df | 1249 | 4 |
| 0.00025 | 25 | 0.0063 | 0.101 | 0.069 | 0.0063 | $ss_{reg}$, $ss_{resid}$ | 0.001 | 0.000 |
| 0.00013 | 25 | 0.0033 | 0.061 | 0.028 | 0.0023 | | | |

Regression results are calculated using the function LINEST

| | | | | |
|---|---|---|---|---|
| Blank | 0.033 | $r^2$= | 0.997 | |
| | | m= | 0.099 | |
| | | 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 |
|---|---|---|---|---|---|---|---|---|
| 0.129 | 0.096 | 0.009 | 25 | 98 | 2000 | 0.007 | 0.007 | 7.280 |
| 0.130 | 0.097 | 0.009 | 25 | 98 | 2000 | 0.007 | | |
| 0.124 | 0.091 | 0.009 | 25 | 98 | 2000 | 0.007 | | |

| Assay Date | 4/7/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | Microsome type | recombinant | Microsome ID | MET-0254, | Technician ID | JG | Replicate # | 2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Microsome Dilution Details | | |
|---|---|---|
| Dilution A | 0.098 | mL microsome Stock used |
| | 2 | mL total volume |
| | 20.40816 | dilution factor |
| Dilution B | 0.8 | mL microsome Dilution A used |
| | 35 | mL total volume |
| | 43.75 | dilution factor |
| Dilution C (if applicable) | | mL microsome Dilution B used |
| | | mL total volume |
| | NA | dilution factor |
| | 892.8571 | total dilution factor |

| Protein Concentration (stock microsomes, mg/mL): | 7.28 |
|---|---|
| Protein Concentration (dilution added to assay, mg/mL): | 0.008154 |

| Test Chemical Concentrations | |
|---|---|
| Level | Final Concentration (M) |
| 1 | 1.00E-06 |
| 2 | 1.00E-07 |
| 3 | 5.00E-08 |
| 4 | 2.50E-08 |
| 5 | 1.00E-08 |
| 6 | 1.00E-09 |
| | |
| | |

| Assay Date | 4/7/2006 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | Microsome type | recombinant | Microsome ID | MET-0254A Vial 5 | Technician ID | JG | Replicate # | 2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Sample ID | | Calculate DPM in aqueous portion after extraction | | | | | | | Calculate % turnover | | | Calculate nmol $^3H_2O$ formed | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample type | Replicate/Level | Nominal total volume (mL) | Aliq Volume (mL) | Aliq. # | DPM/aliq | DPM/mL | Ave DPM/mL | Total DPM | Volume of substrate solution used/assay tube (mL) | total DPM in assay tube (initial) | % conversion to product | Total DPM corrected for background (Background Tubes) | nmol $^3H_2O$ formed | Volume diluted microsomes used in assay tube (mL) | Final [protein] in assay (mg/mL) | Incubation time (min) | Aromatase activity (nmol estrogen formed/mg protein/min |
| Full activity control | 1 | 2 | 0.5 | 1 | 5845.99 | 11693.98 | 11717.71 | 23435.42 | 0.1 | 151952 | 15.42 | 23382 | 0.0308 | 1 | 0.004 | 15 | 0.2521 |
| | | | 0.5 | 2 | 5870.72 | 11741.44 | | | 0.1 | | | | | 1 | | 15 | |
| | 2 | 2 | 0.5 | 1 | 6020.71 | 12041.42 | 12049.16 | 24098.32 | 0.1 | 151952 | 15.86 | 24015 | 0.0317 | 1 | 0.004 | 15 | 0.2593 |
| | | | 0.5 | 2 | 6028.45 | 12056.9 | | | 0.1 | | | | | 1 | | 15 | |
| | 3 | 2 | 0.5 | 1 | 6191.06 | 12382.12 | 12345.49 | 24690.98 | 0.1 | 151952 | 16.25 | 24608 | 0.0325 | 1 | 0.004 | 15 | 0.2657 |
| | | | 0.5 | 2 | 6154.43 | 12308.86 | | | 0.1 | | | | | 1 | | 15 | |
| | 4 | 2 | 0.5 | 1 | 5602.72 | 11205.44 | 11148.92 | 22297.84 | 0.1 | 151952 | 14.67 | 22214 | 0.0293 | 1 | 0.004 | 15 | 0.2395 |
| | | | 0.5 | 2 | 5546.2 | 11092.4 | | | 0.1 | | | | | 1 | | 15 | |
| Background control | 1 | 2 | 0.5 | 1 | 22.34 | 44.68 | 44.45 | 88.9 | 0.1 | 151952 | 0.06 | 5 | 0.0000 | 1 | 0.004 | 15 | 0.0001 |
| | | | 0.5 | 2 | 22.11 | 44.22 | | | 0.1 | | | | | 1 | | 15 | |
| | 2 | 2 | 0.5 | 1 | 19.06 | 38.12 | 38.1 | 76.2 | 0.1 | 151952 | 0.05 | -7 | 0.0000 | 1 | 0.004 | 15 | -0.0001 |
| | | | 0.5 | 2 | 19.04 | 38.08 | | | 0.1 | | | | | 1 | | 15 | |
| | 3 | 2 | 0.5 | 1 | 18.67 | 37.34 | 38.18 | 76.36 | 0.1 | 151952 | 0.05 | -7 | 0.0000 | 1 | 0.004 | 15 | -0.0001 |
| | | | 0.5 | 2 | 19.01 | 38.02 | | | 0.1 | | | | | 1 | | 15 | |
| | 4 | 2 | 0.5 | 1 | 23.33 | 50.66 | 46.06 | 92.12 | 0.1 | 151952 | 0.06 | 9 | 0.0000 | 1 | 0.004 | 15 | 0.0001 |
| | | | 0.5 | 2 | 20.73 | 41.46 | | | 0.1 | | | | | 1 | | 15 | |
| Positive control | 1 | 2 | 0.5 | 1 | | | | | 0.1 | 151952 | | | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 2 | 2 | 0.5 | 1 | | | | | 0.1 | 151952 | | | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 3 | 2 | 0.5 | 1 | | | | | 0.1 | 151952 | | | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 4 | 2 | 0.5 | 1 | | | | | 0.1 | 151952 | | | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| Negative Control | 1 | 2 | 0.5 | 1 | | | | | 0.1 | 151952 | | | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 2 | 2 | 0.5 | 1 | | | | | 0.1 | 151952 | | | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 3 | 2 | 0.5 | 1 | | | | | 0.1 | 151952 | | | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 4 | 2 | 0.5 | 1 | | | | | 0.1 | 151952 | | | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| 4-OH ASDN | 1-1 | 2 | 0.5 | 1 | 412.62 | 825.24 | 786.17 | 1572.34 | 0.1 | 151952 | 1.03 | 1489 | 0.0020 | 1 | 0.004 | 15 | 0.0161 |
| | | | 0.5 | 2 | 373.55 | 747.1 | | | 0.1 | | | | | 1 | | 15 | |
| | 1-2 | 2 | 0.5 | 1 | 404.65 | 809.3 | 801.25 | 1602.5 | 0.1 | 151952 | 1.05 | 1519 | 0.0020 | 1 | 0.004 | 15 | 0.0164 |
| | | | 0.5 | 2 | 396.6 | 793.2 | | | 0.1 | | | | | 1 | | 15 | |
| | 1-3 | 2 | 0.5 | 1 | 420.21 | 840.42 | 837.52 | 1675.04 | 0.1 | 151952 | 1.10 | 1582 | 0.0021 | 1 | 0.004 | 15 | 0.0172 |
| | | | 0.5 | 2 | 417.31 | 834.62 | | | 0.1 | | | | | 1 | | 15 | |
| | 2-1 | 2 | 0.5 | 1 | 2245.52 | 4491.04 | 4484.91 | 8969.82 | 0.1 | 151952 | 5.90 | 8886 | 0.0117 | 1 | 0.004 | 15 | 0.0960 |
| | | | 0.5 | 2 | 2239.39 | 4478.78 | | | 0.1 | | | | | 1 | | 15 | |
| | 2-2 | 2 | 0.5 | 1 | 2201.1 | 4402.2 | 4434.87 | 8869.74 | 0.1 | 151952 | 5.84 | 8786 | 0.0116 | 1 | 0.004 | 15 | 0.0949 |
| | | | 0.5 | 2 | 2233.77 | 4467.54 | | | 0.1 | | | | | 1 | | 15 | |
| | 2-3 | 2 | 0.5 | 1 | 2210.98 | 4421.96 | 4453.75 | 8907.5 | 0.1 | 151952 | 5.86 | 8824 | 0.0117 | 1 | 0.004 | 15 | 0.0953 |
| | | | 0.5 | 2 | 2242.77 | 4485.54 | | | 0.1 | | | | | 1 | | 15 | |
| | 3-1 | 2 | 0.5 | 1 | 3331.13 | 6662.26 | 6666.08 | 13332.16 | 0.1 | 151952 | 8.77 | 13249 | 0.0175 | 1 | 0.004 | 15 | 0.1431 |
| | | | 0.5 | 2 | 3334.95 | 6669.9 | | | 0.1 | | | | | 1 | | 15 | |
| | 3-2 | 2 | 0.5 | 1 | 3376.92 | 6752.84 | 6699.82 | 13381.64 | 0.1 | 151952 | 8.81 | 13298 | 0.0176 | 1 | 0.004 | 15 | 0.1436 |
| | | | 0.5 | 2 | 3314.8 | 6629.6 | | | 0.1 | | | | | 1 | | 15 | |
| | 3-3 | 2 | 0.5 | 1 | 3171.1 | 6342.2 | 6326.05 | 12652.1 | 0.1 | 151952 | 8.33 | 12569 | 0.0166 | 1 | 0.004 | 15 | 0.1357 |
| | | | 0.5 | 2 | 3154.95 | 6309.9 | | | 0.1 | | | | | 1 | | 15 | |
| | 4-1 | 2 | 0.5 | 1 | 4258.19 | 8516.38 | 8424.6 | 16849.2 | 0.1 | 151952 | 11.09 | 16766 | 0.0221 | 1 | 0.004 | 15 | 0.1810 |
| | | | 0.5 | 2 | 4166.41 | 8332.82 | | | 0.1 | | | | | 1 | | 15 | |
| | 4-2 | 2 | 0.5 | 1 | 4069.74 | 8139.48 | 8171.53 | 16343.06 | 0.1 | 151952 | 10.76 | 16260 | 0.0215 | 1 | 0.004 | 15 | 0.1756 |
| | | | 0.5 | 2 | 4101.79 | 8203.58 | | | 0.1 | | | | | 1 | | 15 | |
| | 4-3 | 2 | 0.5 | 1 | 4113.4 | 8226.8 | 8196.07 | 16392.14 | 0.1 | 151952 | 10.79 | 16309 | 0.0215 | 1 | 0.004 | 15 | 0.1761 |
| | | | 0.5 | 2 | 4082.67 | 8165.34 | | | 0.1 | | | | | 1 | | 15 | |
| | 5-1 | 2 | 0.5 | 1 | 5101.71 | 10203.42 | 10133.81 | 20267.62 | 0.1 | 151952 | 13.34 | 20184 | 0.0267 | 1 | 0.004 | 15 | 0.2179 |
| | | | 0.5 | 2 | 5032.1 | 10064.2 | | | 0.1 | | | | | 1 | | 15 | |
| | 5-2 | 2 | 0.5 | 1 | 5231.93 | 10463.86 | 10457.2 | 20914.4 | 0.1 | 151952 | 13.76 | 20831 | 0.0275 | 1 | 0.004 | 15 | 0.2249 |
| | | | 0.5 | 2 | 5225.27 | 10450.54 | | | 0.1 | | | | | 1 | | 15 | |
| | 5-3 | 2 | 0.5 | 1 | 5109.34 | 10218.68 | 10245.01 | 20490.02 | 0.1 | 151952 | 13.48 | 20407 | 0.0269 | 1 | 0.004 | 15 | 0.2203 |
| | | | 0.5 | 2 | 5135.67 | 10271.34 | | | 0.1 | | | | | 1 | | 15 | |
| | 6-1 | 2 | 0.5 | 1 | 5916.27 | 11832.54 | 11749.74 | 23499.48 | 0.1 | 151952 | 15.47 | 23416 | 0.0309 | 1 | 0.004 | 15 | 0.2528 |
| | | | 0.5 | 2 | 5833.47 | 11666.94 | | | 0.1 | | | | | 1 | | 15 | |
| | 6-2 | 2 | 0.5 | 1 | 5749.42 | 11498.84 | 11533.23 | 23066.46 | 0.1 | 151952 | 15.18 | 22983 | 0.0304 | 1 | 0.004 | 15 | 0.2482 |
| | | | 0.5 | 2 | 5787.81 | 11575.62 | | | 0.1 | | | | | 1 | | 15 | |
| | 6-3 | 2 | 0.5 | 1 | 5822.85 | 11645.7 | 11598.13 | 23196.26 | 0.1 | 151952 | 15.27 | 23113 | 0.0305 | 1 | 0.004 | 15 | 0.2496 |
| | | | 0.5 | 2 | 5773.28 | 11550.56 | | | 0.1 | | | | | | | | |

| Assay Date | 4/7/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | Microsome type | recombinant | Microsome ID | MET-0254A Vi | Technician ID | JG | Replicate # | 2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Control Type | Portion | Average | SD |
|---|---|---|---|
| Full activity | Beginning | 0.2557 | 0.0051 |
| Full activity | End | 0.2528 | 0.0183 |
| Full activity | Overall | 0.2543 | 0.0111 |
| Background | Beginning | 0.0000 | 9.69655E-05 |
| Background | End | 0.0000 | 0.000120329 |
| Background | Overall | 0.0000 | 8.98412E-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 | 0.0161 |
| 4-OH ASDN | 1 | 2 | 1.00E-06 | -6.00 | 0.0164 |
| 4-OH ASDN | 1 | 3 | 1.00E-06 | -6.00 | 0.0172 |
| 4-OH ASDN | 2 | 1 | 1.00E-07 | -7.00 | 0.0960 |
| 4-OH ASDN | 2 | 2 | 1.00E-07 | -7.00 | 0.0949 |
| 4-OH ASDN | 2 | 3 | 1.00E-07 | -7.00 | 0.0953 |
| 4-OH ASDN | 3 | 1 | 5.00E-08 | -7.30 | 0.1431 |
| 4-OH ASDN | 3 | 2 | 5.00E-08 | -7.30 | 0.1436 |
| 4-OH ASDN | 3 | 3 | 5.00E-08 | -7.30 | 0.1357 |
| 4-OH ASDN | 4 | 1 | 2.50E-08 | -7.60 | 0.1810 |
| 4-OH ASDN | 4 | 2 | 2.50E-08 | -7.60 | 0.1756 |
| 4-OH ASDN | 4 | 3 | 2.50E-08 | -7.60 | 0.1761 |
| 4-OH ASDN | 5 | 1 | 1.00E-08 | -8.00 | 0.2179 |
| 4-OH ASDN | 5 | 2 | 1.00E-08 | -8.00 | 0.2249 |
| 4-OH ASDN | 5 | 3 | 1.00E-08 | -8.00 | 0.2203 |
| 4-OH ASDN | 6 | 1 | 1.00E-09 | -9.00 | 0.2528 |
| 4-OH ASDN | 6 | 2 | 1.00E-09 | -9.00 | 0.2482 |
| 4-OH ASDN | 6 | 3 | 1.00E-09 | -9.00 | 0.2496 |

| Percent of control values | | | | |
|---|---|---|---|---|
| | | Replicate | | |
| Level | Log[test substance] | 1 | 2 | 3 |
| 1 | -6.00 | 6.32 | 6.45 | 6.76 |
| 2 | -7.00 | 37.74 | 37.31 | 37.47 |
| 3 | -7.30 | 56.26 | 56.47 | 53.38 |
| 4 | -7.60 | 71.20 | 69.05 | 69.26 |
| 5 | -8.00 | 85.72 | 88.46 | 86.66 |
| 6 | -9.00 | 99.44 | 97.60 | 98.16 |

| Aliquot # | Weight of aliquot (g) | DPM/Aliq. | DPM/g soln. | |
|---|---|---|---|---|
| 1 | 0.0200 | 32604.79 | 1630240 | |
| 2 | 0.0201 | 32886.6 | 1636149 | |
| 3 | 0.0201 | 33499.13 | 1666623 | |
| 4 | 0.0198 | 33549.01 | 1694394 | |
| 5 | 0.0198 | 33548.07 | 1694347 | |
| | | | Average DPM/g soln | 1664351 |
| | | | SD | 30686 |
| | | | CV | 1.84 |
| | | | µCi/g soln | 0.750 |

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 | 8.1903 g |
| Mass of dilution B used in substrate prep | 4.5895 g |
| Concentration of nonradiolabeled ASDN in substrate soln. | 0.571565 µg/g |

Calculation of Substrate Solution Specific Activity

1) Calculate µg [$^3$H]ASDN/g soln. = 0.00849 µg/g soln.
µg/g soln.

a. µCi/g soln 0.750
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.

= 0.571565 + 0.00849
= 0.580052 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.292 µCi/µg ASDN

821771 dpm/nmol

| Assay Date | 4/8/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | | |
|---|---|---|---|---|---|---|---|
| Technician ID | JG | Replicate # | 3 | Microsome type | recombinant | Microsome ID | MET-0254A Vial 6 |

| Standards: | 1.42 | 0.95 | 0.71 | 0.47 | 0.24 | 0.12 | 0 |
|---|---|---|---|---|---|---|---|

Protein stock (mg BSA): 10

Total volume of stock (mL):

Protein stock ID:

Samples:

| 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 | 0.00000 | 25 | 0.0000 | | | | m, b | |
| 0.95 | 40 | 100 | 0.00000 | 25 | 0.0000 | | | | $se_m$, $se_b$ | |
| 0.71 | 30 | 100 | 0.00000 | 25 | 0.0000 | | | | $r^2$, $se_y$ | |
| 0.47 | 20 | 100 | 0.00000 | 25 | 0.0000 | | | | F, df | |
| 0.24 | 10 | 100 | 0.00000 | 25 | 0.0000 | | | | $ss_{reg}$, $ss_{resid}$ | |
| 0.12 | 5.2 | 100 | 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 | 4/8/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | | |
|---|---|---|---|---|---|---|---|
| Technician ID | JG | Replicate # | 3 | Microsome type | recombinant | Microsome ID | MET-0254A Vial 6 |

| Standards: | 1.5 | 1 | 0.75 | 0.5 | 0.25 | 0.13 | Blk | Protein stock (mg/10 mL) | Protein stock ID |
|---|---|---|---|---|---|---|---|---|---|
| | 0.3909 | 0.2972 | 0.2381 | 0.1751 | 0.1053 | 0.0662 | 0.0353 | 25 | |
| | 0.4261 | 0.3005 | 0.2408 | 0.1750 | 0.1039 | 0.0707 | 0.0333 | | |
| | 0.4162 | 0.3100 | 0.2411 | 0.1763 | 0.1113 | 0.0630 | 0.0377 | | |

Samples:

| | |
|---|---|
| | 0.1204 |
| | 0.1220 |
| | 0.1067 |

| mg Protein per µL | µL Standard Used | mg Protein Measured | $A_{raw}$ | $A_{adj}$ | Curve Output |
|---|---|---|---|---|---|
| 0.00150 | 25 | 0.0375 | 0.411 | 0.376 | 0.0365 |
| 0.00100 | 25 | 0.0250 | 0.303 | 0.267 | 0.0257 |
| 0.00075 | 25 | 0.0188 | 0.240 | 0.205 | 0.0195 |
| 0.00050 | 25 | 0.0125 | 0.175 | 0.140 | 0.0131 |
| 0.00025 | 25 | 0.0063 | 0.107 | 0.071 | 0.0063 |
| 0.00013 | 25 | 0.0033 | 0.067 | 0.031 | 0.0023 |

| Variables | Regression results | |
|---|---|---|
| m, b | 0.099 | -0.001 |
| $se_m$, $se_b$ | 0.003 | 0.001 |
| $r^2$, $se_y$ | 0.996 | 0.001 |
| F, df | 957 | 4 |
| $ss_{reg}$, $ss_{resid}$ | 0.001 | 0.000 |

Regression results are calculated using the function LINEST

Blank 0.035

$r^2$= 0.996
m= 0.099
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 |
|---|---|---|---|---|---|---|---|---|
| 0.120 | 0.085 | 0.008 | 25 | 98 | 2000 | 0.006 | 0.006 | 5.882 |
| 0.122 | 0.087 | 0.008 | 25 | 98 | 2000 | 0.006 | | |
| 0.107 | 0.071 | 0.006 | 25 | 98 | 2000 | 0.005 | | |

| Assay Date | 4/8/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | Microsome type | recombinant | Microsome ID | MET-0254 | Technician ID | JG | Replicate # | 3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Microsome Dilution Details | | |
|---|---|---|
| Dilution A | 0.098 | mL microsome Stock used |
| | 2 | mL total volume |
| | 20.40816 | dilution factor |
| Dilution B | 1 | mL microsome Dilution A used |
| | 35 | mL total volume |
| | 35 | dilution factor |
| Dilution C (if applicable) | | mL microsome Dilution B used |
| | | mL total volume |
| | NA | dilution factor |
| | 714.2857 | total dilution factor |

| | |
|---|---|
| Protein Concentration (stock microsomes, mg/mL): | 5.882 |
| Protein Concentration (dilution added to assay, mg/mL): | 0.008235 |

| Test Chemical Concentrations | |
|---|---|
| Level | Final Concentration (M) |
| 1 | 1.00E-06 |
| 2 | 1.00E-07 |
| 3 | 5.00E-08 |
| 4 | 2.50E-08 |
| 5 | 1.00E-08 |
| 6 | 1.00E-09 |
| | |
| | |

| Assay Date | 4/8/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | Microsome type | recombinant | Microsome ID | MET-0264A Vial 6 | Technician ID | JG | Replicate # | 3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Sample ID | | Calculate DPM in aqueous portion after extraction | | | | | | | Calculate % turnover | | | Calculate nmol $^3H_2O$ formed | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample type | Replicate/Level | Nominal total volume (mL) | Aliq Volume (mL) | Aliq. # | DPM/aliq | DPM/mL | Ave DPM/mL | Total DPM | Volume of substrate solution used/assay tube (mL) | total DPM in assay tube (initial) | % conversion to product | Total DPM corrected for background (Background Tubes) | nmol $^3H_2O$ formed | Volume diluted microsomes used in assay tube (mL) | Final [protein] in assay (mg/mL) | Incubation time (min) | Aromatase activity (nmol estrogen formed/mg protein/min |
| Full activity control | 1 | 2 | 0.5 | 1 | 8496.17 | 16992.34 | 16924.14 | 33848.28 | 0.1 | 166435 | 20.34 | 33781 | 0.0411 | 1 | 0.004 | 15 | 0.3326 |
| | | | 0.5 | 2 | 8427.97 | 16855.94 | | | 0.1 | | | | | 1 | | 15 | |
| | 2 | 2 | 0.5 | 1 | 8204.68 | 16409.36 | 16455.48 | 32910.96 | 0.1 | 166435 | 19.77 | 32823 | 0.0398 | 1 | 0.004 | 15 | 0.3234 |
| | | | 0.5 | 2 | 8250.8 | 16501.6 | | | 0.1 | | | | | 1 | | 15 | |
| | 3 | 2 | 0.5 | 1 | 8386.78 | 16773.56 | 16606.69 | 33213.38 | 0.1 | 166435 | 19.96 | 33126 | 0.0403 | 1 | 0.004 | 15 | 0.3263 |
| | | | 0.5 | 2 | 8219.91 | 16439.82 | | | 0.1 | | | | | 1 | | 15 | |
| | 4 | 2 | 0.5 | 1 | 8115.92 | 16231.84 | 16296.34 | 32592.68 | 0.1 | 166435 | 19.59 | 32505 | 0.0395 | 1 | 0.004 | 15 | 0.3202 |
| | | | 0.5 | 2 | 8180.42 | 16360.84 | | | 0.1 | | | | | 1 | | 15 | |
| Background control | 1 | 2 | 0.5 | 1 | 27.37 | 54.74 | 55.08 | 110.16 | 0.1 | 166435 | 0.07 | 23 | 0.0000 | 1 | 0.004 | 15 | 0.0002 |
| | | | 0.5 | 2 | 27.71 | 55.42 | | | 0.1 | | | | | 1 | | 15 | |
| | 2 | 2 | 0.5 | 1 | 18.85 | 37.7 | 39.95 | 79.9 | 0.1 | 166435 | 0.04 | -14 | 0.0000 | 1 | 0.004 | 15 | -0.0001 |
| | | | 0.5 | 2 | 19.1 | 38.2 | | | 0.1 | | | | | 1 | | 15 | |
| | 3 | 2 | 0.5 | 1 | 19.45 | 38.9 | 40.44 | 80.88 | 0.1 | 166435 | 0.05 | -7 | 0.0000 | 1 | 0.004 | 15 | -0.0001 |
| | | | 0.5 | 2 | 20.99 | 41.98 | | | 0.1 | | | | | 1 | | 15 | |
| | 4 | 2 | 0.5 | 1 | 26.5 | 53.5 | 42.49 | 84.98 | 0.1 | 166435 | 0.05 | -3 | 0.0000 | 1 | 0.004 | 15 | 0.0000 |
| | | | 0.5 | 2 | 15.68 | 31.36 | | | 0.1 | | | | | 1 | | 15 | |
| Positive control | 1 | 2 | 0.5 | 1 | | | | | 0.1 | 166435 | | | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 2 | 2 | 0.5 | 1 | | | | | 0.1 | 166435 | | | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 3 | 2 | 0.5 | 1 | | | | | 0.1 | 166435 | | | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 4 | 2 | 0.5 | 1 | | | | | 0.1 | 166435 | | | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| Negative Control | 1 | 2 | 0.5 | 1 | | | | | 0.1 | 166435 | | | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 2 | 2 | 0.5 | 1 | | | | | 0.1 | 166435 | | | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 3 | 2 | 0.5 | 1 | | | | | 0.1 | 166435 | | | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 4 | 2 | 0.5 | 1 | | | | | 0.1 | 166435 | | | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| 4-OH ASDN | 1-1 | 2 | 0.5 | 1 | 515.59 | 1031.18 | 1017.02 | 2034.04 | 0.1 | 166435 | 1.22 | 1947 | 0.0024 | 1 | 0.004 | 15 | 0.0162 |
| | | | 0.5 | 2 | 501.43 | 1002.86 | | | 0.1 | | | | | 1 | | 15 | |
| | 1-2 | 2 | 0.5 | 1 | 575.65 | 1151.3 | 1141.29 | 2282.58 | 0.1 | 166435 | 1.37 | 2195 | 0.0027 | 1 | 0.004 | 15 | 0.0216 |
| | | | 0.5 | 2 | 565.64 | 1131.28 | | | 0.1 | | | | | 1 | | 15 | |
| | 1-3 | 2 | 0.5 | 1 | 579.72 | 1158.44 | 1168.68 | 2337.36 | 0.1 | 166435 | 1.40 | 2250 | 0.0027 | 1 | 0.004 | 15 | 0.0222 |
| | | | 0.5 | 2 | 588.96 | 1177.92 | | | 0.1 | | | | | 1 | | 15 | |
| | 2-1 | 2 | 0.5 | 1 | 3024.78 | 6049.56 | 6087.65 | 12175.3 | 0.1 | 166435 | 7.32 | 12088 | 0.0147 | 1 | 0.004 | 15 | 0.1161 |
| | | | 0.5 | 2 | 3062.87 | 6125.74 | | | 0.1 | | | | | 1 | | 15 | |
| | 2-2 | 2 | 0.5 | 1 | 3272.34 | 6544.68 | 6437.85 | 12875.7 | 0.1 | 166435 | 7.74 | 12788 | 0.0156 | 1 | 0.004 | 15 | 0.1230 |
| | | | 0.5 | 2 | 3165.51 | 6331.02 | | | 0.1 | | | | | 1 | | 15 | |
| | 2-3 | 2 | 0.5 | 1 | 3242.1 | 6484.2 | 6472.96 | 12945.92 | 0.1 | 166435 | 7.78 | 12858 | 0.0156 | 1 | 0.004 | 15 | 0.1237 |
| | | | 0.5 | 2 | 3230.86 | 6461.72 | | | 0.1 | | | | | 1 | | 15 | |
| | 3-1 | 2 | 0.5 | 1 | 4341.7 | 8683.4 | 8717.4 | 17434.8 | 0.1 | 166435 | 10.48 | 17347 | 0.0211 | 1 | 0.004 | 15 | 0.1709 |
| | | | 0.5 | 2 | 4375.7 | 8751.4 | | | 0.1 | | | | | 1 | | 15 | |
| | 3-2 | 2 | 0.5 | 1 | 4483.04 | 8966.08 | 8945.11 | 17890.22 | 0.1 | 166435 | 10.75 | 17803 | 0.0217 | 1 | 0.004 | 15 | 0.1754 |
| | | | 0.5 | 2 | 4462.07 | 8924.14 | | | 0.1 | | | | | 1 | | 15 | |
| | 3-3 | 2 | 0.5 | 1 | 4544.4 | 9088.8 | 9093.47 | 18186.94 | 0.1 | 166435 | 10.93 | 18099 | 0.0220 | 1 | 0.004 | 15 | 0.1783 |
| | | | 0.5 | 2 | 4549.07 | 9098.14 | | | 0.1 | | | | | 1 | | 15 | |
| | 4-1 | 2 | 0.5 | 1 | 5642.97 | 11285.94 | 11261.17 | 22522.34 | 0.1 | 166435 | 13.53 | 22435 | 0.0273 | 1 | 0.004 | 15 | 0.2210 |
| | | | 0.5 | 2 | 5618.2 | 11236.4 | | | 0.1 | | | | | 1 | | 15 | |
| | 4-2 | 2 | 0.5 | 1 | 6401.3 | 12802.6 | 12675.49 | 25350.98 | 0.1 | 166435 | 15.23 | 25294 | 0.0307 | 1 | 0.004 | 15 | 0.2489 |
| | | | 0.5 | 2 | 6274.19 | 12548.38 | | | 0.1 | | | | | 1 | | 15 | |
| | 4-3 | 2 | 0.5 | 1 | 5905.29 | 11810.58 | 11820.66 | 23641.32 | 0.1 | 166435 | 14.20 | 23554 | 0.0287 | 1 | 0.004 | 15 | 0.2320 |
| | | | 0.5 | 2 | 5915.37 | 11830.74 | | | 0.1 | | | | | 1 | | 15 | |
| | 5-1 | 2 | 0.5 | 1 | 7132.77 | 14265.54 | 14368.16 | 28736.32 | 0.1 | 166435 | 17.27 | 28649 | 0.0349 | 1 | 0.004 | 15 | 0.2822 |
| | | | 0.5 | 2 | 7235.39 | 14470.78 | | | 0.1 | | | | | 1 | | 15 | |
| | 5-2 | 2 | 0.5 | 1 | 7959.29 | 15918.58 | 15955.6 | 31911.2 | 0.1 | 166435 | 19.17 | 31824 | 0.0387 | 1 | 0.004 | 15 | 0.3135 |
| | | | 0.5 | 2 | 7996.31 | 15992.62 | | | 0.1 | | | | | 1 | | 15 | |
| | 5-3 | 2 | 0.5 | 1 | 7103.32 | 14206.64 | 14078.41 | 28156.82 | 0.1 | 166435 | 16.92 | 28069 | 0.0342 | 1 | 0.004 | 15 | 0.2765 |
| | | | 0.5 | 2 | 6975.09 | 13950.18 | | | 0.1 | | | | | 1 | | 15 | |
| | 6-1 | 2 | 0.5 | 1 | 8113.39 | 16226.78 | 16223.64 | 32447.28 | 0.1 | 166435 | 19.50 | 32360 | 0.0394 | 1 | 0.004 | 15 | 0.3188 |
| | | | 0.5 | 2 | 8110.25 | 16220.5 | | | 0.1 | | | | | 1 | | 15 | |
| | 6-2 | 2 | 0.5 | 1 | 8561.33 | 17192.66 | 17114.16 | 34228.32 | 0.1 | 166435 | 20.57 | 34141 | 0.0415 | 1 | 0.004 | 15 | 0.3363 |
| | | | 0.5 | 2 | 8922.83 | 17045.66 | | | 0.1 | | | | | 1 | | 15 | |
| | 6-3 | 2 | 0.5 | 1 | 7973.09 | 15946.18 | 15837.11 | 31674.22 | 0.1 | 166435 | 19.03 | 31587 | 0.0384 | 1 | 0.004 | 15 | 0.3112 |
| | | | 0.5 | 2 | 7864.02 | 15728.04 | | | 0.1 | | | | | | | | |

| Assay Date | 4/8/2005 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | Microsome type | recombinant | Microsome ID | MET-0254A Vi | Technician ID | JG | Replicate # | 3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Control Type | Portion | Average | SD |
|---|---|---|---|
| Full activity | Beginning | 0.3280 | 0.0065 |
| Full activity | End | 0.3233 | 0.0043 |
| Full activity | Overall | 0.3256 | 0.0053 |
| Background | Beginning | 0.0000 | 0.000252591 |
| Background | End | 0.0000 | 2.8561E-05 |
| Background | Overall | 0.0000 | 0.000155622 |
| 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 | 0.0192 |
| 4-OH ASDN | 1 | 2 | 1.00E-06 | -6.00 | 0.0216 |
| 4-OH ASDN | 1 | 3 | 1.00E-06 | -6.00 | 0.0222 |
| 4-OH ASDN | 2 | 1 | 1.00E-07 | -7.00 | 0.1191 |
| 4-OH ASDN | 2 | 2 | 1.00E-07 | -7.00 | 0.1260 |
| 4-OH ASDN | 2 | 3 | 1.00E-07 | -7.00 | 0.1267 |
| 4-OH ASDN | 3 | 1 | 5.00E-08 | -7.30 | 0.1709 |
| 4-OH ASDN | 3 | 2 | 5.00E-08 | -7.30 | 0.1754 |
| 4-OH ASDN | 3 | 3 | 5.00E-08 | -7.30 | 0.1783 |
| 4-OH ASDN | 4 | 1 | 2.50E-08 | -7.60 | 0.2210 |
| 4-OH ASDN | 4 | 2 | 2.50E-08 | -7.60 | 0.2489 |
| 4-OH ASDN | 4 | 3 | 2.50E-08 | -7.60 | 0.2320 |
| 4-OH ASDN | 5 | 1 | 1.00E-08 | -8.00 | 0.2822 |
| 4-OH ASDN | 5 | 2 | 1.00E-08 | -8.00 | 0.3135 |
| 4-OH ASDN | 5 | 3 | 1.00E-08 | -8.00 | 0.2765 |
| 4-OH ASDN | 6 | 1 | 1.00E-09 | -9.00 | 0.3188 |
| 4-OH ASDN | 6 | 2 | 1.00E-09 | -9.00 | 0.3363 |
| 4-OH ASDN | 6 | 3 | 1.00E-09 | -9.00 | 0.3112 |

| Percent of control values | | | | |
|---|---|---|---|---|
| | Log[test | Replicate | | |
| Level | substance] | 1 | 2 | 3 |
| 1 | -6.00 | 5.89 | 6.64 | 6.81 |
| 2 | -7.00 | 36.57 | 38.69 | 38.90 |
| 3 | -7.30 | 52.48 | 53.86 | 54.76 |
| 4 | -7.60 | 67.87 | 76.43 | 71.26 |
| 5 | -8.00 | 86.67 | 96.28 | 84.92 |
| 6 | -9.00 | 97.90 | 103.29 | 95.56 |

# Appendix G

## Unsuccessful Replicate Spreadsheets (12/28/04 through 12/30/04)

| Aliquot # | Weight of aliquot (g) | DPM/Aliq. | DPM/g soln. | |
|---|---|---|---|---|
| 1 | 0.0192 | 30113.86 | 1568430 | |
| 2 | 0.0193 | 30878.99 | 1599948 | |
| 3 | 0.0196 | 31230.81 | 1593409 | |
| 4 | 0.0204 | 31707.46 | 1554287 | |
| 5 | 0.0195 | 31609.32 | 1620991 | |
| | | | Average DPM/g soln | 1587413 |
| | | | SD | 26354 |
| | | | CV | 1.66 |
| | | | µCi/g soln | 0.715 |

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 | 8.209 g |
| Mass of dilution B used in substrate prep | 4.6139 g |
| Concentration of nonradiolabeled ASDN in substrate soln. | 0.573295 µg/g |

Calculation of Substrate Solution Specific Activity

1) Calculate µg [$^3$H]ASDN/g soln. = 0.00809 µg/g soln.
µg/g soln.

| | |
|---|---|
| a. µCi/g soln | 0.715 |
| 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.

= 0.573295 + 0.00809
= 0.581389 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.230 µCi/µg ASDN

781980 dpm/nmol

| Assay Date | 12/28/2004 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | | |
|---|---|---|---|---|---|---|---|
| Technician ID | JG | Replicate # | -- | Microsome type | recombinant | Microsome ID | MET-0254A Vial 1 |

| Standards: | 1.42 | 0.95 | 0.71 | 0.47 | 0.24 | 0.12 | 0 | Protein stock (mg BSA) | Total volume of stock (mL) | Protein stock ID |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0.3862 | 0.2843 | 0.2164 | 0.1604 | 0.0788 | 0.0531 | 0.0266 | 23.649 | 10 | |
| | 0.3957 | 0.3023 | 0.2354 | 0.1607 | 0.0843 | 0.0529 | 0.0215 | | | |
| | 0.3849 | 0.3011 | 0.2113 | 0.1682 | 0.0830 | 0.0547 | 0.0275 | | | |

Samples:

| | | |
|---|---|---|
| | 0.1697 | 0.0513 |
| | 0.1810 | 0.0504 |
| | 0.1775 | 0.0512 |

| 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 | 0.389 | 0.364 | 0.0339 | m, b | 0.094 | 0.000 |
| 0.95 | 40 | 100 | 0.00095 | 25 | 0.0236 | 0.296 | 0.271 | 0.0252 | $se_m$, $se_b$ | 0.005 | 0.001 |
| 0.71 | 30 | 100 | 0.00071 | 25 | 0.0177 | 0.222 | 0.197 | 0.0182 | $r^2$, $se_y$ | 0.991 | 0.001 |
| 0.47 | 20 | 100 | 0.00047 | 25 | 0.0118 | 0.163 | 0.138 | 0.0127 | F, df | 420 | 4 |
| 0.24 | 10 | 100 | 0.00024 | 25 | 0.0059 | 0.082 | 0.057 | 0.0052 | $ss_{reg}$, $ss_{resid}$ | 0.001 | 0.000 |
| 0.12 | 5.2 | 100 | 0.00012 | 25 | 0.0031 | 0.054 | 0.028 | 0.0025 | | | |

Regression results are calculated using the function LINEST

Blank 0.025

$r^2$= 0.991
m= 0.094
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 |
|---|---|---|---|---|---|---|---|---|
| 0.170 | 0.145 | 0.013 | 25 | 1 | 1 | 0.001 | 0.001 | 0.558 |
| 0.181 | 0.156 | 0.014 | 25 | 1 | 1 | 0.001 | | |
| 0.178 | 0.152 | 0.014 | 25 | 1 | 1 | 0.001 | | |
| 0.051 | 0.026 | 0.002 | 25 | 65 | 3965 | 0.006 | 0.005 | 5.444 |
| 0.050 | 0.025 | 0.002 | 25 | 65 | 3965 | 0.005 | | |
| 0.051 | 0.026 | 0.002 | 25 | 65 | 3965 | 0.005 | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Assay Date | 12/28/2004 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | | 6 | | | |
| Technician ID | JG | Replicate # | -- | Microsome type | | recombinant | Microsome ID | MET-0254A Vial 1 | |
| 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 | ######## | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | Microsome type | recombinar | Microsome ID | MET-0254 | Technician ID | JG | Replicate # | -- |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Microsome Dilution Details | | |
|---|---|---|
| Dilution A | 0.0653 | mL microsome Stock used |
| | 3.9653 | mL total volume |
| | 60.72435 | 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 |
| | 607.2435 | total dilution factor |

| | |
|---|---|
| Protein Concentration (stock microsomes, mg/mL): | 5.444 |
| Protein Concentration (dilution added to assay, mg/mL): | 0.008965 |

| 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 | 12/26/2004 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | Microsome type | recombinant | Microsome ID | MET-0254A Vial 1 | Technician ID | JC | Replicate # | .. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| 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 | 9766 | 19531.46 | 19792.5 | 39585.0 | 0.1 | 158741 | 24.94 | 39477 | 0.0505 | 1 | 0.004 | 15 | 0.3754 |
| | | | 0.5 | 2 | 10027 | 20054.14 | | | 0.1 | | | | | 1 | | 15 | |
| | 2 | 2 | 0.5 | 1 | 8836 | 17671.46 | 17674.43 | 35348.86 | 0.1 | 158741 | 22.27 | 35240 | 0.0451 | 1 | 0.004 | 15 | 0.3351 |
| | | | 0.5 | 2 | 8839 | 17677.4 | | | 0.1 | | | | | 1 | | 15 | |
| | 3 | 2 | 0.5 | 1 | 8779 | 17557.76 | 17804.99 | 35609.98 | 0.1 | 158741 | 22.43 | 35501 | 0.0454 | 1 | 0.004 | 15 | 0.3376 |
| | | | 0.5 | 2 | 9026 | 18052.22 | | | 0.1 | | | | | 1 | | 15 | |
| | 4 | 2 | 0.5 | 1 | 8703 | 17406.76 | 17421.06 | 34842.12 | 0.1 | 158741 | 21.95 | 34733 | 0.0444 | 1 | 0.004 | 15 | 0.3303 |
| | | | 0.5 | 2 | 8718 | 17435.36 | | | 0.1 | | | | | 1 | | 15 | |
| Background control | 1 | 2 | 0.5 | 1 | 31 | 62.6 | 63.46 | 126.92 | 0.1 | 158741 | 0.08 | 18 | 0.0000 | 1 | 0.004 | 15 | 0.0002 |
| | | | 0.5 | 2 | 32 | 64.32 | | | 0.1 | | | | | 1 | | 15 | |
| | 2 | 2 | 0.5 | 1 | 27 | 53 | 52.31 | 104.62 | 0.1 | 158741 | 0.07 | -4 | 0.0000 | 1 | 0.004 | 15 | 0.0000 |
| | | | 0.5 | 2 | 26 | 51.62 | | | 0.1 | | | | | 1 | | 15 | |
| | 3 | 2 | 0.5 | 1 | 24 | 48.04 | 56.14 | 112.28 | 0.1 | 158741 | 0.07 | 3 | 0.0000 | 1 | 0.004 | 15 | 0.0000 |
| | | | 0.5 | 2 | 32 | 64.24 | | | 0.1 | | | | | 1 | | 15 | |
| | 4 | 2 | 0.5 | 1 | 24 | 48.18 | 46.01 | 92.02 | 0.1 | 158741 | 0.06 | -17 | 0.0000 | 1 | 0.004 | 15 | -0.0002 |
| | | | 0.5 | 2 | 22 | 43.84 | | | 0.1 | | | | | 1 | | 15 | |
| Positive control | 1 | 2 | 0.5 | 1 | | | | | 0.1 | 158741 | | #VALUE! | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 2 | 2 | 0.5 | 1 | | | | | 0.1 | 158741 | | #VALUE! | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 3 | 2 | 0.5 | 1 | | | | | 0.1 | 158741 | | #VALUE! | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 4 | 2 | 0.5 | 1 | | | | | 0.1 | 158741 | | #VALUE! | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| Negative Control | 1 | 2 | 0.5 | 1 | | | | | 0.1 | 158741 | | #VALUE! | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 2 | 2 | 0.5 | 1 | | | | | 0.1 | 158741 | | #VALUE! | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 3 | 2 | 0.5 | 1 | | | | | 0.1 | 158741 | | #VALUE! | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 4 | 2 | 0.5 | 1 | | | | | 0.1 | 158741 | | #VALUE! | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| 4-OH ASDN | 1-1 | 2 | 0.5 | 1 | 588 | 1176.48 | 1229.9 | 2459.8 | 0.1 | 158741 | 1.55 | 2351 | 0.0030 | 1 | 0.004 | 15 | 0.0224 |
| | | | 0.5 | 2 | 642 | 1283.32 | | | 0.1 | | | | | 1 | | 15 | |
| | 1-2 | 2 | 0.5 | 1 | 617 | 1233.74 | 1278.37 | 2556.74 | 0.1 | 158741 | 1.61 | 2448 | 0.0031 | 1 | 0.004 | 15 | 0.0233 |
| | | | 0.5 | 2 | 662 | 1323 | | | 0.1 | | | | | 1 | | 15 | |
| | 1-3 | 2 | 0.5 | 1 | 600 | 1200.16 | 1189.12 | 2378.24 | 0.1 | 158741 | 1.50 | 2289 | 0.0029 | 1 | 0.004 | 15 | 0.0216 |
| | | | 0.5 | 2 | 589 | 1178.08 | | | 0.1 | | | | | 1 | | 15 | |
| | 2-1 | 2 | 0.5 | 1 | 3516 | 7032.96 | 7054.11 | 14108.22 | 0.1 | 158741 | 8.89 | 13999 | 0.0179 | 1 | 0.004 | 15 | 0.1331 |
| | | | 0.5 | 2 | 3538 | 7075.28 | | | 0.1 | | | | | 1 | | 15 | |
| | 2-2 | 2 | 0.5 | 1 | 3594 | 7187.8 | 7178.79 | 14357.58 | 0.1 | 158741 | 9.04 | 14249 | 0.0182 | 1 | 0.004 | 15 | 0.1355 |
| | | | 0.5 | 2 | 3585 | 7169.78 | | | 0.1 | | | | | 1 | | 15 | |
| | 2-3 | 2 | 0.5 | 1 | 3542 | 7084.74 | 7134.56 | 14269.12 | 0.1 | 158741 | 8.99 | 14160 | 0.0181 | 1 | 0.004 | 15 | 0.1347 |
| | | | 0.5 | 2 | 3592 | 7184.38 | | | 0.1 | | | | | 1 | | 15 | |
| | 3-1 | 2 | 0.5 | 1 | 4769 | 9537.94 | 9352.76 | 18705.52 | 0.1 | 158741 | 11.78 | 18597 | 0.0238 | 1 | 0.004 | 15 | 0.1768 |
| | | | 0.5 | 2 | 4584 | 9167.58 | | | 0.1 | | | | | 1 | | 15 | |
| | 3-2 | 2 | 0.5 | 1 | 10899 | 21397.32 | 21621.81 | 43243.62 | 0.1 | 158741 | 27.24 | 43135 | 0.0592 | 1 | 0.004 | 15 | 0.4102 |
| | | | 0.5 | 2 | 10923 | 21846.3 | | | 0.1 | | | | | 1 | | 15 | |
| | 3-3 | 2 | 0.5 | 1 | 4940 | 9878.62 | 9865.5 | 19731 | 0.1 | 158741 | 12.43 | 19622 | 0.0251 | 1 | 0.004 | 15 | 0.1866 |
| | | | 0.5 | 2 | 4926 | 9851.38 | | | 0.1 | | | | | 1 | | 15 | |
| | 4-1 | 2 | 0.5 | 1 | 5786 | 11571.6 | 11558.7 | 23117.4 | 0.1 | 158741 | 14.56 | 23008 | 0.0294 | 1 | 0.004 | 15 | 0.2188 |
| | | | 0.5 | 2 | 5773 | 11545.8 | | | 0.1 | | | | | 1 | | 15 | |
| | 4-2 | 2 | 0.5 | 1 | 6353 | 12706.8 | 13003.08 | 26006.16 | 0.1 | 158741 | 16.38 | 25897 | 0.0331 | 1 | 0.004 | 15 | 0.2463 |
| | | | 0.5 | 2 | 6650 | 13300.36 | | | 0.1 | | | | | 1 | | 15 | |
| | 4-3 | 2 | 0.5 | 1 | 6222 | 12443.34 | 12638.17 | 25276.34 | 0.1 | 158741 | 15.92 | 25167 | 0.0322 | 1 | 0.004 | 15 | 0.2393 |
| | | | 0.5 | 2 | 6417 | 12833 | | | 0.1 | | | | | 1 | | 15 | |
| | 5-1 | 2 | 0.5 | 1 | 7896 | 15792.16 | 15884.98 | 31769.96 | 0.1 | 158741 | 20.01 | 31661 | 0.0405 | 1 | 0.004 | 15 | 0.3011 |
| | | | 0.5 | 2 | 7989 | 15977.8 | | | 0.1 | | | | | 1 | | 15 | |
| | 5-2 | 2 | 0.5 | 1 | 7756 | 15512.72 | 15800.95 | 31601.9 | 0.1 | 158741 | 19.91 | 31493 | 0.0403 | 1 | 0.004 | 15 | 0.2995 |
| | | | 0.5 | 2 | 8045 | 16089.18 | | | 0.1 | | | | | 1 | | 15 | |
| | 5-3 | 2 | 0.5 | 1 | 7699 | 15398.54 | 15708.24 | 31416.48 | 0.1 | 158741 | 19.79 | 31308 | 0.0400 | 1 | 0.004 | 15 | 0.2977 |
| | | | 0.5 | 2 | 8009 | 16017.94 | | | 0.1 | | | | | 1 | | 15 | |
| | 6-1 | 2 | 0.5 | 1 | 8910 | 17819.58 | 17891.67 | 35783.34 | 0.1 | 158741 | 22.54 | 35674 | 0.0456 | 1 | 0.004 | 15 | 0.3392 |
| | | | 0.5 | 2 | 8982 | 17963.76 | | | 0.1 | | | | | 1 | | 15 | |
| | 6-2 | 2 | 0.5 | 1 | 8285 | 16569.06 | 16826.56 | 33653.12 | 0.1 | 158741 | 21.33 | 33744 | 0.0432 | 1 | 0.004 | 15 | 0.3209 |
| | | | 0.5 | 2 | 8642 | 17284.06 | | | 0.1 | | | | | 1 | | 15 | |
| | 6-3 | 2 | 0.5 | 1 | 8757 | 17514.3 | 17532.05 | 35064.1 | 0.1 | 158741 | 22.09 | 34955 | 0.0447 | 1 | 0.004 | 15 | 0.3324 |
| | | | 0.5 | 2 | 8775 | 17549.8 | | | 0.1 | | | | | | | | |

| Assay Date | 12/28/2004 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | Microsome 6 type | recombinant | Microsome ID | MET-0254A Vi | Technician ID | JG | Replicate # | -- |
|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Control Type | Portion | Average | SD |
|---|---|---|---|
| Full activity | Beginning | 0.3553 | 0.0285 |
| Full activity | End | 0.3339 | 0.0052 |
| Full activity | Overall | 0.3446 | 0.0208 |
| Background | Beginning | 0.0001 | 0.00014995 |
| Background | End | -0.0001 | 0.000136233 |
| Background | Overall | 0.0000 | 0.000138628 |
| 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 | 0.0224 |
| 4-OH ASDN | 1 | 2 | 1.00E-06 | -6.00 | 0.0233 |
| 4-OH ASDN | 1 | 3 | 1.00E-06 | -6.00 | 0.0216 |
| 4-OH ASDN | 2 | 1 | 1.00E-07 | -7.00 | 0.1331 |
| 4-OH ASDN | 2 | 2 | 1.00E-07 | -7.00 | 0.1355 |
| 4-OH ASDN | 2 | 3 | 1.00E-07 | -7.00 | 0.1347 |
| 4-OH ASDN | 3 | 1 | 5.00E-08 | -7.30 | 0.1768 |
| 4-OH ASDN | 3 | 2 | 5.00E-08 | -7.30 | 0.4102 |
| 4-OH ASDN | 3 | 3 | 5.00E-08 | -7.30 | 0.1866 |
| 4-OH ASDN | 4 | 1 | 2.50E-08 | -7.60 | 0.2188 |
| 4-OH ASDN | 4 | 2 | 2.50E-08 | -7.60 | 0.2463 |
| 4-OH ASDN | 4 | 3 | 2.50E-08 | -7.60 | 0.2393 |
| 4-OH ASDN | 5 | 1 | 1.00E-08 | -8.00 | 0.3011 |
| 4-OH ASDN | 5 | 2 | 1.00E-08 | -8.00 | 0.2995 |
| 4-OH ASDN | 5 | 3 | 1.00E-08 | -8.00 | 0.2977 |
| 4-OH ASDN | 6 | 1 | 1.00E-09 | -9.00 | 0.3392 |
| 4-OH ASDN | 6 | 2 | 1.00E-09 | -9.00 | 0.3209 |
| 4-OH ASDN | 6 | 3 | 1.00E-09 | -9.00 | 0.3324 |

| | Percent of control values | | | |
|---|---|---|---|---|
| | Log[test | Replicate | | |
| Level | substance] | 1 | 2 | 3 |
| 1 | -6.00 | 6.49 | 6.75 | 6.26 |
| 2 | -7.00 | 38.63 | 39.32 | 39.08 |
| 3 | -7.30 | 51.32 | 119.03 | 54.15 |
| 4 | -7.60 | 63.49 | 71.46 | 69.45 |
| 5 | -8.00 | 87.37 | 86.91 | 86.39 |
| 6 | -9.00 | 98.45 | 93.12 | 96.46 |

| Aliquot # | Weight of aliquot (g) | DPM/Aliq. | DPM/g soln. | |
|---|---|---|---|---|
| 1 | 0.0196 | 32280.49 | 1646964 | |
| 2 | 0.0197 | 33650.59 | 1708152 | |
| 3 | 0.0195 | 35059.55 | 1797926 | |
| 4 | 0.0196 | 33984.6 | 1733908 | |
| 5 | 0.0197 | 34102.87 | 1731110 | |
| | | | Average DPM/g soln | 1723612 |
| | | | SD | 54311 |
| | | | CV | 3.15 |
| | | | µCi/g soln | 0.776 |

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

| ASDN solution | mg ASDN added | total volume (mL) | dilution factor | [ASDN] in solution (µg/mL) |
|---|---|---|---|---|
| Stock | 10.1 | 10 | | 1010.00 |
| Dilution A | | | 100 | 10.10 |
| Dilution B | | | 10 | 1.01 |

Calculation of concentration nonradiolabeled ASDN in substrate solution

| | |
|---|---|
| Total g substrate solution | 8.0993 g |
| Mass of dilution B used in substrate prep | 4.5101 g |
| Concentration of nonradiolabeled ASDN in substrate soln. | 0.562419 µg/g |

Calculation of Substrate Solution Specific Activity

1) Calculate µg [$^3$H]ASDN/g soln. = 0.00879 µg/g soln.
µg/g soln.

| | |
|---|---|
| a. µCi/g soln | 0.776 |
| 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.

= 0.562419 + 0.00879
= 0.571208 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.359 µCi/µg ASDN

864208 dpm/nmol

| | | | | | | |
|---|---|---|---|---|---|---|
| Assay Date | 12/29/2004 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | |
| Technician ID | JG | Replicate # | -- | Microsome type | recombinant | Microsome ID: Met-0254A Vial 2 |

| Standards: | 1.42 | 0.95 | 0.71 | 0.47 | 0.24 | 0.12 | 0 |
|---|---|---|---|---|---|---|---|
| | 0.3792 | 0.2918 | 0.2191 | 0.1582 | 0.0931 | 0.0550 | 0.0234 |
| | 0.3951 | 0.2988 | 0.2255 | 0.1626 | 0.0875 | 0.0583 | 0.0246 |
| | 0.4040 | 0.2916 | 0.2324 | 0.1674 | 0.0934 | 0.0580 | 0.0226 |

| Protein stock (mg BSA) | Total volume of stock (mL) | Protein stock ID |
|---|---|---|
| 23.649 | 10 | |

| Samples: | BSA Control | Microsomes |
|---|---|---|
| | 0.1655 | 0.0477 |
| | 0.1775 | 0.0492 |
| | 0.1772 | 0.0506 |

| 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 | 0.393 | 0.369 | 0.0342 |
| 0.95 | 40 | 100 | 0.00095 | 25 | 0.0236 | 0.294 | 0.271 | 0.0248 |
| 0.71 | 30 | 100 | 0.00071 | 25 | 0.0177 | 0.226 | 0.202 | 0.0183 |
| 0.47 | 20 | 100 | 0.00047 | 25 | 0.0118 | 0.163 | 0.139 | 0.0124 |
| 0.24 | 10 | 100 | 0.00024 | 25 | 0.0059 | 0.091 | 0.068 | 0.0056 |
| 0.12 | 5.2 | 100 | 0.00012 | 25 | 0.0031 | 0.057 | 0.034 | 0.0023 |

| Variables | Regression results | |
|---|---|---|
| m, b | 0.095 | -0.001 |
| $se_m$, $se_b$ | 0.004 | 0.001 |
| $r^2$, $se_y$ | 0.994 | 0.001 |
| F, df | 669 | 4 |
| $ss_{reg}$, $ss_{resid}$ | 0.001 | 0.000 |

Regression results are calculated using the function LINEST

Blank 0.024

$r^2$= 0.994
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 |
|---|---|---|---|---|---|---|---|---|---|
| BSA Control | 0.166 | 0.142 | 0.013 | 25 | 1 | 1 | 0.001 | 0.001 | 0.535 |
| BSA Control | 0.178 | 0.154 | 0.014 | 25 | 1 | 1 | 0.001 | | |
| BSA Control | 0.177 | 0.154 | 0.014 | 25 | 1 | 1 | 0.001 | | |
| Microsomes | 0.048 | 0.024 | 0.001 | 25 | 65 | 3965 | 0.004 | 0.004 | 3.861 |
| Microsomes | 0.049 | 0.026 | 0.002 | 25 | 65 | 3965 | 0.004 | | |
| Microsomes | 0.051 | 0.027 | 0.002 | 25 | 65 | 3965 | 0.004 | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Assay Date | 12/29/2004 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | | 6 | | | |
| Technician ID | JG | Replicate # | -- | Microsome type | | recombinant | Microsome ID | Met-0254A 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 — $r^2$= 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 | ######## | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | Microsome type | recombinat | Microsome ID | Met-0254A | Technician ID | JG | Replicate # | -- |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Microsome Dilution Details | | |
|---|---|---|
| Dilution A | 0.0653 | mL microsome Stock used |
| | 3.9653 | mL total volume |
| | 60.72435 | 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 |
| | 607.2435 | total dilution factor |

| Protein Concentration (stock microsomes, mg/mL): | 3.861 |
|---|---|
| Protein Concentration (dilution added to assay, mg/mL): | 0.006358 |

| 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 | 12/29/2004 | Test Chemical ID | 4-OH ASON | # Concentrations tested | 6 | Microsome type | recombinant | Microsome ID | Met-0254A Vial 2 | Technician ID | JG | Replicate # | -- |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| 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 | 8364 | 16727.16 | 16729.53 | 33459.06 | 0.1 | 172361 | 19.41 | 33458 | 0.0367 | 1 | 0.003 | 15 | 0.4056 |
| | | | 0.5 | 2 | 8365 | 16731.9 | | | 0.1 | | | | | 1 | | 15 | |
| | 2 | 2 | 0.5 | 1 | 8432 | 16863.38 | 17026.39 | 34066.78 | 0.1 | 172361 | 19.76 | 34052 | 0.0394 | 1 | 0.003 | 15 | 0.4131 |
| | | | 0.5 | 2 | 8595 | 17189.4 | | | 0.1 | | | | | 1 | | 15 | |
| | 3 | 2 | 0.5 | 1 | 7762 | 15523.18 | 15773.59 | 31547.18 | 0.1 | 172361 | 18.30 | 31543 | 0.0365 | 1 | 0.003 | 15 | 0.3827 |
| | | | 0.5 | 2 | 8012 | 16024 | | | 0.1 | | | | | 1 | | 15 | |
| | 4 | 2 | 0.5 | 1 | 7814 | 15627.96 | 15804.24 | 31608.48 | 0.1 | 172361 | 18.34 | 31604 | 0.0366 | 1 | 0.003 | 15 | 0.3834 |
| | | | 0.5 | 2 | 7990 | 15980.52 | | | 0.1 | | | | | 1 | | 15 | |
| Background control | 1 | 2 | 0.5 | 1 | 0 | 0 | 5.59 | 11.18 | 0.1 | 172361 | 0.01 | 7 | 0.0000 | 1 | 0.003 | 15 | 0.0001 |
| | | | 0.5 | 2 | 6 | 11.18 | | | 0.1 | | | | | 1 | | 15 | |
| | 2 | 2 | 0.5 | 1 | 1 | 2.52 | 1.26 | 2.52 | 0.1 | 172361 | 0.00 | -2 | 0.0000 | 1 | 0.003 | 15 | 0.0000 |
| | | | 0.5 | 2 | 0 | 0 | | | 0.1 | | | | | 1 | | 15 | |
| | 3 | 2 | 0.5 | 1 | 0 | 0 | 2.16 | 4.36 | 0.1 | 172361 | 0.00 | 0 | 0.0000 | 1 | 0.003 | 15 | 0.0000 |
| | | | 0.5 | 2 | 2 | 4.36 | | | 0.1 | | | | | 1 | | 15 | |
| | 4 | 2 | 0.5 | 1 | 0 | 0 | 0 | 0 | 0.1 | 172361 | 0.00 | -5 | 0.0000 | 1 | 0.003 | 15 | -0.0001 |
| | | | 0.5 | 2 | 0 | 0 | | | 0.1 | | | | | 1 | | 15 | |
| Positive control | 1 | 2 | 0.5 | 1 | | | | | 0.1 | 172361 | | #VALUE! | #VALUE! | 1 | 0.003 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 2 | 2 | 0.5 | 1 | | | | | 0.1 | 172361 | | #VALUE! | #VALUE! | 1 | 0.003 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 3 | 2 | 0.5 | 1 | | | | | 0.1 | 172361 | | #VALUE! | #VALUE! | 1 | 0.003 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 4 | 2 | 0.5 | 1 | | | | | 0.1 | 172361 | | #VALUE! | #VALUE! | 1 | 0.003 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| Negative Control | 1 | 2 | 0.5 | 1 | | | | | 0.1 | 172361 | | #VALUE! | #VALUE! | 1 | 0.003 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 2 | 2 | 0.5 | 1 | | | | | 0.1 | 172361 | | #VALUE! | #VALUE! | 1 | 0.003 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 3 | 2 | 0.5 | 1 | | | | | 0.1 | 172361 | | #VALUE! | #VALUE! | 1 | 0.003 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 4 | 2 | 0.5 | 1 | | | | | 0.1 | 172361 | | #VALUE! | #VALUE! | 1 | 0.003 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| 4-OH ASON | 1-1 | 2 | 0.5 | 1 | 508 | 1009.06 | 1033.22 | 2066.44 | 0.1 | 172361 | 1.20 | 2062 | 0.0024 | 1 | 0.003 | 15 | 0.0250 |
| | | | 0.5 | 2 | 529 | 1057.38 | | | 0.1 | | | | | 1 | | 15 | |
| | 1-2 | 2 | 0.5 | 1 | 487 | 973.74 | 1003.27 | 2006.54 | 0.1 | 172361 | 1.16 | 2002 | 0.0023 | 1 | 0.003 | 15 | 0.0243 |
| | | | 0.5 | 2 | 516 | 1032.8 | | | 0.1 | | | | | 1 | | 15 | |
| | 1-3 | 2 | 0.5 | 1 | 509 | 1018.35 | 1045.74 | 2091.48 | 0.1 | 172361 | 1.21 | 2087 | 0.0024 | 1 | 0.003 | 15 | 0.0253 |
| | | | 0.5 | 2 | 537 | 1073.12 | | | 0.1 | | | | | 1 | | 15 | |
| | 2-1 | 2 | 0.5 | 1 | 3064 | 6107.86 | 6202.61 | 12405.22 | 0.1 | 172361 | 7.20 | 12401 | 0.0143 | 1 | 0.003 | 15 | 0.1505 |
| | | | 0.5 | 2 | 3149 | 6297.36 | | | 0.1 | | | | | 1 | | 15 | |
| | 2-2 | 2 | 0.5 | 1 | 3047 | 6094.74 | 6124.9 | 12249.8 | 0.1 | 172361 | 7.11 | 12245 | 0.0142 | 1 | 0.003 | 15 | 0.1486 |
| | | | 0.5 | 2 | 3078 | 6155.06 | | | 0.1 | | | | | 1 | | 15 | |
| | 2-3 | 2 | 0.5 | 1 | 3007 | 6014.49 | 6063.69 | 12127.38 | 0.1 | 172361 | 7.04 | 12123 | 0.0140 | 1 | 0.003 | 15 | 0.1471 |
| | | | 0.5 | 2 | 3056 | 6112.82 | | | 0.1 | | | | | 1 | | 15 | |
| | 3-1 | 2 | 0.5 | 1 | 4380 | 8760.48 | 8781.81 | 17563.62 | 0.1 | 172361 | 10.19 | 17559 | 0.0203 | 1 | 0.003 | 15 | 0.2130 |
| | | | 0.5 | 2 | 4402 | 8803.16 | | | 0.1 | | | | | 1 | | 15 | |
| | 3-2 | 2 | 0.5 | 1 | 4349 | 8698.44 | 8751.97 | 17503.94 | 0.1 | 172361 | 10.16 | 17499 | 0.0202 | 1 | 0.003 | 15 | 0.2123 |
| | | | 0.5 | 2 | 4403 | 8805.5 | | | 0.1 | | | | | 1 | | 15 | |
| | 3-3 | 2 | 0.5 | 1 | 4244 | 8487.36 | 8483.97 | 16967.94 | 0.1 | 172361 | 9.84 | 16963 | 0.0196 | 1 | 0.003 | 15 | 0.2058 |
| | | | 0.5 | 2 | 4240 | 8480.58 | | | 0.1 | | | | | 1 | | 15 | |
| | 4-1 | 2 | 0.5 | 1 | 5214 | 10428.28 | 10557.03 | 21114.06 | 0.1 | 172361 | 12.25 | 21110 | 0.0244 | 1 | 0.003 | 15 | 0.2561 |
| | | | 0.5 | 2 | 5343 | 10685.78 | | | 0.1 | | | | | 1 | | 15 | |
| | 4-2 | 2 | 0.5 | 1 | 5555 | 11110.46 | 11035.64 | 22071.28 | 0.1 | 172361 | 12.81 | 22067 | 0.0255 | 1 | 0.003 | 15 | 0.2677 |
| | | | 0.5 | 2 | 5480 | 10960.82 | | | 0.1 | | | | | 1 | | 15 | |
| | 4-3 | 2 | 0.5 | 1 | 5559 | 11118.92 | 11013.76 | 22027.52 | 0.1 | 172361 | 12.78 | 22023 | 0.0255 | 1 | 0.003 | 15 | 0.2672 |
| | | | 0.5 | 2 | 5454 | 10908.6 | | | 0.1 | | | | | 1 | | 15 | |
| | 5-1 | 2 | 0.5 | 1 | 6762 | 13523.9 | 13758.6 | 27517.2 | 0.1 | 172361 | 15.96 | 27513 | 0.0318 | 1 | 0.003 | 15 | 0.3338 |
| | | | 0.5 | 2 | 6997 | 13993.3 | | | 0.1 | | | | | 1 | | 15 | |
| | 5-2 | 2 | 0.5 | 1 | 6846 | 13690.34 | 13801.21 | 27602.42 | 0.1 | 172361 | 16.01 | 27598 | 0.0319 | 1 | 0.003 | 15 | 0.3348 |
| | | | 0.5 | 2 | 6956 | 13912.08 | | | 0.1 | | | | | 1 | | 15 | |
| | 5-3 | 2 | 0.5 | 1 | 6627 | 13253.1 | 13423.74 | 26847.48 | 0.1 | 172361 | 15.58 | 26843 | 0.0311 | 1 | 0.003 | 15 | 0.3257 |
| | | | 0.5 | 2 | 6797 | 13594.38 | | | 0.1 | | | | | 1 | | 15 | |
| | 6-1 | 2 | 0.5 | 1 | 7819 | 15638.66 | 15909.84 | 31819.68 | 0.1 | 172361 | 18.48 | 31815 | 0.0368 | 1 | 0.003 | 15 | 0.3860 |
| | | | 0.5 | 2 | 8091 | 16181 | | | 0.1 | | | | | 1 | | 15 | |
| | 6-2 | 2 | 0.5 | 1 | 7778 | 15552.22 | 15756.33 | 31512.66 | 0.1 | 172361 | 18.28 | 31508 | 0.0365 | 1 | 0.003 | 15 | 0.3823 |
| | | | 0.5 | 2 | 7980 | 15960.44 | | | 0.1 | | | | | 1 | | 15 | |
| | 6-3 | 2 | 0.5 | 1 | 7849 | 15697.5 | 15711.09 | 31422.18 | 0.1 | 172361 | 18.23 | 31418 | 0.0364 | 1 | 0.003 | 15 | 0.3812 |
| | | | 0.5 | 2 | 7862 | 15724.68 | | | 0.1 | | | | | | | | |

| Assay Date | 12/29/2004 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | Microsome type | recombinant | Microsome ID | Met-0254A | Via Technician ID | JG | Replicate # | -- |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Control Type | Portion | Average | SD |
|---|---|---|---|
| Full activity | Beginning | 0.4095 | 0.0051 |
| Full activity | End | 0.3831 | 0.0005 |
| Full activity | Overall | 0.3963 | 0.0156 |
| Background | Beginning | 0.0000 | 7.42945E-05 |
| Background | End | 0.0000 | 3.74046E-05 |
| Background | Overall | 0.0000 | 5.81063E-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 | 0.0250 |
| 4-OH ASDN | 1 | 2 | 1.00E-06 | -6.00 | 0.0243 |
| 4-OH ASDN | 1 | 3 | 1.00E-06 | -6.00 | 0.0253 |
| 4-OH ASDN | 2 | 1 | 1.00E-07 | -7.00 | 0.1505 |
| 4-OH ASDN | 2 | 2 | 1.00E-07 | -7.00 | 0.1486 |
| 4-OH ASDN | 2 | 3 | 1.00E-07 | -7.00 | 0.1471 |
| 4-OH ASDN | 3 | 1 | 5.00E-08 | -7.30 | 0.2130 |
| 4-OH ASDN | 3 | 2 | 5.00E-08 | -7.30 | 0.2123 |
| 4-OH ASDN | 3 | 3 | 5.00E-08 | -7.30 | 0.2058 |
| 4-OH ASDN | 4 | 1 | 2.50E-08 | -7.60 | 0.2561 |
| 4-OH ASDN | 4 | 2 | 2.50E-08 | -7.60 | 0.2677 |
| 4-OH ASDN | 4 | 3 | 2.50E-08 | -7.60 | 0.2672 |
| 4-OH ASDN | 5 | 1 | 1.00E-08 | -8.00 | 0.3338 |
| 4-OH ASDN | 5 | 2 | 1.00E-08 | -8.00 | 0.3348 |
| 4-OH ASDN | 5 | 3 | 1.00E-08 | -8.00 | 0.3257 |
| 4-OH ASDN | 6 | 1 | 1.00E-09 | -9.00 | 0.3860 |
| 4-OH ASDN | 6 | 2 | 1.00E-09 | -9.00 | 0.3823 |
| 4-OH ASDN | 6 | 3 | 1.00E-09 | -9.00 | 0.3812 |

| Percent of control values | | | | |
|---|---|---|---|---|
| | Log[test | Replicate | | |
| Level | substance] | 1 | 2 | 3 |
| 1 | -6.00 | 6.31 | 6.13 | 6.39 |
| 2 | -7.00 | 37.97 | 37.49 | 37.11 |
| 3 | -7.30 | 53.76 | 53.58 | 51.93 |
| 4 | -7.60 | 64.63 | 67.56 | 67.42 |
| 5 | -8.00 | 84.23 | 84.49 | 82.18 |
| 6 | -9.00 | 97.40 | 96.46 | 96.19 |

| Aliquot # | Weight of aliquot (g) | DPM/Aliq. | DPM/g soln. | |
|---|---|---|---|---|
| 1 | 0.0196 | 31705.75 | 1617640 | |
| 2 | 0.0198 | 32245.25 | 1628548 | |
| 3 | 0.0199 | 33313.09 | 1674025 | |
| 4 | 0.0197 | 33082.48 | 1679314 | |
| 5 | 0.0200 | 33478.86 | 1673943 | |
| | | | Average DPM/g soln | 1654694 |
| | | | SD | 29184 |
| | | | CV | 1.76 |
| | | | 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 | 8.2422 g |
| Mass of dilution B used in substrate prep | 4.644 g |
| Concentration of nonradiolabeled ASDN in substrate soln. | 0.580345 µg/g |

Calculation of Substrate Solution Specific Activity

1) Calculate µg [$^3$H]ASDN/g soln. = 0.00844 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.

= 0.580345 + 0.00844
= 0.588783 µg ASDN/g soln.

3) Calculate Solution Specific Activity

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

804888 dpm/nmol

| Assay Date | 12/30/2004 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | | |
|---|---|---|---|---|---|---|---|
| Technician ID | JG | Replicate # | -- | Microsome type | recombinant | Microsome ID | MET-0254A Vial 3 |

| | | | | | | | | 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 | |
| | 0.3966 | 0.2908 | 0.2300 | 0.1711 | 0.0903 | 0.0526 | 0.0252 | | | |
| | 0.3948 | 0.3582 | 0.2368 | 0.1711 | 0.0889 | 0.0567 | 0.0219 | | | |
| | 0.4106 | 0.3099 | 0.2357 | 0.1791 | 0.0863 | 0.0563 | 0.0239 | | | |
| Samples: | | | | | | | | | | |
| | | 0.1615 | 0.0520 | | | | | | | |
| | | 0.1648 | 0.0523 | | | | | | | |
| | | 0.1727 | 0.0468 | | | | | | | |

| 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 | 0.401 | 0.377 | 0.0332 | m, b | 0.090 | -0.001 |
| 0.95 | 40 | 100 | 0.00095 | 25 | 0.0236 | 0.320 | 0.296 | 0.0259 | $se_m$, $se_b$ | 0.006 | 0.001 |
| 0.71 | 30 | 100 | 0.00071 | 25 | 0.0177 | 0.234 | 0.211 | 0.0183 | $r^2$, $se_y$ | 0.982 | 0.002 |
| 0.47 | 20 | 100 | 0.00047 | 25 | 0.0118 | 0.174 | 0.150 | 0.0129 | F, df | 220 | 4 |
| 0.24 | 10 | 100 | 0.00024 | 25 | 0.0059 | 0.089 | 0.065 | 0.0052 | $ss_{reg}$, $ss_{resid}$ | 0.001 | 0.000 |
| 0.12 | 5.2 | 100 | 0.00012 | 25 | 0.0031 | 0.055 | 0.032 | 0.0022 | | | |

Regression results are calculated using the function LINEST

| | | | |
|---|---|---|---|
| Blank | 0.024 | $r^2$= | 0.982 |
| | | m= | 0.090 |
| | | 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 |
|---|---|---|---|---|---|---|---|---|
| 0.162 | 0.138 | 0.012 | 25 | 1 | 1 | 0.000 | 0.000 | 0.488 |
| 0.165 | 0.141 | 0.012 | 25 | 1 | 1 | 0.000 | | |
| 0.173 | 0.149 | 0.013 | 25 | 1 | 1 | 0.001 | | |
| 0.052 | 0.028 | 0.002 | 25 | 65 | 3965 | 0.005 | 0.004 | 4.382 |
| 0.052 | 0.029 | 0.002 | 25 | 65 | 3965 | 0.005 | | |
| 0.047 | 0.023 | 0.001 | 25 | 65 | 3965 | 0.004 | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Assay Date | 12/30/2004 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | | 6 | | | |
| Technician ID | JG | Replicate # | -- | Microsome type | | recombinant | Microsome ID | MET-0254A Vial 3 | |
| Standards: | 1.5 | 1 | 0.75 | 0.5 | 0.25 | 0.13 | Blk | Protein stock (mg/10 mL) | Protein stock ID |

Samples:

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

| | $A_{raw}$ | $A_{adj}$ | mg protein measured | µL diluted µSOMES | Vol usome prep. (µL) | Final vol. Diluted usomes (µL) | mg protein/µL Prep. | average mg/µL | mg/mL |
|---|---|---|---|---|---|---|---|---|---|

| Assay Date | ######## | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | Microsome type | recombinat Microsome ID | MET-0254 | Technician ID | JG | Replicate # | -- |
|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Microsome Dilution Details | | |
|---|---|---|
| Dilution A | 0.0653 | mL microsome Stock used |
| | 3.9653 | mL total volume |
| | 60.72435 | 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 |
| | 607.2435 | total dilution factor |

| | |
|---|---|
| Protein Concentration (stock microsomes, mg/mL): | 4.382 |
| Protein Concentration (dilution added to assay, mg/mL): | 0.007216 |

| 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 | 12/30/2004 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | 6 | Microsome type | recombinant | Microsome ID | MET-0254A Vial 3 | Technician ID | JG | Replicate # | -- |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| 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 | 13031 | 26062.62 | 26302.08 | 52604.16 | 0.1 | 165469 | 31.79 | 52474 | 0.0652 | 1 | 0.004 | 15 | 0.6023 |
| | | | 0.5 | 2 | 13271 | 26541.54 | | | 0.1 | | | | | 1 | | 15 | |
| | 2 | 2 | 0.5 | 1 | 13220 | 26440.38 | 26455.42 | 52910.84 | 0.1 | 165469 | 31.98 | 52761 | 0.0656 | 1 | 0.004 | 15 | 0.6058 |
| | | | 0.5 | 2 | 13335 | 26470.46 | | | 0.1 | | | | | 1 | | 15 | |
| | 3 | 2 | 0.5 | 1 | 12741 | 25481.06 | 25565.68 | 51131.36 | 0.1 | 165469 | 30.80 | 51001 | 0.0634 | 1 | 0.004 | 15 | 0.5854 |
| | | | 0.5 | 2 | 12825 | 25650.3 | | | 0.1 | | | | | 1 | | 15 | |
| | 4 | 2 | 0.5 | 1 | 12753 | 25505.94 | 25667.1 | 51334.2 | 0.1 | 165469 | 31.02 | 51204 | 0.0636 | 1 | 0.004 | 15 | 0.5877 |
| | | | 0.5 | 2 | 12914 | 25828.26 | | | 0.1 | | | | | 1 | | 15 | |
| Background control | 1 | 2 | 0.5 | 1 | 34 | 68.72 | 64.37 | 128.74 | 0.1 | 165469 | 0.08 | -1 | 0.0000 | 1 | 0.004 | 15 | 0.0000 |
| | | | 0.5 | 2 | 30 | 59.82 | | | 0.1 | | | | | 1 | | 15 | |
| | 2 | 2 | 0.5 | 1 | 35 | 70.82 | 69.27 | 138.54 | 0.1 | 165469 | 0.08 | -1 | 0.0000 | 1 | 0.004 | 15 | 0.0001 |
| | | | 0.5 | 2 | 34 | 67.72 | | | 0.1 | | | | | 1 | | 15 | |
| | 3 | 2 | 0.5 | 1 | 29 | 56.94 | 61.29 | 122.58 | 0.1 | 165469 | 0.07 | -7 | 0.0000 | 1 | 0.004 | 15 | -0.0001 |
| | | | 0.5 | 2 | 33 | 63.64 | | | 0.1 | | | | | 1 | | 15 | |
| | 4 | 2 | 0.5 | 1 | 27 | 54.66 | 64.87 | 129.74 | 0.1 | 165469 | 0.08 | 0 | 0.0000 | 1 | 0.004 | 15 | 0.0000 |
| | | | 0.5 | 2 | 37 | 74.88 | | | 0.1 | | | | | 1 | | 15 | |
| Positive control | 1 | 2 | 0.5 | 1 | | | | | 0.1 | 165469 | | #VALUE! | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 2 | 2 | 0.5 | 1 | | | | | 0.1 | 165469 | | #VALUE! | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 3 | 2 | 0.5 | 1 | | | | | 0.1 | 165469 | | #VALUE! | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 4 | 2 | 0.5 | 1 | | | | | 0.1 | 165469 | | #VALUE! | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| Negative Control | 1 | 2 | 0.5 | 1 | | | | | 0.1 | 165469 | | #VALUE! | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 2 | 2 | 0.5 | 1 | | | | | 0.1 | 165469 | | #VALUE! | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 3 | 2 | 0.5 | 1 | | | | | 0.1 | 165469 | | #VALUE! | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| | 4 | 2 | 0.5 | 1 | | | | | 0.1 | 165469 | | #VALUE! | #VALUE! | 1 | 0.004 | 15 | #VALUE! |
| | | | 0.5 | 2 | | | | | 0.1 | | | | | 1 | | 15 | |
| 4-OH ASDN | 1-1 | 2 | 0.5 | 1 | 828 | 1655.28 | 1658.14 | 3316.28 | 0.1 | 165469 | 2.00 | 3166 | 0.0040 | 1 | 0.004 | 15 | 0.0366 |
| | | | 0.5 | 2 | 831 | 1661 | | | 0.1 | | | | | 1 | | 15 | |
| | 1-2 | 2 | 0.5 | 1 | 872 | 1743.48 | 1730.21 | 3460.42 | 0.1 | 165469 | 2.09 | 3331 | 0.0041 | 1 | 0.004 | 15 | 0.0382 |
| | | | 0.5 | 2 | 859 | 1716.96 | | | 0.1 | | | | | 1 | | 15 | |
| | 1-3 | 2 | 0.5 | 1 | 863 | 1726.5 | 1753.96 | 3507.92 | 0.1 | 165469 | 2.12 | 3378 | 0.0042 | 1 | 0.004 | 15 | 0.0388 |
| | | | 0.5 | 2 | 891 | 1782.42 | | | 0.1 | | | | | 1 | | 15 | |
| | 2-1 | 2 | 0.5 | 1 | 4932 | 9863.46 | 9866.14 | 19732.28 | 0.1 | 165469 | 11.93 | 19602 | 0.0244 | 1 | 0.004 | 15 | 0.2250 |
| | | | 0.5 | 2 | 4934 | 9868.82 | | | 0.1 | | | | | 1 | | 15 | |
| | 2-2 | 2 | 0.5 | 1 | 4939 | 9877.64 | 9921.97 | 19843.94 | 0.1 | 165469 | 11.99 | 19714 | 0.0245 | 1 | 0.004 | 15 | 0.2263 |
| | | | 0.5 | 2 | 4983 | 9966.3 | | | 0.1 | | | | | 1 | | 15 | |
| | 2-3 | 2 | 0.5 | 1 | 4885 | 9769.14 | 9776.02 | 19552.04 | 0.1 | 165469 | 11.82 | 19422 | 0.0241 | 1 | 0.004 | 15 | 0.2229 |
| | | | 0.5 | 2 | 4891 | 9782.9 | | | 0.1 | | | | | 1 | | 15 | |
| | 3-1 | 2 | 0.5 | 1 | 6957 | 13914.8 | 13880.27 | 27760.54 | 0.1 | 165469 | 16.76 | 27631 | 0.0343 | 1 | 0.004 | 15 | 0.3171 |
| | | | 0.5 | 2 | 6923 | 13845.74 | | | 0.1 | | | | | 1 | | 15 | |
| | 3-2 | 2 | 0.5 | 1 | 7257 | 14513.92 | 14590.69 | 29181.38 | 0.1 | 165469 | 17.64 | 29051 | 0.0361 | 1 | 0.004 | 15 | 0.3335 |
| | | | 0.5 | 2 | 7334 | 14667.46 | | | 0.1 | | | | | 1 | | 15 | |
| | 3-3 | 2 | 0.5 | 1 | 6968 | 13935.72 | 14018.6 | 28037.6 | 0.1 | 165469 | 16.94 | 27906 | 0.0347 | 1 | 0.004 | 15 | 0.3203 |
| | | | 0.5 | 2 | 7051 | 14101.88 | | | 0.1 | | | | | 1 | | 15 | |
| | 4-1 | 2 | 0.5 | 1 | 9047 | 18094.1 | 18134.66 | 36269.32 | 0.1 | 165469 | 21.92 | 36139 | 0.0449 | 1 | 0.004 | 15 | 0.4148 |
| | | | 0.5 | 2 | 9086 | 18175.22 | | | 0.1 | | | | | 1 | | 15 | |
| | 4-2 | 2 | 0.5 | 1 | 9535 | 19069.4 | 19000.63 | 38001.26 | 0.1 | 165469 | 22.97 | 37871 | 0.0471 | 1 | 0.004 | 15 | 0.4347 |
| | | | 0.5 | 2 | 9466 | 18931.86 | | | 0.1 | | | | | 1 | | 15 | |
| | 4-3 | 2 | 0.5 | 1 | 9260 | 18872.06 | 18425.38 | 36850.76 | 0.1 | 165469 | 22.27 | 36721 | 0.0456 | 1 | 0.004 | 15 | 0.4215 |
| | | | 0.5 | 2 | 9139 | 18278.7 | | | 0.1 | | | | | 1 | | 15 | |
| | 5-1 | 2 | 0.5 | 1 | 10477 | 20954.98 | 21016.22 | 42032.44 | 0.1 | 165469 | 25.40 | 41903 | 0.0521 | 1 | 0.004 | 15 | 0.4810 |
| | | | 0.5 | 2 | 10539 | 21077.46 | | | 0.1 | | | | | 1 | | 15 | |
| | 5-2 | 2 | 0.5 | 1 | 11193 | 22386.18 | 22363.01 | 44726.02 | 0.1 | 165469 | 27.03 | 44596 | 0.0554 | 1 | 0.004 | 15 | 0.5119 |
| | | | 0.5 | 2 | 11170 | 22339.84 | | | 0.1 | | | | | 1 | | 15 | |
| | 5-3 | 2 | 0.5 | 1 | 11184 | 22367.18 | 22482.1 | 44864.2 | 0.1 | 165469 | 27.17 | 44834 | 0.0557 | 1 | 0.004 | 15 | 0.5146 |
| | | | 0.5 | 2 | 11299 | 22597.04 | | | 0.1 | | | | | 1 | | 15 | |
| | 6-1 | 2 | 0.5 | 1 | 13008 | 26015.84 | 25761.05 | 51522.1 | 0.1 | 165469 | 31.14 | 51392 | 0.0639 | 1 | 0.004 | 15 | 0.5899 |
| | | | 0.5 | 2 | 12753 | 25506.26 | | | 0.1 | | | | | 1 | | 15 | |
| | 6-2 | 2 | 0.5 | 1 | 12672 | 25344.76 | 25486.46 | 50972.92 | 0.1 | 165469 | 30.81 | 50843 | 0.0632 | 1 | 0.004 | 15 | 0.5836 |
| | | | 0.5 | 2 | 12814 | 25628.16 | | | 0.1 | | | | | 1 | | 15 | |
| | 6-3 | 2 | 0.5 | 1 | 12785 | 25570.12 | 25777.53 | 51555.18 | 0.1 | 165469 | 31.16 | 51425 | 0.0639 | 1 | 0.004 | 15 | 0.5903 |
| | | | 0.5 | 2 | 12993 | 25985.06 | | | 0.1 | | | | | 1 | | 15 | |

| Assay Date | 12/30/2004 | Test Chemical ID | 4-OH ASDN | # Concentrations tested | | Microsome 6 type | recombinant | Microsome ID | MET-0254A Vi | Technician ID | JG | Replicate # | -- |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| Control Type | Portion | Average | SD |
|---|---|---|---|
| Full activity | Beginning | 0.6041 | 0.0025 |
| Full activity | End | 0.5866 | 0.0016 |
| Full activity | Overall | 0.5953 | 0.0102 |
| Background | Beginning | 0.0000 | 7.9538E-05 |
| Background | End | 0.0000 | 5.81114E-05 |
| Background | Overall | 0.0000 | 7.54416E-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 | 0.0366 |
| 4-OH ASDN | 1 | 2 | 1.00E-06 | -6.00 | 0.0382 |
| 4-OH ASDN | 1 | 3 | 1.00E-06 | -6.00 | 0.0388 |
| 4-OH ASDN | 2 | 1 | 1.00E-07 | -7.00 | 0.2250 |
| 4-OH ASDN | 2 | 2 | 1.00E-07 | -7.00 | 0.2263 |
| 4-OH ASDN | 2 | 3 | 1.00E-07 | -7.00 | 0.2229 |
| 4-OH ASDN | 3 | 1 | 5.00E-08 | -7.30 | 0.3171 |
| 4-OH ASDN | 3 | 2 | 5.00E-08 | -7.30 | 0.3335 |
| 4-OH ASDN | 3 | 3 | 5.00E-08 | -7.30 | 0.3203 |
| 4-OH ASDN | 4 | 1 | 2.50E-08 | -7.60 | 0.4148 |
| 4-OH ASDN | 4 | 2 | 2.50E-08 | -7.60 | 0.4347 |
| 4-OH ASDN | 4 | 3 | 2.50E-08 | -7.60 | 0.4215 |
| 4-OH ASDN | 5 | 1 | 1.00E-08 | -8.00 | 0.4810 |
| 4-OH ASDN | 5 | 2 | 1.00E-08 | -8.00 | 0.5119 |
| 4-OH ASDN | 5 | 3 | 1.00E-08 | -8.00 | 0.5146 |
| 4-OH ASDN | 6 | 1 | 1.00E-09 | -9.00 | 0.5899 |
| 4-OH ASDN | 6 | 2 | 1.00E-09 | -9.00 | 0.5836 |
| 4-OH ASDN | 6 | 3 | 1.00E-09 | -9.00 | 0.5903 |

| | | Percent of control values | | |
|---|---|---|---|---|
| | Log[test | Replicate | | |
| Level | substance] | 1 | 2 | 3 |
| 1 | -6.00 | 6.14 | 6.42 | 6.51 |
| 2 | -7.00 | 37.79 | 38.01 | 37.45 |
| 3 | -7.30 | 53.27 | 56.01 | 53.81 |
| 4 | -7.60 | 69.68 | 73.02 | 70.80 |
| 5 | -8.00 | 80.79 | 85.98 | 86.44 |
| 6 | -9.00 | 99.09 | 98.03 | 99.15 |

# Appendix H

## Statistical Analysis Report (BioSTAT Consultants, Inc.)

# Statistical Analysis Summary
# WIL Research Laboratories, LLC
# Study Protocol – 431010

1 Statistical Methodology
1.1 Analysis Methodology
2 Results
3 Statistical Analysis Summaries
Table 3.1: Analysis of Variance Comparisons Among Concentration Response Curve Fits
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

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.

The SAS procedure Proc Mixed was used for analysis using the following statements:

```
proc mixed covtest;
class replicate;
model mean = /solution cl;
random replicate;
repeated/group=replicate;
parms (1) (STD1) (STD2) (STD3)/hold=2,3,4;
run;
```

where STD1, STD2 and STD3 are the standard error for each replicate provided by PRISM.

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. The fixed effect factor in the model was portion (beginning or end). Random effect factors in the model included replicate and the replicate by portion interaction.

The SAS procedure Proc Mixed was used for analysis using the following statements:

```
proc mixed covtest;
class portion replicate;
model value = portion;
random replicate portion*replicate;
lsmeans portion/cl;
run;
```

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 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

| Parameter | Statistic | Replicate 1 | Replicate 2 | Replicate 3 | Overall |
|---|---|---|---|---|---|
| Slope (ß) | Prism Best Fit Value (S.E.) | -0.993 (0.020) | -0.969 (0.013) | -0.987 (0.030) | -- |
| | Prism 95% CI (lower, upper) | (-1.035, -0.951) | (-0.997, -0.941) | (-1.050, -0.923) | -- |
| | LSM (LSM s.e.) | -- | -- | -- | -0.977 (0.010) |
| | 95% CI (lower, upper) | -- | -- | -- | (-1.021, -0.934) |
| log10IC50 ($\mu$) | Prism Best Fit Value (S.E.) | -7.136 (0.011) | -7.213 (0.007) | -7.207 (0.016) | -- |
| | Prism 95% CI (lower, upper) | (-7.159, -7.113) | (-7.228, -7.198) | (-7.240, -7.174) | -- |
| | LSM (LSM s.e.) | -- | -- | -- | -7.185 (0.025) |
| | 95% CI (lower, upper) | -- | -- | -- | (-7.293, -7.078) |

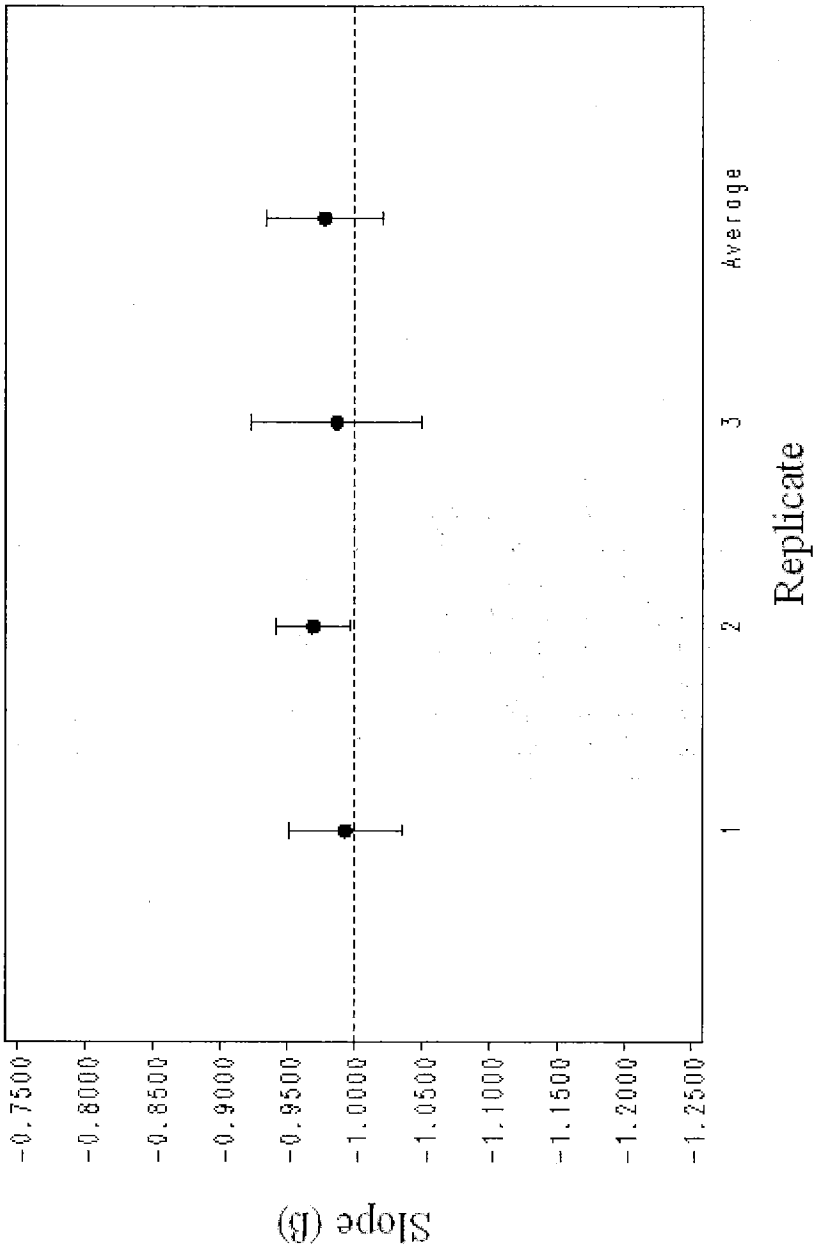



**Figure 3.2: Estimates for Slope and Confidence Intervals for all Replicates**

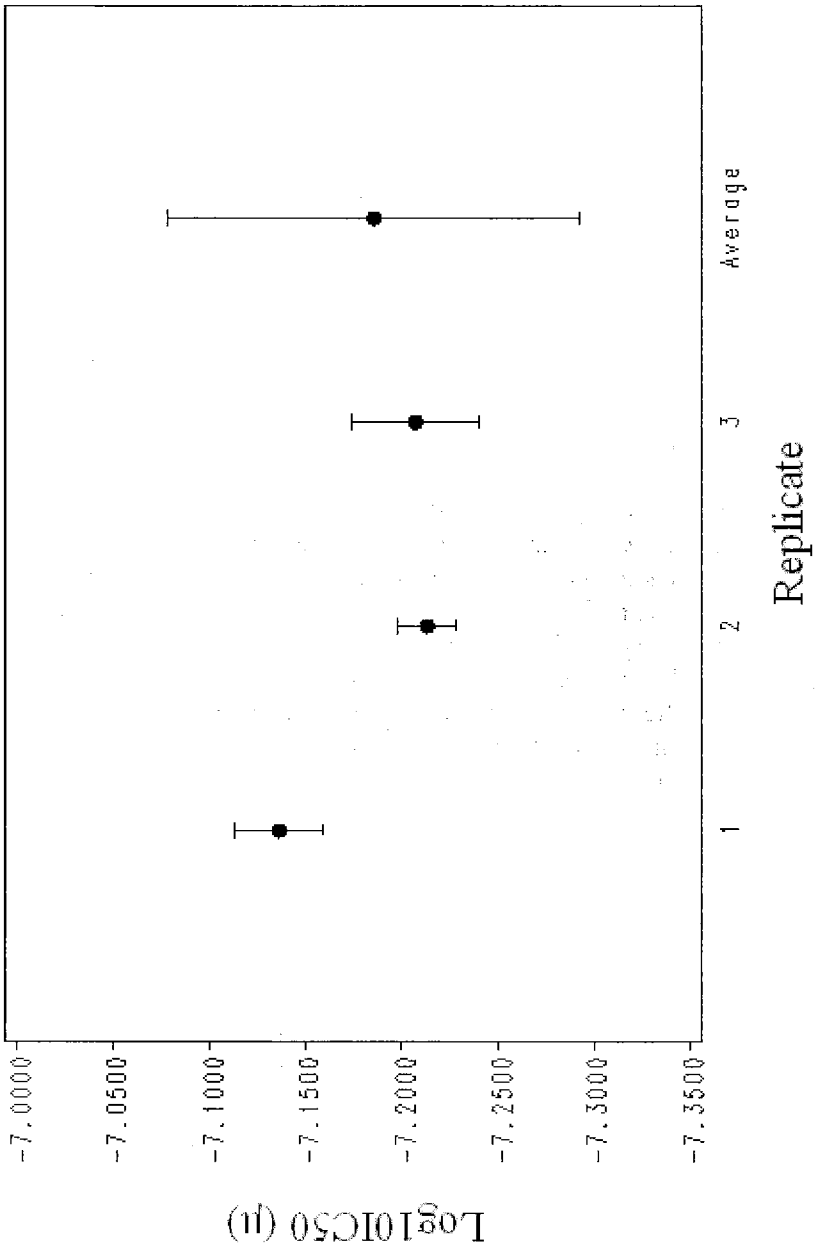



**Figure 3.3: Estimates for Log10IC50 and Confidence Intervals for all Replicates**

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

| | | | Replicate |
|---|---|---|---|
| Activity | Portion | Statistic | Overall |
| Background | Beginning | LSM (LSM s.e.) | 0.013 (0.016) |
| | | 95% CI (lower, upper) | (-0.057, 0.083) |
| | End | LSM (LSM s.e.) | -0.013 (0.016) |
| | | 95% CI (lower, upper) | (-0.083, 0.057) |
| | ANOVA P-Values | Portion = 0.3741 | |
| | | Replicate = . | |
| | | Portion*Replicate = . | |
| Full Enzyme | Beginning | LSM (LSM s.e.) | 100.256 (1.106) |
| | | 95% CI (lower, upper) | (95.496, 105.016) |
| | End | LSM (LSM s.e.) | 99.744 (1.106) |
| | | 95% CI (lower, upper) | (94.984, 104.504) |
| | ANOVA P-Values | Portion = 0.7744 | |
| | | Replicate = . | |
| | | Portion*Replicate = . | |

# APPENDIX D

## Interlaboratory Statistical Analysis Report

# DRAFT INTERLABORATORY STATISTICAL ANALYSIS REPORT

on

# RECOMBINANT AROMATASE VALIDATION STUDY
# 4-OH ASDN POSITIVE CONTROL INHIBITOR STUDY

EPA CONTRACT NUMBER 68-W-01-023
WORK ASSIGNMENT 4-17, TASK 3

December 9, 2005

Prepared for

U.S. ENVIRONMENTAL PROTECTION AGENCY
ENDOCRINE DISRUPTOR SCREENING PROGRAM
WASHINGTON, D.C.

Prepared by

BATTELLE
505 King Avenue
Columbus, Ohio 43201

## Summary and Conclusions

The principal results of the inter-laboratory analysis are summarized below.

1. The estimates for $\log_{10}IC_{50}$ were consistent among the three laboratories. The coefficient of variation among laboratories was about 4% whether replicate 1 in Laboratory C was included or excluded. The averages and associated 95% CIs were about same including and excluding replicate 1 in Laboratory C. The variance among laboratories was about 1.4 times higher than the average within laboratory variance when replicate 1 in Laboratory C was included, and was about 0.8 times the average within laboratory variance when replicate 1 in Laboratory C was excluded.

2. The estimated slope for Laboratory B was slightly smaller than those for Laboratories A and C, whether replicate 1 in Laboratory C was included or excluded. The coefficient of variation among laboratories was less than 5% whether replicate 1 in Laboratory C was included or excluded. The variance among laboratories was about four times higher than the average within laboratory variance when replicate 1 in Laboratory C was included and was about three times higher than the average within laboratory variance when replicate 1 in Laboratory C was excluded.

3. Average background activity control was not significantly different at the end and at the beginning of each replicate, for each laboratory or across the three laboratories. The estimated variance among the laboratories was zero or near zero.

4. Averaged across replicates, full enzyme activity control was significantly lower at the end than at the beginning in Laboratories A and C whether replicate 1 in Laboratory C was included or excluded. Full enzyme activity control was not significantly lower at the end than at the beginning in Laboratory B or combined across the three laboratories. The estimated variance among the laboratories was nearly three times of the average within laboratory variance when replicate 1 in Laboratory C was included and was nearly two times of the average within laboratory variance when replicate 1 in Laboratory C was excluded.

## Introduction and Background

Task 3 of Work Assignment 4-17, the Recombinant Aromatase Validation Study, involves three individual laboratories (labeled as A, B, and C) independently carrying out the recombinant aromatase assay with positive control inhibitor 4-OH ASDN and centrally prepared microsomes according to a common protocol. An "intra-laboratory" statistical analysis was carried out based on each laboratory's test data according to a common statistical analysis plan. The "inter-laboratory" statistical analysis discussed in this report combines summary values developed in each intra-laboratory analysis and assesses the relationships among them, the extent of inter-laboratory variation, and overall consensus estimates. This report discusses the methods used and the results obtained from combining the intra-laboratory statistical analysis results.

The intra-laboratory analyses were performed on the "percent of control" responses for the recombinant aromatase assays at each of the laboratories. The inter-laboratory analysis is based on the $\log_{10}IC_{50}$ and slope parameters of the concentration response curve fits determined in the intra-laboratory analyses. The inter-laboratory analysis also compares across the three laboratories the full enzyme activity control and the background activity control responses at the beginnings and the ends of the replicates.

## Test Organization

Recombinant assay aromatase activity levels were determined for the full enzyme activity control, the background activity control, and for six graded concentrations of the positive control inhibitor 4-OH ASDN. Three replicates of the positive control inhibitor study were carried out at each laboratory, and an additional replicate was carried out at Laboratory C. Within each replicate three repetitions were run at each of the 4-OH ASDN log (base 10) concentrations -6, -7, -7.3, -7.6, -8, and -9. In addition two repeat tubes of the full enzyme activity and background activity controls were run prior to the 4-OH ASDN runs and two repeat tubes of the full enzyme activity and background activity controls were run following the 4-OH ASDN runs.

Intra-laboratory statistical analyses were carried out on the "percent of control" responses. Percent of control is defined as the ratio of the background adjusted aromatase activity in the tube under consideration to the average background adjusted aromatase activity among the four full enzyme activity control tubes within the replicate, times 100. The average percent of control among the four full enzyme activity control tubes is necessarily 100 percent within each replicate. The average percent of control among the four background activity control tubes is necessarily 0 percent within each replicate.

Nominally for an inhibitor the percent of control activity values vary between approximately 0% near the high inhibition concentrations and approximately 100% near the low inhibition concentrations, but this may vary with the inhibitor.

Intra-laboratory statistical analyses were performed based on a common analysis plan. The following results were reported in each intra-laboratory analysis.

1. Concentration curve fits within each replicate to describe the trend in the percent of control activity across varying inhibitor concentrations of test substance 4-OH ASDN.

2. Estimates of the $\log_{10}IC_{50}$ concentration, slope, and associated standard errors within each replicate.

3. Average $\log_{10}IC_{50}$ concentration, average slope, and associated standard errors across replicates.

4. Comparisons between the full enzyme activity and background activity controls obtained at the beginning and those obtained at the end of each replicate.

Laboratories A and B reported results based on three replicates. Laboratory C reported results based on replicates 1 to 4 as well as results based on replicates 2 to 4. The reported standard errors of the average results across replicates for Laboratories A and C incorporated the among replicate component of variation. The reported standard error for laboratory B did not.

The "inter-laboratory" statistical analysis combines summary values developed in each of the intra-laboratory analyses to assess relationships among the results at each laboratory, the extent of laboratory-to-laboratory variation, and overall consensus estimates among the laboratories with associated variability estimates (incorporating laboratory-to-laboratory variability). The inter-laboratory analysis is based on the average $\log_{10}IC_{50}$ and slope parameters of the concentration response curve fits determined by each of the test laboratories, as reported in the intra-laboratory analyses. The inter-laboratory analysis also compares among laboratories the full enzyme activity control results and the background activity control results obtained at the end of each replicate with those obtained at the beginning.

The objectives of the inter-laboratory statistical analysis are to:

- Determine the average values and the variabilities among laboratories for the parameters mentioned above.
- Determine the coefficients of variation among laboratories for the $\log_{10}IC_{50}$ and the slope parameters.
- Estimate the ratio of the among laboratory variation to the average within laboratory variation for the parameters mentioned above.

The inter-laboratory analyses were performed on two versions of the data:

- Including all the data
- Excluding replicate 1 from laboratory C.

## Statistical Analysis Methods

Statistical analyses were carried out for each of the four endpoints discussed above in the Test Organization section: $\log_{10}IC_{50}$, slope, portion effect (beginning minus end) for background activity control, and portion effect for full enzyme activity control.

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

variation. The degrees of freedom associated with the overall weighted average was calculated as

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

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

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

It should be noted that when calculating the within laboratory mean $\log_{10}IC_{50}$ and slope across replicates, Laboratories A and C incorporated the replicate-to-replicate component of variation in the standard errors of the averages, while laboratory B did not. Also Laboratories A and C calculated the differences between beginning and end (beginning minus end) when comparing the full enzyme activity and background activity controls obtained at the beginning and those obtained at the end of each replicate, whereas laboratory B reported only beginning and end values. The sums of beginning and end values must be equal to 0 for the background activity controls and 200 for full enzyme activity controls. Therefore, for laboratory B the differences were calculated as $-2 \times$ (End values) for the background activity controls and as 200 $-$ 2 x (End values) for the full enzyme activity controls. The associated standard errors for these differences were calculated as $2 \times$ (Standard errors associated with the end values).

To describe the variability among the individual laboratory values relative to the overall average value, coefficients of variation (CV) and their associated 95% confidence intervals (95% CIs) were calculated for the $\log_{10}IC_{50}$ and the slope parameters. The coefficient of variation is defined as the standard deviation of the effect response divided by its mean. The methods for calculating the CV and the associated 95% CI were different depending on the underlying assumption about the distribution of the endpoint parameter.

For $\log_{10}IC_{50}$, the measurements were assumed to be approximately log normally distributed. The CV therefore is expressed as

$$CV = [\exp(S^2)-1]^{1/2}$$

where $S^2$ is the total variance among the three laboratories. $S^2$ is approximated by $3(se)^2$ where se is the standard error of the pooled mean estimate. This would be exact if the within laboratory variances were equal across laboratories.

The 95% confidence interval is based on the chi square distribution and is calculated as

$$[(\exp (df*S^2/(\chi^2_{df,\,0.975}))- 1)^{1/2}, (\exp(df*S^2/(\chi^2_{df,\,0.025}))- 1)^{1/2}]$$

where df is the estimated degree of freedom among the three laboratories.

For slope ($\beta$), the measurements are assumed to be approximately normal. The CV therefore is expressed as

$$CV=S/\beta_{avg}$$

where $S^2$ is the total variance among the three laboratories, defined as above and $S = \sqrt{S^2}$. The endpoints of the confidence interval for CV are based on the noncentral t distribution (Lehman, 1986).

To describe the variability among laboratories relative to variability within laboratories the ratio of the variance between laboratories to the average variance within laboratories was calculated as

$$R=S^2_{lab}\,[1/3(s_1^2 + s_2^2 + s_3^2)]$$

where $S^2_{lab}$ is the variance among the three laboratories and ($s_1^2$, $s_2^2$, $s_3^2$) are the squares of the within laboratory standard errors at the three laboratories. A confidence interval for this ratio is based on the F-distribution with ($\nu_{lab}$, $\nu_{wi}$) degree of freedom

$$[R/F^{-1}(0.975), R/F^{-1}(0.025)]$$

where $\nu_{lab}=2$ and $\nu_{wi}$ is based on Satterthwaite's approximation

$$\nu_{wi} \approx [(s_1^2 + s_2^2 + s_3^2)^2]/[s_1^4/\nu_1 + s_2^4/\nu_2 + s_3^4/\nu_3].$$

This ratio is calculated for each of the four endpoint parameters.

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

## Statistical Analysis Results

Table 1 displays the estimated parameter values and the associated within laboratory 95% confidence intervals about these values. It also displays the overall mean values across laboratories and their associated 95% confidence intervals, incorporating among laboratory variation based on the random effects analysis of variance. The overall mean was calculated

with and without replicate 1 for Laboratory C. These means and confidence intervals are shown in Figures 1 through 8. Each figure includes reference lines corresponding to the overall average. The estimated CVs and their associated 95% confidence intervals for the overall means for the $\log_{10}IC_{50}$ and slope parameters are also presented in Table 1.

Table 2 displays the within laboratory variances and their associated degrees of freedom for each laboratory. These are the squares of the within laboratory standard errors associated with the estimated parameter values. Table 2 also displays the laboratory to laboratory random variation and the squares of the standard errors of the overall mean values, as well as their associated degrees of freedom. The ratios of the among laboratory variances to the unweighted average within laboratory variances are also displayed, with their associated 95% confidence intervals.

The estimates for $\log_{10}IC_{50}$ were similar among the three laboratories, with or without replicate 1 in laboratory C (Table 1). For Laboratory C, the estimated variance without replicate 1 was about twice that with replicate 1 included (Table 2). The average values and the associated 95% CIs for $\log_{10}IC_{50}$ across the three laboratories were about same whether replicate 1 for Laboratory C was included or excluded. The variance among laboratories was about 1.4 times higher than the pooled average within laboratory variance when replicate 1 for Laboratory C was included and about 0.8 times the average within laboratory variance when replicate 1 for Laboratory C was excluded. The confidence intervals about these ratios were wide because of the small number of degrees of freedom (2) associated with the among laboratory variance. The coefficient of variation among laboratories was about 4%, whether replicate 1 in Laboratory C was included or excluded.

The estimated slope for Laboratory B was 7% to 11% smaller than those for Laboratories A and C whether replicate 1 in Laboratory C was included or was excluded. For Laboratory C, the estimated variance when replicate 1 was excluded was about twice that when replicate 1 was included. The average value and the associated 95% confidence interval for slope across the three laboratories remained about the same whether replicate 1 in Laboratory C was included or excluded. The among laboratory variance was about four times the average within laboratory variance when replicate 1 in Laboratory C was included and about three times the average within laboratory variance when replicate 1 in Laboratory C was excluded. The coefficient of variation among laboratories was slightly less than 5% whether replicate 1 in Laboratory C was included or excluded.

No significant differences (beginning minus end) existed for the background activity controls, for any laboratory or across the three laboratories combined (Table 1). The estimated variance among the laboratories was negligible (Table 2).

Full enzyme activity control was significantly higher at the beginning than at the end for Laboratories A and C but not for Laboratory B or combined across the three laboratories (Table 1). This is the case whether replicate 1 in Laboratory C was included or as excluded. The estimated variance among the laboratories was slightly less than three times the average within

laboratory variance when replicate 1 in Laboratory C was included and slightly less than twice the average within laboratory variance when replicate 1 in Laboratory C was excluded.

## References

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

Lehmann, E. *Testing Statistical Hypotheses.* John Wiley & Sons, Inc., 1986, p. 352-3.

**Table 1. Parameter Estimate and the 95% Confidence Interval for the Percent of Control Responses for Recombinant Aromatase Assay**

| Parameter | Estimate and 95% Confidence Interval | | | | CV(%) and 95% CI[3] |
|---|---|---|---|---|---|
| | Lab A[1] | Lab B[1] | Lab C[1] | Average[2, 4] | |
| **Rep 1-4 for Lab C** | | | | | |
| $Log_{10}IC_{50}$ | -7.107 (-7.176, -7.038) | -7.185 (-7.293, -7.077) | -7.083 (-7.199, -6.967) | -7.127 (-7.203, -7.051) | 4.014 (2.245, 16.082) |
| Slope | -0.877 (-0.960, -0.794) | -0.977 (-1.020, -0.934) | -0.911 (-0.984, -0.839) | -0.926 (-1.002, -0.850) | 4.725 (2.459, 30.120) |
| Difference (Beginning Minus End) for Background Activity Control | 0.084 (-0.179, 0.346 ) | 0.026 (-0.112, 0.164) | 0.100 (-0.055, 0.255) | 0.041 (-0.019, 0.100) | |
| Difference (Beginning Minus End) for Full Enzyme Activity Control | 9.354 (3.426, 15.282) | 0.512 (-9.005, 10.030) | 10.844 (6.116, 15.572) | 6.827 (-1.578, 15.231) | |
| **Rep 2-4 for Lab C** | | | | | |
| $Log_{10}IC_{50}$ | -7.107 (-7.176, -7.038) | -7.185 (-7.293, -7.077) | -7.090 (-7.310, -6.870) | -7.132 (-7.201, -7.063) | 4.026 (2.342, 13.195) |
| Slope | -0.877 (-0.960, -0.794) | -0.977 (-1.020, -0.934) | -0.912 (-1.056, -0.768) | -0.927 (-1.004, -0.850) | 4.940 (2.570, 31.529) |
| Difference (Beginning Minus End) for Background Activity Control | 0.084 (-0.179, 0.346) | 0.026 (-0.112, 0.164) | 0.111 (-0.107, 0.328) | 0.037 (-0.024, 0.099) | |
| Difference (Beginning Minus End) for Full Enzyme Activity Control | 9.354 (3.426, 15.282) | 0.512 (-9.005, 10.030) | 8.247 (4.110, 12.383) | 5.975 (-1.270, 13.221) | |

1. The estimates and 95% confidence intervals are based on the intra-laboratory analyses for the three participating laboratories. Laboratory C provided results separately for replicates 1 to 4 and for replicates 2 to 4.

2. The overall effects and confidence intervals in this table were estimated using a one-way random effects analysis of variance, with heterogeneous variances among the three laboratories. The variances for each laboratory were specified as the squares of the within laboratory standard errors.

3. CV was calculated for the average results for the $\log_{10}IC_{50}$ and the slope parameters.

4. Averages were calculated:
   - including all three replicates for laboratories A and B and replicates 1 to 4 for laboratory C
   - including all three replicates for laboratories A and B and replicates 2 to 4 for laboratory C

**Table 2. Variance Components and Ratio of Between and Within Laboratories Variances. Recombinant Aromatase Assay Percent of Control**

| Parameter | Within Laboratory Variance[1] | | | | Among Laboratory Variance[3] and (p-value) (df=2) | Mean Variance[4, 5] | Ratio and 95% CI[6] |
|---|---|---|---|---|---|---|---|
| | Lab A | Lab B | Lab C | Pooled Average[2] | | | |
| **Rep 1-4 for Lab C** | | | | | | | |
| **$Log_{10}IC_{50}$** | 0.0002 /df=1.67 | 0.0006 /df=2 | 0.0013 /df=3.04 | 0.0007 /df=5.69 | 0.0010 (p=0.2329) | 0.0005 /df=2.82 | 1.4250 (0.1886, 56.0338) |
| **Slope** | 0.0005 /df=2.34 | 0.0001 /df=2 | 0.0005 /df=3.16 | 0.0004 /df=6.40 | 0.0016 (p=0.1478) | 0.0006 /df=3.32 | 4.1032 (0.5915, 161.427) |
| **Difference (Beginning Minus End) for Background Activity Control** | 0.0139 /df=10 | 0.0010 /df=2 | 0.0052 /df=14 | 0.0067 /df=18.66 | 1.61E-22 (p=1.0000) | 0.0008 /df=18.66 | 2.40E-20 (5.31E-21, 9.48E-19) |
| **Difference (Beginning Minus End) for Full Enzyme Activity Control** | 7.0783 /df=10 | 4.8929 /df=2 | 4.8585 /df=14 | 5.6099 /df=15.17 | 16.1907 (p=0.1757) | 7.2509 /df=3.11 | 2.8861 (0.6075, 113.804) |
| **Rep 2-4 for Lab C** | | | | | | | |
| **$Log_{10}IC_{50}$** | 0.0002 /df=1.67 | 0.0006 /df=2 | 0.0026 /df=1.98 | 0.0011 /df=3.21 | 0.0009 (p=0.2366) | 0.0005 /df=3.44 | 0.8166 (0.0568, 32.0000) |
| **Slope** | 0.0005 /df=2.34 | 0.0001 /df=2 | 0.0010 /df=1.91 | 0.0005 /df=3.96 | 0.0016 (p=0.1510) | 0.0007 /df=3.57 | 3.0161 (0.2803, 118.369) |
| **Difference (Beginning Minus End) for Background Activity Control** | 0.0139 /df=10 | 0.0010 /df=2 | 0.0095 /df=10 | 0.0081 /df=20.68 | 0 (p=1.0000) | 0.0009 /df=20.68 | 0 (0,0) |
| **Difference (Beginning Minus End) for Full Enzyme Activity Control** | 7.0783 /df=10 | 4.8929 /df=2 | 3.4466 /df=10 | 5.1393 /df=13.08 | 10.1661 (p=0.2075) | 5.0542 /df=2.93 | 1.9781 (0.3992, 77.9803) |

1. The within laboratory variance for each laboratory is the square of the standard error associated with parameter estimate, which was reported in the intra-laboratory analyses for each of the three participating laboratories. Laboratory C provided results for replicates 1 to 4 and for replicates 2 to 4.

2. Pooled average for the within laboratory variances is the unweighted average of the within laboratory variances among the three laboratories. Associated degrees of freedom were based on Satterthwaite's approximation

3. Variance among laboratories is based on a one-way random effects analysis of variance model with heterogeneous variances among the three laboratories, equal to the squares of the within laboratory standard errors.

4. Mean variance is the square of the standard error of the pooled weighted mean value. It includes both within and among laboratory variation.

5. Degrees of freedom for the (mean) overall effect variance were estimated as $2*((1/K)*\sum(S_L^2 + S_i^2))^2/(var(S_L^2)+(2/K^2)*\sum(S_i^4/df_i))$, where $S_L^2$ is the among laboratory variance, $S_i^2$ and $df_i$ are the reported variance and degrees of freedom for laboratory i, $var(S_L^2)$ is the variance of $S_L^2$, and K is the number of laboratories (Hartung and Makambi, 2001).

6. Ratio of the among laboratory variance and the pooled average within laboratory variance.

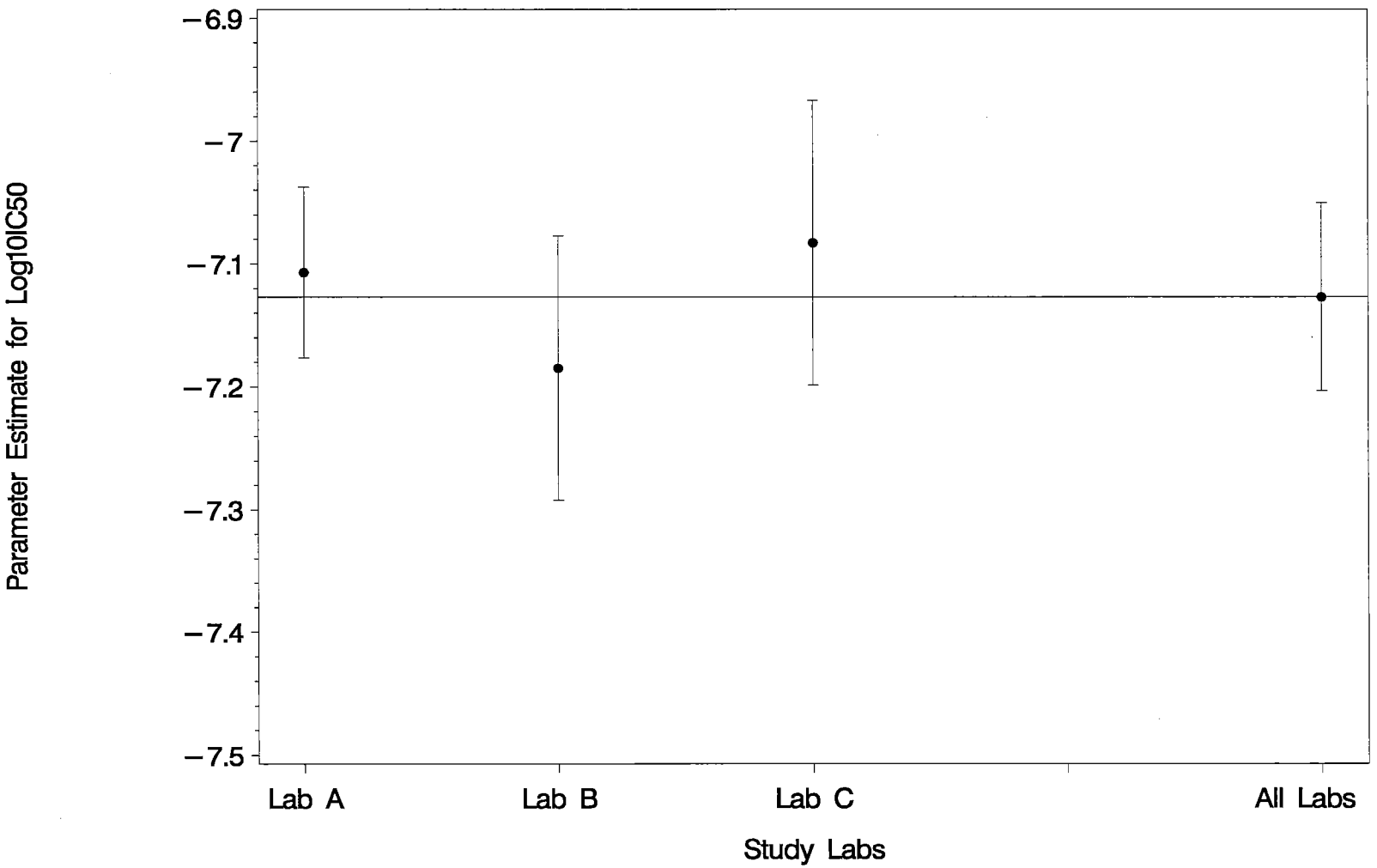



**Figure 1.** **Parameter Estimates and Their Associated 95% Confidence Intervals for $Log_{10}IC_{50}$ in Recombinant Aromatase Assay, Across Laboratories and by Each Laboratory. The Solid Reference Line Corresponds to the Average Across Laboratories. Laboratory C Includes Replicates 1-4.**

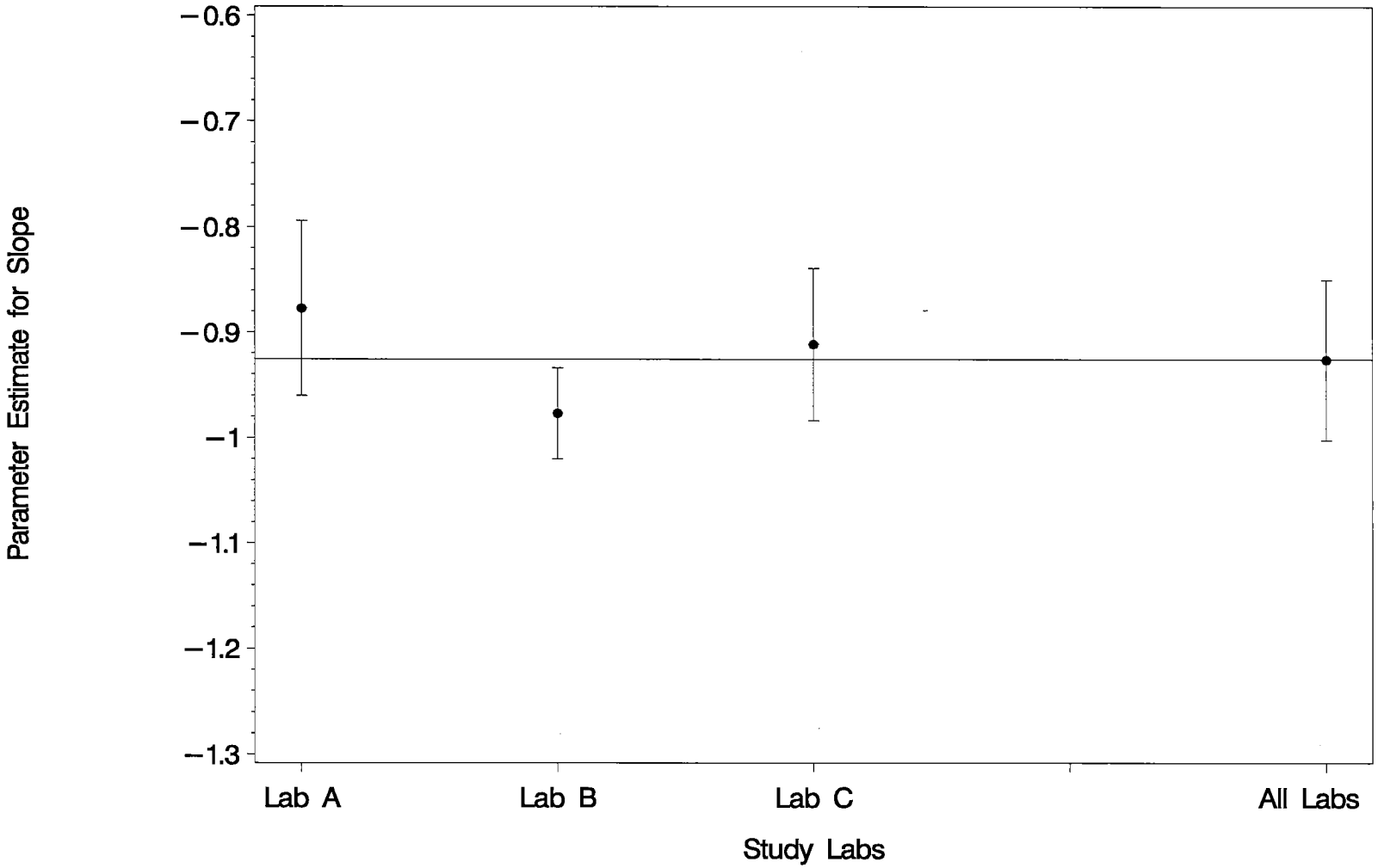



**Figure 2.** **Parameter Estimates and Their Associated 95% Confidence Intervals for Slope in the Recombinant Aromatase Assay, by Laboratory and Across Laboratories. The Solid Reference Line Corresponds to the Average Across Laboratories. Laboratory C Includes Replicates 1-4.**

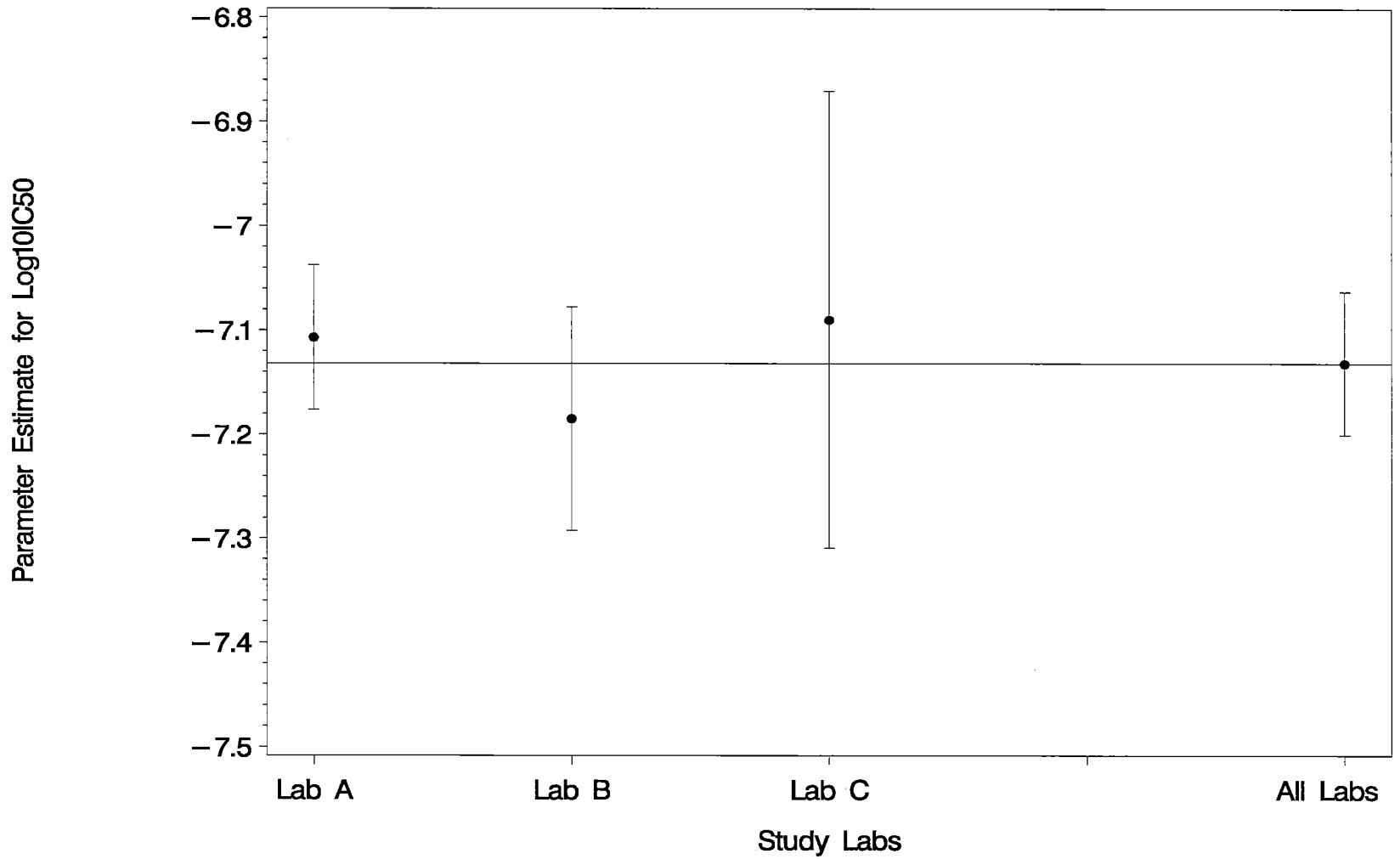



**Figure 3.** **Parameter Estimates and Their Associated 95% Confidence Intervals for $Log_{10}IC_{50}$ in Recombinant Aromatase Assay, Across Laboratories and by Each Laboratory. The Solid Reference Line Corresponds to the Average Across Laboratories. Laboratory C Includes Replicates 2-4.**

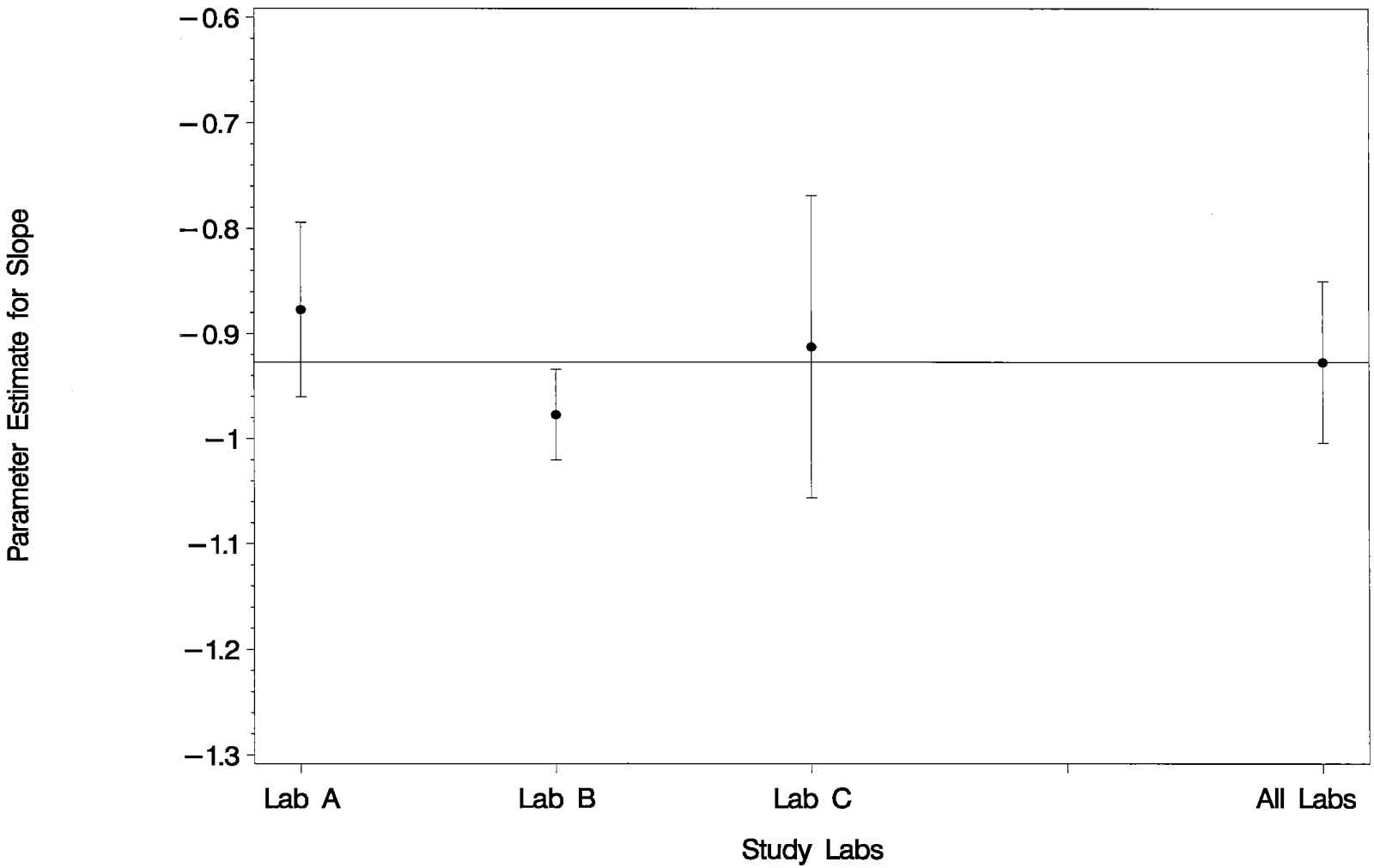



**Figure 4.** **Parameter Estimates and Their Associated 95% Confidence Intervals for Slope in the Recombinant Aromatase Assay, by Laboratory and Across Laboratories. The Solid Reference Line Corresponds to the Average Across Laboratories. Laboratory C Includes Replicates 2-4.**

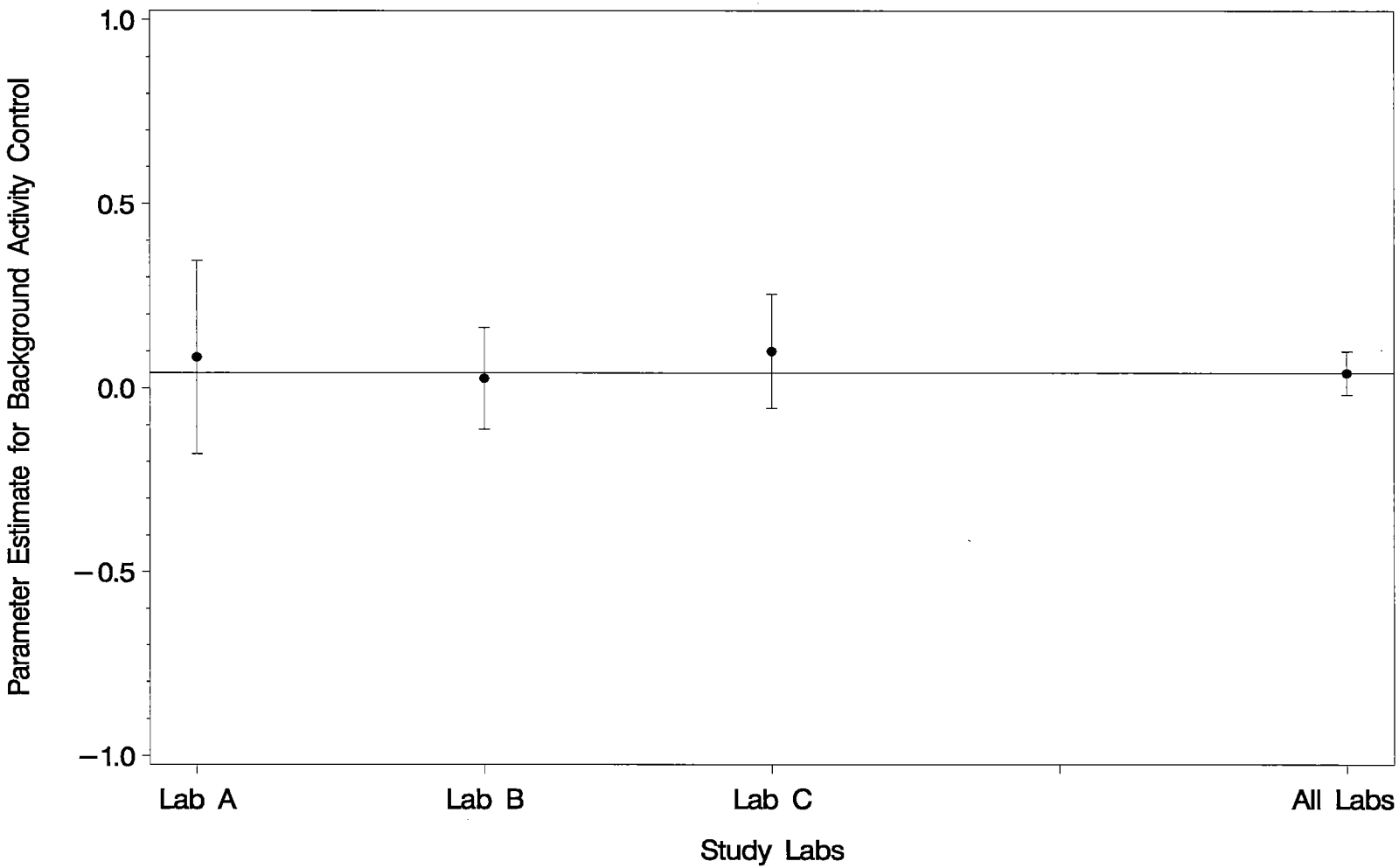



**Figure 5.** **Parameter Estimates and Their Associated 95% Confidence Intervals for Difference (Beginning Minus End) for Background Activity Controls in the Recombinant Aromatase Assay, by Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average Across Laboratories. Laboratory C Includes Replicates 1-4.**

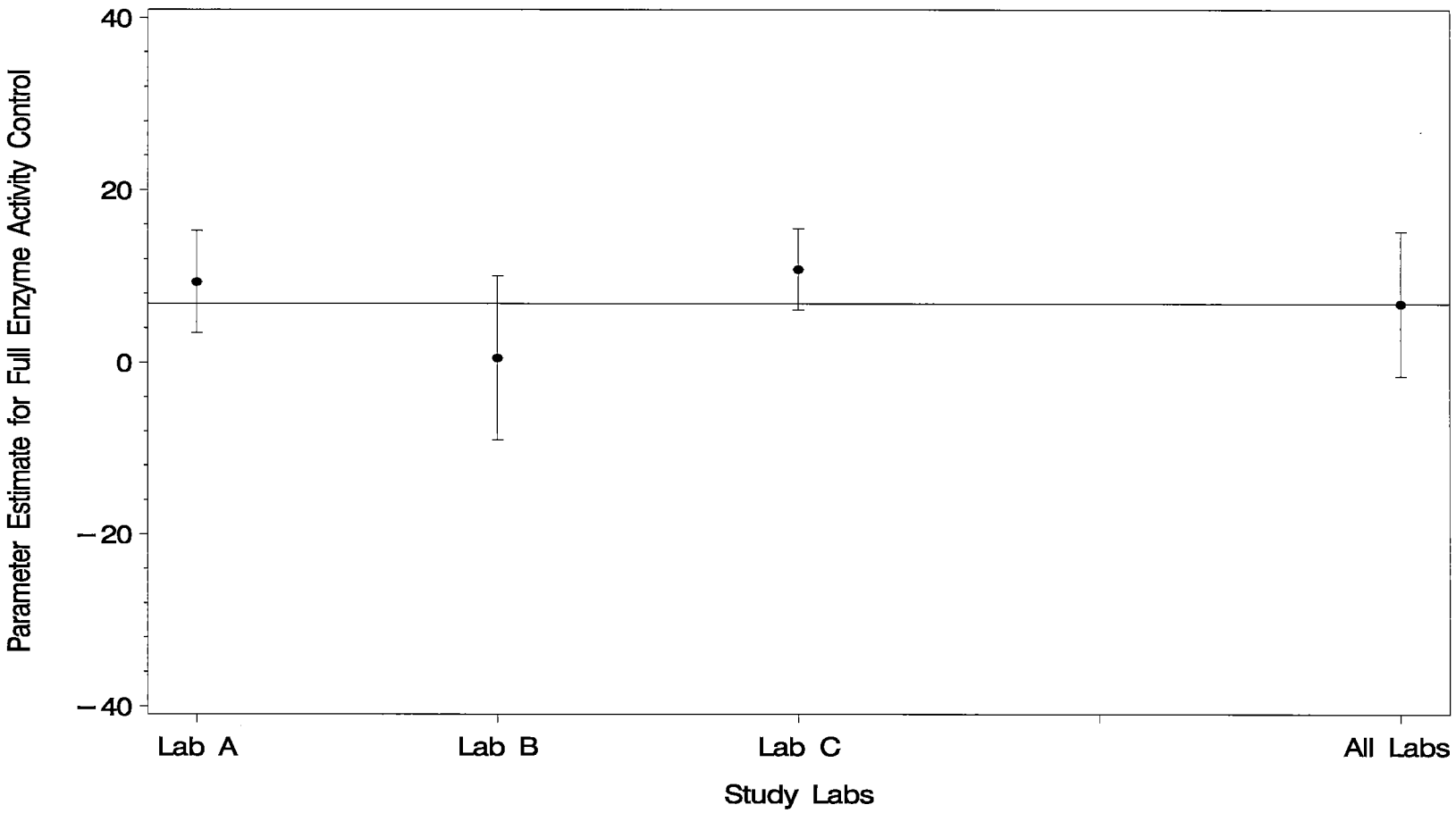



**Figure 6.** **Parameter Estimates and Their Associated 95% Confidence Intervals for Difference (Beginning Minus End) for Full Enzyme Activity Controls in the Recombinant Aromatase Assay, by Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average Across Laboratories. Laboratory C Includes Replicates 1-4.**

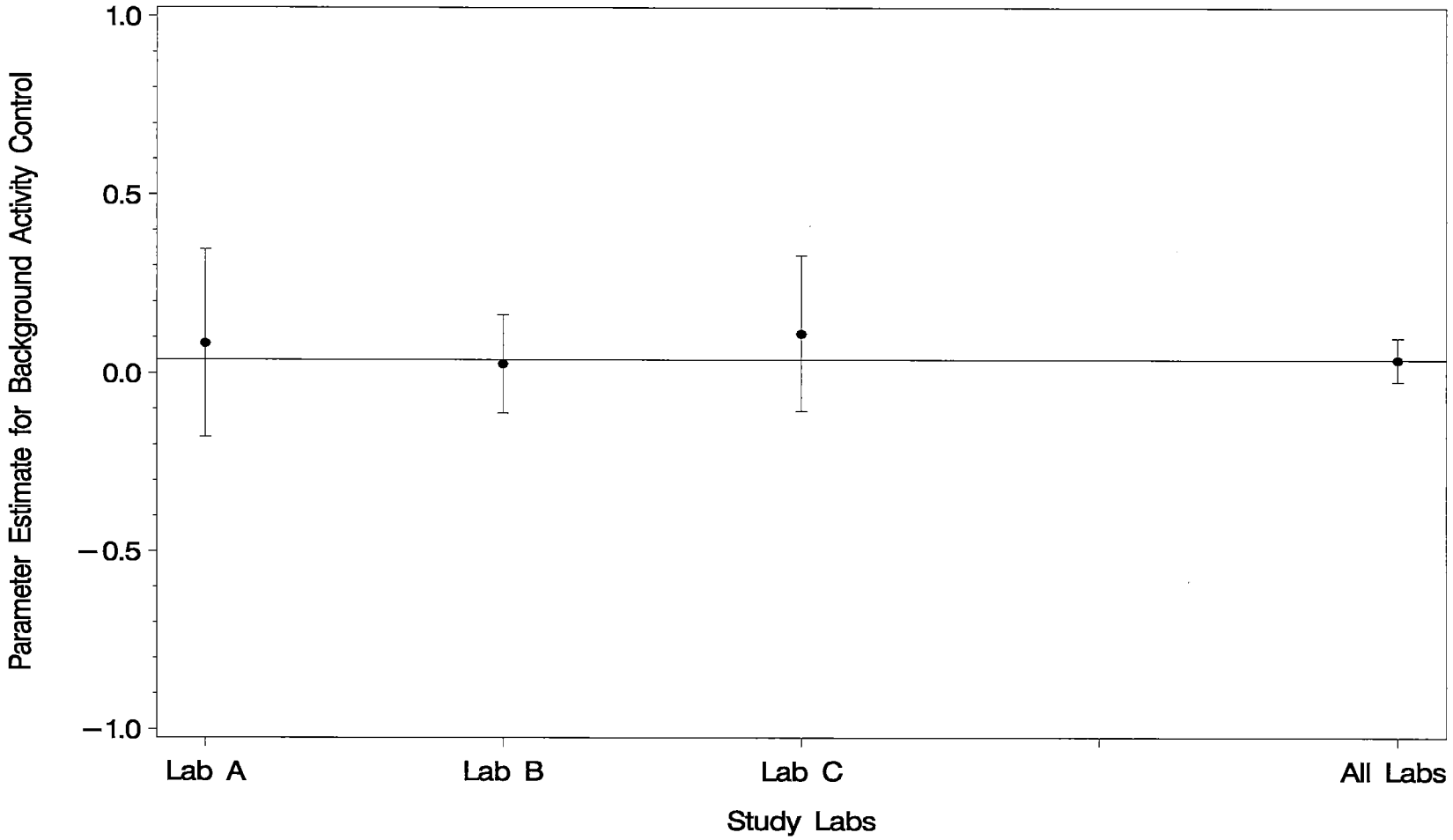



**Figure 7.** **Parameter Estimates and Their Associated 95% Confidence Intervals for Difference (Beginning Minus End) for Background Activity Controls in the Recombinant Aromatase Assay, by Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average Across Laboratories. Laboratory C Includes Replicates 2-4.**

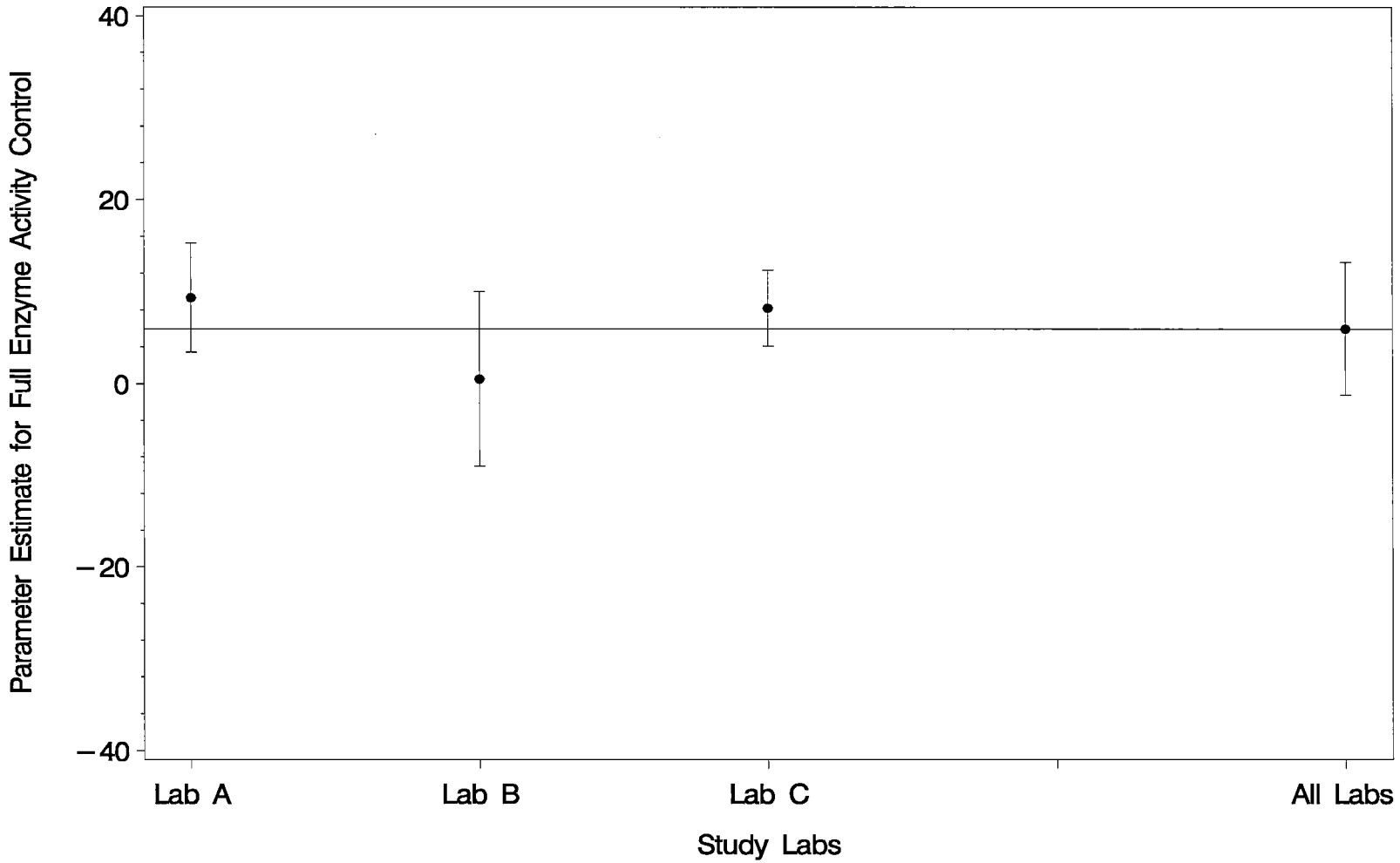



**Figure 8.** **Parameter Estimates and Their Associated 95% Confidence Intervals for Difference (Beginning Minus End) for Full Enzyme Activity Controls in the Recombinant Aromatase Assay, by Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average Across Laboratories. Laboratory C Includes Replicates 2-4.**