

FINAL REPORT

on

**MICROSOMAL AROMATASE PREVALIDATION SUPPLEMENTARY STUDY:
DETERMINE DAY-TO-DAY AND TECHNICIAN VARIABILITY**

**EPA CONTRACT NUMBER 68-W-01-023
WORK ASSIGNMENT 4-10, Task 3**

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FINAL TASK REPORT

Microsomal Aromatase Prevalidation Supplementary Study

WA 4-10 Task 3: Determine Day-to-Day and Technician Variability

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FINAL REPORT

Title: **Microsomal Aromatase Prevalidation Supplementary Study: WA 4-10 Task 3: Determine Day-to-Day and Technician Variability**

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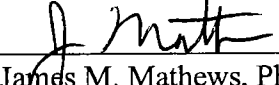
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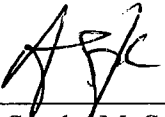
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Quality Assurance Statement

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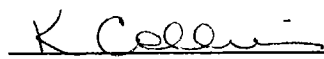
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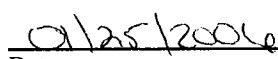
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This study was audited by the Sciences and Engineering – Health Sciences Quality Assurance Unit and the results of the inspections and audits were reported to the task leader and management as identified below. To the best of our knowledge, the reported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

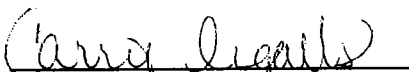
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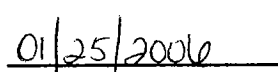

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1.0 OBJECTIVES

The objectives of this Work Assignment are to:

- ◆ investigate the high variability found in Work Assignment (WA) 2-24 for the study of 4-hydroxyandrostenedione in the recombinant assay,
- ◆ generate complete inhibition curves for econazole and ketoconazole, and
- ◆ compare two different methods for measuring aromatase activity. The first method is an indirect one that measures the formation of tritiated water formed by the aromatization of radiolabeled 4-hydroxyandrostendione. The second method measures estrone formation by radioimmunoassay.

The purpose of this Work Assignment is to perform a series of prevalidation studies to address the problems originally identified from WA 2-24 before beginning the interlaboratory validation studies and to compare two different methods for measuring aromatase activity.

This task report includes data from WA 4-10 Task 3: Determine Day-to-Day and Technician Variability. The experiments reported herein are described in the protocol (RTI-900-AN) Sections 2.0 and 3.0.

2.0 MATERIALS AND METHODS

2.1 Substrate

2.1.1 Identity

The substrate for the aromatase assay is androstenedione (ASDN). Non-radiolabeled and radiolabeled ASDN were used. The non-radiolabeled ASDN (Lot # 072K1134) was obtained from Sigma, St. Louis, MO and had a reported purity of 99%. The radiolabeled androstenedione ([1 β -³H]-androstenedione, [³H]ASDN, Lot # 3474115), was obtained from Perkin Elmer Life Science, Boston and had a reported specific activity of 25.3 Ci/mmol. Radiochemical purity was reported by the supplier to be > 97%.

2.1.2 Radiochemical Purity

The radiochemical purity of the [^3H]ASDN was determined using high performance liquid chromatography (HPLC) and liquid scintillation counting. The HPLC system consisted 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 were collected using Waters Millennium³² Client/Server Chromatography Data System Software, Version 4.0.

The HPLC method used a Zorbax SB-C18 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 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). A reference standard of nonradiolabeled ASDN was analyzed by the same method and coelution of the nonradiolabeled and radiolabeled ASDN was confirmed.

2.2 Test Substances

2.2.1 4-Hydroxyandrostenedione (4-OH ASDN)

CAS No.: 566-48-3
Molecular Formula/Weight: $\text{C}_{19}\text{H}_{26}\text{O}_3$; 302.4 g/mol
Supplier: Sigma
Lot No: 063K4069
Purity: 99%
Storage Conditions: 2-8°C

2.2.2 Ketoconazole

CAS No.: 65277-42-1
Molecular Formula/Weight: $\text{C}_{26}\text{H}_{28}\text{Cl}_2\text{N}_4\text{O}_4$; 531.48 g/mol
Supplier: Spectrum
Lot No.: QL0352
Purity: 99.73%
Storage Conditions: Room temperature

2.2.3 Econazole

CAS No.: 24169-02-6 (econazole nitrate)
Molecular Formula/Weight: $C_{18}H_{15}Cl_3N_2O \cdot HNO_3$; 444.7 g/mol
Supplier: Sigma
Lot No.: 71K1902
Purity: 99%
Storage Conditions: Room temperature

Solutions of the test substances were prepared fresh each day of use. Vehicle for 4-OH ASDN and ketoconazole was EtOH and that for econazole was DMSO.

2.3 Microsome Preparation

2.3.1 Human Placental

Human placental microsomes prepared for use on WA 2-24 were used for the current work. No additional microsome preparations were made. Microsomes were thawed rapidly in a $37 \pm 1^\circ\text{C}$ water bath, rehomogenized using a Potter Elvehjem homogenizer and then kept on ice until used.

2.3.2 Recombinant

Human CYP19 + P450 Reductase SUPERSOMES™ (Lot 4) were obtained from Gentest. They were stored at $<-70^\circ\text{C}$. The stated total protein content was 4.6 mg/mL in 100 mM potassium phosphate (pH 7.4). The stated aromatase activity (with testosterone as substrate) was 240 nmol β -estradiol/(min x mg protein); 3.5 pmol β -estradiol/pmol P450/ min.

The human recombinant microsomes were thawed rapidly in a $37 \pm 1^\circ\text{C}$ water bath, rehomogenized using a Potter Elvehjem homogenizer and then kept on ice until used.

2.4 Protein Determination

The protein concentration of the microsomal preparations was determined on each day of use of the microsomes in the aromatase assay. A 6-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 μ L aliquot of unknown or standard, 125 μ L 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 min to allow for color development. The absorbances are stable for about 1 h. Each sample (unknown and standards) 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 curve developed using the protein standards.

2.5 Cytochrome P450 Aromatase (CYP19) Activity

Aromatase activity of the current human placenta and human recombinant microsome stock (Lot 3), in addition to that of the new Lot 4 of the recombinant microsomes, was determined prior to their use in IC₅₀ determination studies to demonstrate that they had sufficient activity to conduct the current experiments. Each preparation was run in triplicate tubes in the aromatase assay using the optimized conditions determined in WA 2-24 as presented in Table 1.

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

The assays were performed in 13x100 mm test tubes maintained at 37 $\pm$ 1°C in a shaking water bath. Propylene glycol (100 μ L), [³H]ASDN, NADPH, and buffer (0.1 M sodium phosphate buffer, pH 7.4) were combined in the test tubes (total volume 1 mL). The tubes were placed at 37 $\pm$ 1°C in the water bath for five min 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 min. The incubations were stopped by the addition of methylene chloride

(2.0 mL); the tubes were vortex-mixed for ca. 5 s and placed on ice. The tubes were then vortex-mixed an additional 20-25 s. The tubes were then centrifuged using a Beckman GS-6R centrifuge with GH-3.8 rotor for 10 min at a setting of 1000 rpm. The methylene chloride layer was removed and discarded; the aqueous layers were extracted again with methylene chloride (2 mL). This extraction procedure was performed one additional time, each time discarding the methylene chloride layer. The aqueous layers were transferred to vials and duplicate aliquots (0.5 mL) were transferred to 20-mL liquid scintillation counting vials. Liquid scintillation cocktail (Ultima Gold, Packard, 10 mL) was added to each counting vial and shaken to mix the solution. The radiochemical content of each aliquot was determined by liquid scintillation spectrometry (LSS).

2.6 Determine Day-to-Day and Technician Variability

2.6.1 Assay Conduct

Day-to-day and technician variabilities of the aromatase assay were examined. The assay was conducted with both microsome types (human placental and human recombinant) using the optimized conditions determined in WA 2-24 and presented above in Table 1, with the addition of varying concentrations of the aromatase inhibitor, 4-OH ASDN. Appropriate controls (defined below) were included with each assay set. A Study (Table 2) was defined as a set of assays using a single microsome type with varying concentrations (in triplicate) of the inhibitor (with appropriate controls) conducted by a single technician on a given day. Three technicians conducted Studies utilizing each microsome type on three days each. Each assay was conducted independently, except that a common stock vial of microsomes (by type) and inhibitor solutions was used for each day.

Table 2. Study Definitions

	Recombinant assay with 4-OH ASDN	Placental assay with 4-OH ASDN
Technician 1	Studies 1, 2, 3	Studies 4, 5, 6
Technician 2	Studies 7, 8, 9	Studies 10, 11, 12
Technician 3	Studies 13, 14, 15	Studies 16, 17, 18

Studies were conducted on the following schedule.

Table 3. Actual Study Schedule

Date	Active Technicians ^a	Microsome Type	Study
April 1, 2004	1 3	Placental, recombinant Recombinant	1, 4 13
April 5, 2004	1 2 3	Placental, recombinant Placental, recombinant Recombinant	2, 5 7, 10 14
April 7, 2004	1 2 3	Placental, recombinant Placental, recombinant Recombinant	3, 6 8, 11 15
April 9, 2004	2 3	Placental, recombinant Placental	9, 12 16
April 12, 2004	3	Placental	17*
April 13, 2004	3	Placental	18
April 22, 2004	3	Placental	17*

^a Technician 3 was less experienced with the assay and therefore, was assigned to run only one Study per day.

* The first day Study 17 was run, there was an error in the preparation of the 4-OH ASDN solutions. The study was rerun on another day. Data from the first run (4/12/2004) were not used.

Each study tested the response of aromatase activity to the presence of eight concentrations of 4-OH ASDN. Each concentration of 4-OH ASDN was run in triplicate tubes in each Study. The concentrations of 4-OH ASDN used were (final in incubation): 10^{-6} , 5 and 2.5×10^{-7} , 10^{-7} , 5 and 2.5×10^{-8} , 10^{-8} and 10^{-9} M. In addition, positive and negative control samples (as in WA 2-24) were included for each study. Positive controls contained substrate, NADPH, propylene glycol, buffer, vehicle (20 μ L EtOH) and microsomes. Negative controls contained all positive control assay components except NADPH. Four positive and four negative controls were included with each Study and were treated the same as the other samples. The controls sets were split so that two tubes (of each positive and negative controls) were run at the beginning and two at the end of each study set.

The assay was conducted as described in Section 2.5 with the following modification. 4-OH ASDN (or vehicle) was added to the mixture of propylene glycol, substrate, NADPH and buffer in a volume of 20 μ L prior to preincubation of that mixture. The volume of buffer used was adjusted so the total incubation volume remained at 2 mL.

2.6.2 Data Analysis

Raw LSS data (CPM) were converted to DPM using Process2 (Version 2.1 09251998) software. DPM data and protein assay data were entered into a validated Excel spreadsheet. 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 specific activity (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 negative 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). Enzyme activity was expressed in nmol (mg protein)⁻¹min⁻¹ and was calculated by dividing the amount of ³H₂O formed (nmol) by the product of mg microsomal protein used times the incubation time, e.g. 15 min. Average activity in the positive 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 positive control activity and multiplying by 100.

IC₅₀ was 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:

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

Where:

X is the logarithm of concentration

Y is the percent activity

Bottom is the lower plateau

Top is the upper plateau

2.6.3 Statistical Analysis

2.6.3.1 Quality Control Comparisons. Positive and negative control activities were combined across batches and an analysis of variance was carried out. The factors in the analysis were microsome type, batch, portion of batch (beginning or end) and the interaction between batch and portion of batch. The response was enzymatic activity. The estimated variance between the two replicate runs/batch was based on the pooled value across the quadruplicate replicate runs in a given Study for a given microsome type.

2.6.3.2 Variability Assessment. Differences among technicians and day-to-day within technicians for enzymatic activity at various concentration levels of 4-OH ASDN were estimated. The variance components were inspected across the assays to determine their comparability. Analyses were based on mixed effects analysis of variance models using PROC MIXED or PROC GLM in SAS Version 8.2. Inhibitor concentrations and technician were treated as fixed effects; day-to-day variation was treated as a random effect. Variation between replicates within a single assay was examined using the Levene's test for homogeneity of variance. Graphical analyses were used to examine the changes in the variances across technician, day and inhibitor concentration.

2.6.3.3 Concentration response curve fits. Analysis of variance tests were carried out on $\log_{10}(\text{IC}_{50})$, slope and associated within day standard errors. Technician-to-technician variation was determined for each of the parameters and was tested for significance.

3.0 RESULTS

3.1 Substrate Purity

The radiochemical purity of [^3H]ASDN (Lot # 3474115) was shown by HPLC to be 97.6%.

3.2 Aromatase Activity in Stock Microsome Preparations

Aromatase activities of the human placental microsomes prepared as part of WA 2-24, the human recombinant microsomes used in WA 2-24 (Lot 3) and a new batch of human recombinant microsomes (Lot 4) were determined as described in Section 2.5 and are presented in Table 4.

Table 4. Aromatase Activity of Stock Microsomes

Microsome Type	Aromatase Activity (nmol/mg/min, mean $\pm$ SD)
Human Placental	0.0710 $\pm$ 0.0037
Recombinant (Lot 3)	0.4615 $\pm$ 0.0047
Recombinant (Lot 4)	0.4543 $\pm$ 0.0046

These activity levels meet the acceptance criteria for aromatase activity of >0.05 nmol/mg/min for placental and > 0.375 nmol/mg/min for recombinant microsomes. The stocks were determined to also have sufficient protein content for conduct of the assays. Because there was not enough of the Lot 3 recombinant microsomes remaining for conduct of all of the studies, it was decided to run all recombinant experiments using the Lot 4 microsomes.

3.3 Day-to-Day and Technician Variability

3.3.1 Detailed Data Tables

The aromatase assay was conducted by three technicians on three days each for each microsome type (placental and recombinant) as described in Section 2.5. The detailed individual technician and day-to-day results for each study are presented in Appendix A.

3.3.2 Control Sample Results

Means and standard deviations for the control activities are presented in Table 5. Within a technician and day, positive control values from samples run at the end of the assay set were always slightly ($\sim 10\%$) lower than those run at the beginning of the assay set. Within a particular assay day, when each technician used the same stock of microsomes, but prepared their own dilutions, positive control values varied (average coefficient of variance was 15%)

from technician-to-technician. This may have been due to slight differences in microsome dilution preparation. Most differences in positive control aromatase activities were minor and even more so when normalized to percent control activities when the aromatase activities are converted to percent of control activities for the IC_{50} determination.

The analysis of variance results for the control samples are presented in Table 6. The analysis of the control samples shows no significant variance in the negative control activities by technician, day, portion (beginning vs. end) and no significant variance in the interaction between day and portion. Mean negative control activities were near zero for all studies. In a few cases, higher negative control activities were measured (see Tables A-1 and A-2), probably due to inadvertent cross contamination of the negative control tubes with small amounts of NADPH. Positive control values show significant variance by technician, day and portion but no significant variance in the interaction between day and portion.

Table 5. Control Activities Mean and Standard Deviations

Microsome Type	Control Type	Technician	Portion of Batch (Day)	Day 1		Day 2		Day 3	
				Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
Placental	Negative	1	Begin	1.108e-02	3.137e-02	-4.716e-03	1.167e-05	-9.299e-05	0.000e+00
		1	End	-1.108e-02	2.840e-05	4.716e-03	9.831e-03	9.299e-05	2.630e-04
		2	Begin	1.475e-05	4.172e-05	1.239e-06	1.752e-05	5.284e-05	3.956e-05
		2	End	-1.475e-05	1.788e-05	-1.239e-06	2.804e-05	-5.284e-05	4.396e-06
		3	Begin	-6.032e-06	4.777e-05	2.358e-05	4.669e-05	3.554e-05	1.256e-05
		3	End	6.032e-06	1.706e-05	-2.358e-05	4.669e-05	-3.554e-05	1.319e-04
	Positive	1	Begin	5.211e-02	5.274e-05	4.575e-02	4.756e-04	6.574e-02	2.060e-03
		1	End	4.935e-02	2.260e-03	4.239e-02	8.987e-04	6.018e-02	9.549e-04
		2	Begin	5.028e-02	1.213e-03	5.397e-02	1.577e-04	6.029e-02	2.027e-03
		2	End	4.501e-02	1.887e-03	4.967e-02	1.998e-04	5.642e-02	4.836e-04
		3	Begin	5.054e-02	1.536e-03	4.564e-02	5.870e-04	5.380e-02	1.423e-03
		3	End	4.850e-02	5.118e-05	4.071e-02	1.004e-03	5.004e-02	9.737e-05
Recombinant	Negative	1	Begin	2.103e-04	3.278e-04	7.036e-05	3.731e-04	-1.266e-04	8.952e-05
		1	End	-2.103e-04	2.428e-05	-7.036e-05	9.951e-05	1.266e-04	1.611e-04
		2	Begin	-4.309e-04	3.921e-04	1.238e-05	3.501e-05	-4.831e-06	2.733e-05
		2	End	4.309e-04	1.526e-03	-1.238e-05	3.035e-04	4.831e-06	1.367e-05
		3	Begin	-7.643e-03	1.612e-04	6.853e-06	1.938e-05	-3.857e-06	5.454e-05
		3	End	7.643e-03	2.212e-02	-6.853e-06	3.877e-05	3.857e-06	8.727e-05
	Positive	1	Begin	4.020e-01	7.357e-03	4.171e-01	4.229e-04	5.219e-01	2.548e-02
		1	End	3.693e-01	1.938e-02	3.951e-01	7.724e-03	4.752e-01	1.303e-02
		2	Begin	3.262e-01	9.941e-03	3.753e-01	3.793e-03	4.270e-01	5.343e-03
		2	End	2.849e-01	4.854e-03	2.997e-01	2.778e-03	3.952e-01	4.920e-04
		3	Begin	3.035e-01	1.488e-02	2.588e-01	1.363e-02	4.683e-01	9.436e-03
		3	End	2.846e-01	1.585e-02	2.554e-01	1.389e-02	4.209e-01	2.589e-02

Table 6. ANOVA Results for the Control Activities

Microsome Type	Control Type	Source	DF	Type I SS	Mean Square	F Value	Pr > F
Placental	Negative	Technician	2	3.257E-33	1.628E-33	0.00	1.0000
		Day(Technician)	4	8.844E-33	2.211E-33	0.00	1.0000
		Portion	1	1.816E-05	1.816E-05	0.30	0.5908
		Day*Portion	2	1.751E-04	8.755E-05	1.43	0.2586
	Positive	Technician	2	1.544E-04	7.718E-05	52.16	<.0001
		Day(Technician)	4	2.918E-04	7.295E-05	49.31	<.0001
		Portion	1	1.430E-04	1.430E-04	96.62	<.0001
		Day*Portion	2	1.833E-06	9.165E-07	0.62	0.5466
Recombinant	Negative	Technician	2	8.563E-36	4.282E-36	0.00	1.0000
		Day(Technician)	4	9.380E-36	2.345E-36	0.00	1.0000
		Portion	1	2.780E-05	2.780E-05	1.04	0.3190
		Day*Portion	2	5.468E-05	2.734E-05	1.02	0.3763
	Positive	Technician	2	6.487E-02	3.243E-02	123.46	<.0001
		Day(Technician)	4	1.513E-02	3.783E-03	14.40	<.0001
		Portion	1	1.137E-02	1.137E-02	43.26	<.0001
		Day*Portion	2	1.974E-04	9.868E-05	0.38	0.6908

3.3.3 Inhibitor Results

Differences among technicians and day-to-day within technicians for enzymatic activity at various inhibitor concentrations were estimated and are presented in Tables 7 and 8. No significant variance of activity was found among technicians. There were significant day-to-day variances within technicians for both microsome types. These effects may be due to slight differences in microsome dilution preparation as discussed above. Levene's test for homogeneity of variance detected significant variances between replicates within a single assay for technician, inhibitor concentration, and day, and all interactions between technician, inhibitor concentration and day for the placental data. Significant differences were also found for all of those parameters in the recombinant assay. The magnitude of these variances appeared to be small compared with the activities, though, and no pattern to the variances was obvious, so evaluation of weighting schemes for use in the nonlinear curve fitting (next paragraph) was not pursued at this time.

Table 7. Technician-to-Technician Variability

Type	Effect	Num DF	Den DF	F Value	Pr > F
Placental	Technician	2	6	0.48	0.6386
Placental	Level	7	200	1216.00	<.0001
Recombinant	Technician	2	6	1.98	0.2187
Recombinant	Level	7	200	685.73	<.0001

Table 8. Day-to-Day Variability

Type	Cov Parm	Subject	Estimate	Standard Error	Z Value	Pr > Z
Placental	Day	Technician	0.000015	8.887E-6	1.69	0.0451
Placental	Residual		8.049E-6	0	.	.
Recombinant	Day	Technician	0.000623	0.000376	1.66	0.0487
Recombinant	Residual		0.000673	0.000067	10.00	<.0001

Graphs of aromatase activity as percent of control versus log[4-OH ASDN] are presented in Figures 1 and 2 for placental and recombinant microsomes, respectively. This data is also presented in Tables 9 and 10. Percent of control values for Technician 3, Day 2 were well above 100% at the lower inhibitor concentrations. No cause for this was readily apparent. IC₅₀ values for the inhibition of aromatase activity by 4-OH ASDN in placental and recombinant microsomes, respectively are presented in Tables 11 and 12. No significant technician-to-technician variation was detected for log(IC₅₀), slope or their associated standard errors for either microsome type (Tables 13 and 14).

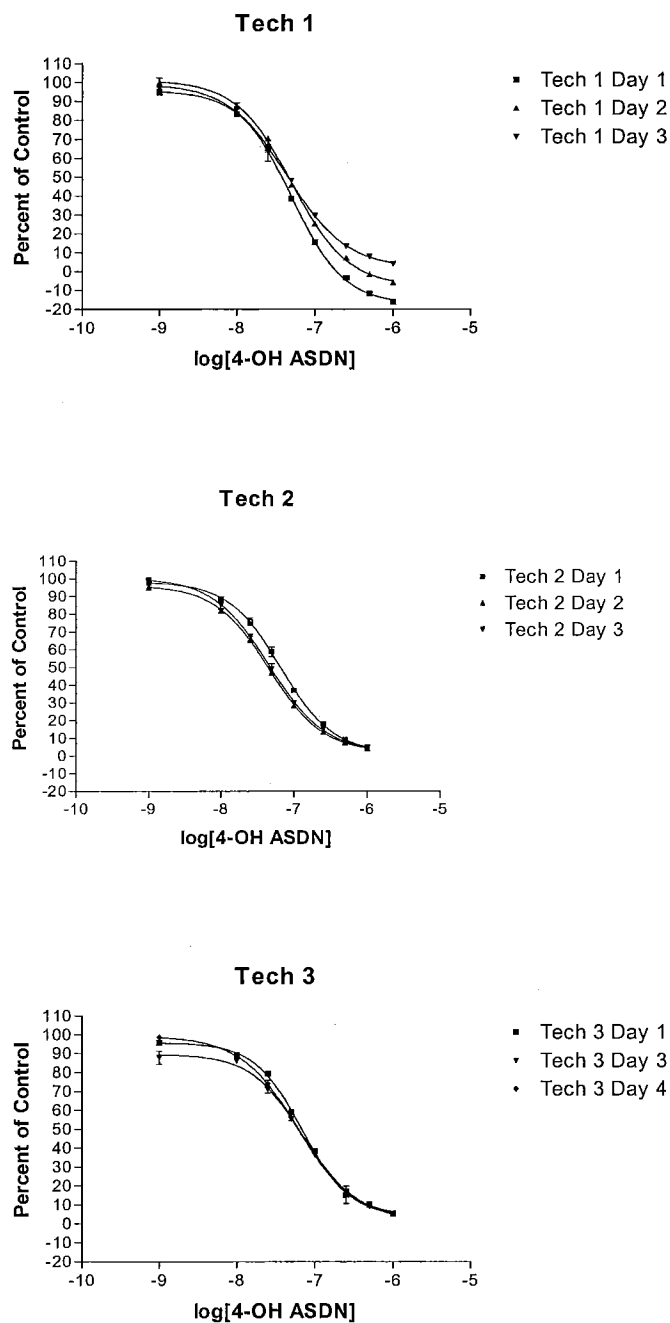


Figure 1. Placental Aromatase 4-OH ASDN Inhibition Curves

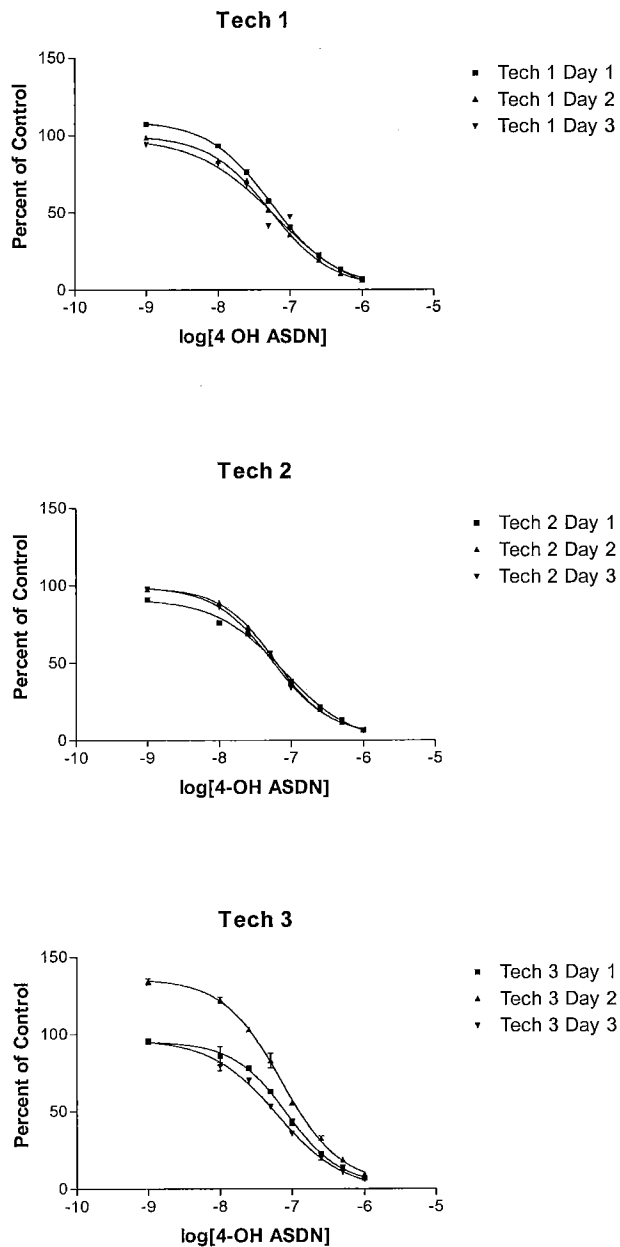


Figure 2. Recombinant Aromatase 4-OH ASDN Inhibition Curves

Table 9. Placental Percent of Control Values

Level	log[4-OH ASDN]	Day								
		1			2			3		
		Replicate								
		1	2	3	1	2	3	1	2	3
		Technician 1								
1	-6.0	-15.7	-16.1	-16.2	-6.1	-5.5	-5.9	4.2	4.5	4.0
2	-6.3	-11.1	-11.7	-12.4	-1.4	-1.4	-1.7	7.5	8.3	7.0
3	-6.6	-3.2	-3.7	-3.4	7.6	7.7	6.2	13.7	13.4	13.2
4	-7.0	16.1	15.5	15.1	23.8	26.3	25.7	30.8	29.8	28.3
5	-7.3	37.9	39.1	38.5	44.9	47.7	44.9	50.1	47.3	46.6
6	-7.6	65.4	68.4	61.8	69.0	70.6	71.5	70.5	52.8	68.9
7	-8.0	81.6	84.2	84.1	90.7	86.0	85.9	87.9	85.6	84.4
8	-9.0	97.9	92.4	94.5	98.6	104.7	97.1	98.3	98.9	95.2
		Technician 2								
1	-6.0	5.1	5.1	5.0	4.6	4.3	4.2	4.9	5.0	4.4
2	-6.3	9.5	8.4	9.4	7.3	7.6	7.6	8.4	7.5	7.9
3	-6.6	18.0	18.8	17.1	13.5	13.7	13.2	15.9	15.0	14.9
4	-7.0	39.4	35.5	36.2	28.2	28.8	28.4	31.6	28.9	29.1
5	-7.3	64.0	57.5	55.0	48.8	45.4	46.1	55.0	46.7	45.9
6	-7.6	79.7	75.0	72.6	68.1	66.9	62.4	69.3	66.3	66.4
7	-8.0	91.1	87.2	87.3	80.4	84.6	81.1	90.0	85.2	82.0
8	-9.0	101.3	97.0	95.9	99.0	94.2	93.3	99.7	101.2	96.7
		Technician 3								
1	-6.0	5.2	5.4	5.3	5.4	5.6	5.6	5.3	5.3	5.2
2	-6.3	10.5	9.9	9.5	10.6	10.8	9.7	9.2	9.2	9.9
3	-6.6	20.1	6.1	19.7	17.3	17.8	16.5	18.3	16.6	16.9
4	-7.0	39.8	37.6	38.3	35.7	35.7	37.5	39.0	37.5	35.4
5	-7.3	60.3	57.4	59.4	53.5	56.4	56.1	58.2	57.7	52.8
6	-7.6	80.6	78.5	78.9	73.1	67.4	71.9	77.1	73.8	70.9
7	-8.0	90.9	87.9	89.4	86.4	84.9	87.0	88.1	88.2	87.8
8	-9.0	94.3	98.4	95.0	89.0	93.4	81.4	98.3	99.7	98.0

Table 10. Recombinant Percent of Control Data

Level	log[4-OH ASDN]	Day								
		1			2			3		
		Replicate								
		1	2	3	1	2	3	1	2	3
		Technician 1								
1	-6.0	7.3	6.7	7.0	6.0	6.2	5.8	6.9	6.3	6.3
2	-6.3	14.0	12.9	12.8	10.9	10.2	10.4	12.2	11.4	11.2
3	-6.6	23.4	21.9	22.0	19.3	19.2	19.1	20.6	20.5	19.4
4	-7.0	42.0	41.6	37.8	36.2	35.0	35.8	38.3	54.1	50.1
5	-7.3	60.0	57.2	55.3	50.8	51.5	53.2	53.6	35.1	35.7
6	-7.6	77.3	78.0	73.6	69.2	67.9	75.0	71.7	67.7	64.7
7	-8.0	94.9	89.5	95.4	82.5	85.8	82.6	85.6	80.4	78.6
8	-9.0	111.4	106.4	103.8	95.6	96.5	103.7	97.1	90.5	94.5
		Technician 2								
1	-6.0	6.8	7.0	6.0	7.4	5.9	6.4	6.9	5.9	6.4
2	-6.3	13.3	14.3	11.1	13.1	10.9	10.7	11.2	10.4	13.8
3	-6.6	21.5	22.4	20.1	22.0	18.9	19.1	19.6	19.5	21.6
4	-7.0	39.4	37.8	36.7	41.2	34.7	34.7	34.2	32.8	35.2
5	-7.3	61.6	54.0	53.7	64.7	53.0	51.5	53.3	53.7	57.1
6	-7.6	67.8	70.0	68.8	81.5	69.3	68.9	71.4	64.2	74.9
7	-8.0	78.0	73.7	76.3	98.0	85.3	83.6	83.6	88.1	86.8
8	-9.0	93.0	93.5	86.5	101.1	98.0	93.7	95.5	94.7	103.9
		Technician 3								
1	-6.0	6.7	7.4	6.7	9.4	9.4	9.7	6.9	5.7	5.8
2	-6.3	14.8	15.2	12.4	21.6	17.1	17.6	11.5	9.8	10.9
3	-6.6	23.0	24.1	20.7	35.3	30.4	32.1	18.9	23.1	18.5
4	-7.0	42.9	46.7	39.6	56.1	55.4	54.7	36.1	35.0	36.5
5	-7.3	61.2	64.3	63.8	91.9	76.0	81.2	52.2	53.9	53.3
6	-7.6	78.2	78.3	77.8	103.4	105.0	101.3	71.5	70.3	68.9
7	-8.0	92.3	92.1	73.6	123.6	124.5	118.6	82.4	73.9	81.2
8	-9.0	98.0	96.7	91.9	133.3	131.3	137.8	96.9	97.1	92.9

Table 11. IC₅₀s for the Inhibition of Aromatase Activity in Human Placental Microsomes by 4-OH ASDN

Date	Technician	Study	IC ₅₀	Technician Mean ± SD ^a
April 1, 2004	1	4	51.1	
April 5, 2004	1	5	51.1	
April 7, 2004	1	6	44.5	48.9 ± 3.8
April 5, 2004	2	10	66.1	
April 7, 2004	2	11	45.9	
April 9, 2004	2	12	45.8	52.6 ± 11.7
April 9, 2004	3	16	68.9	
April 22, 2004	3	17*	68.3	
April 13, 2004	3	18	61.9	66.4 ± 3.9

^a Mean ± SD by technician

* Data are from second run of Study 17 because there was an error in solution preparation for the first run of that study.

Table 12. IC₅₀s for the Inhibition of Aromatase Activity in Human Recombinant Microsomes by 4-OH ASDN

Date	Technician	Study	IC ₅₀ (nM)	Mean ± SD ^a
April 1, 2004	1	1	55.8	
April 5, 2004	1	2	54.9	
April 7, 2004	1	3	65.3	58.7 ± 5.8
April 5, 2004	2	7	78.5	
April 7, 2004	2	8	60.2	
April 9, 2004	2	9	54.7	64.5 ± 12.5
April 1, 2004	3	13	83.5	
April 5, 2004	3	14	71.4	
April 7, 2004	3	15	62.3	72.4 ± 10.6

^a Mean ± SD by technician

Table 13. ANOVA for Log(IC₅₀)

Obs	type	Dependent	DF	MS	FValue	ProbF
1	Placental	LOGIC ₅₀	2	0.01496330	4.35	0.0680
2	Recombinant	LOGIC ₅₀	2	0.00611337	1.48	0.3004
3	Placental	SE_LogIC ₅₀	2	0.00000039	0.01	0.9922
4	Recombinant	SE_LogIC ₅₀	2	0.00054003	0.39	0.6944

Table 14. ANOVA Results for Hill Slope

Obs	type	Dependent	Source	DF	MS	FValue	ProbF
1	Placental	HILLSLOPE	Technician	2	0.01359451	1.42	0.3124
2	Recombinant	HILLSLOPE	Technician	2	0.01806777	1.30	0.3407
3	Placental	SE_Slope	Technician	2	0.00027909	0.43	0.6695
4	Recombinant	SE_Slope	Technician	2	0.00017053	0.06	0.9466

4.0 DISCUSSION/CONCLUSIONS

Preliminary aromatase activity values determined for previously thawed and refrozen stocks of human placental and human recombinant microsomes showed that aromatase activity was well preserved (113% and 98% of previously determined average activities for placental and recombinant preparations, respectively) through at least one freeze/thaw cycle.

The aromatase assay was conducted using both microsome types in order to examine technician-to-technician and day-to-day variation in enzyme activity. Aromatase activity was also measured in the presence of eight different levels of the aromatase inhibitor, 4-OH ASDN, and variance was assessed. IC_{50} s were calculated for 4-OH ASDN in each microsome type and these were examined for technician-to-technician variation.

Statistically significant variances in positive control activities were seen for technician-to-technician, day-to-day, and portion (beginning vs. end) interactions. The source of this variation is not clear, but it may be related to small discrepancies in the preparation of the final microsomal protein dilutions. The protein concentrations used in the assay were very low, and small variances in actual protein concentration could have a large effect on apparent aromatase activity. Generally the activities of the two portion replicates of a given Study were very similar, and therefore, it does not appear that the variances are due to an experimental error. Also, the positive control activities for a given day were generally comparable to those activities measured in the presence of the lowest concentration of 4-OH ASDN for a given day.

Technician-to-technician variation in the aromatase activity of either microsome type in the presence of varying concentrations of 4-OH ASDN was not statistically significant. Day-to-day variance within a technician was statistically significant ($p < 0.05$) for both microsome types.

IC_{50} values were calculated for each Study by nonlinear regression of the percent of control activity remaining at each of the 4-OH ASDN concentrations. An analysis of variance was carried out on the $\log(IC_{50})$, slope and their associated standard errors for technician-to-technician variation. No significant technician-to-technician variation was found in any of those

parameters. The mean IC_{50} for the human placental microsome assay was 56.0 ± 10.3 nM (range 44.5-68.9 nM) and that for the human recombinant microsome assay was 65.2 ± 10.5 nM (range 54.7-83.5 nM). These IC_{50} s are comparable to the literature values of 30-50 nM cited in the WA 2-24 protocol and those previously determined in WA 2-24, which were 41 and 53 nM for placental and recombinant preparations, respectively.

APPENDIX A

Detailed Aromatase Assay Data Tables

Table A-1. Placental Aromatase Assay Technician 1, Day 1

[illegible]

Table A-1. Placental Aromatase Assay Technician 1, Day 1 (Continued)

[illegible]

Table A-1. Placental Aromatase Assay Technician 1, Day 1 (Continued)

[illegible]

Table A-1. Placental Aromatase Assay Technician 1, Day 1 (Continued)

[illegible]

Table A-2. Placental Aromatase Assay Technician 1, Day 2

[illegible]

Table A-2. Placental Aromatase Assay Technician 1, Day 2 (continued)

[illegible]

Table A-2. Placental Aromatase Assay Technician 1, Day 2 (continued)

[illegible]

Table A-2. Placental Aromatase Assay Technician 1, Day 2 (continued)

[illegible]

Table A-3. Placental Aromatase Assay Technician 1, Day 3

[illegible]

Table A-3. Placental Aromatase Assay Technician 1, Day 3 (Continued)

[illegible]

Table A-3. Placental Aromatase Assay Technician 1, Day 3 (Continued)

[illegible]

Table A-3. Placental Aromatase Assay Technician 1, Day 3 (Continued)

[illegible]

Table A-4. Placental Aromatase Assay Technician 2, Day 1

[illegible]

Table A-4. Placental Aromatase Assay Technician 2, Day 1 (Continued)

[illegible]

Table A-4. Placental Aromatase Assay Technician 2, Day 1 (Continued)

[illegible]

Table A-4. Placental Aromatase Assay Technician 2, Day 1 (Continued)

[illegible]

Table A-5. Placental Aromatase Assay Technician 2, Day 2

[illegible]

Table A-5. Placental Aromatase Assay Technician 2, Day 2 (Continued)

[illegible]

Table A-5. Placental Aromatase Assay Technician 2, Day 2 (Continued)

[illegible]

Table A-5. Placental Aromatase Assay Technician 2, Day 2 (Continued)

[illegible]

Table A-6. Placental Aromatase Assay Technician 2, Day 3

[illegible]

Table A-6. Placental Aromatase Assay Technician 2, Day 3 (Continued)

[illegible]

Table A-6. Placental Aromatase Assay Technician 2, Day 3 (Continued)

[illegible]

Table A-6. Placental Aromatase Assay Technician 2, Day 3 (Continued)

[illegible]

Table A-7. Placental Aromatase Assay Technician 3. Day 1

[illegible]

Table A-7. Placental Aromatase Assay Technician 3, Day 1 (Continued)

[illegible]

Table A-7. Placental Aromatase Assay Technician 3, Day 1 (Continued)

[illegible]

Table A-7. Placental Aromatase Assay Technician 3, Day 1 (Continued)

[illegible]

Table A-8. Placental Aromatase Assay Technician 3, Day 2

[illegible]

Table A-8. Placental Aromatase Assay Technician 3, Day 2 (Continued)

Sample Info		Calculate DPM in aqueous portion after extraction							Calculate % turnover				Calculate final assay protein concentration									
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	Total DPM in assay tube (initial)	% conversion to product	Total DPM corrected for background (Blank Tubes)	nmol ³ H ₂ O formed	[protein] stock microsomes (mg/mL)	Volume of stock microsomes used in dilution (μL)	Final volume of diluted microsomes (μL)	[protein] in dilution (mg/mL)	Volume diluted microsomes	Final [protein] in assay (mg/mL)	Incubation time (min)	Aromatase activity (fmol estrogen formed/mg protein/min)	
4-OH ASDN	1-1	2	0.5	1	548	1096	1106	2212	0.1	210285	1.05	2108	0.0020	0.208	3600	50000	0.015	1	0.007	15	0.0089	
			0.5	2	558	1116			0.1													
	1-2	2	0.5	1	563	1126	1160	2320	0.1	210285	1.10	2216	0.0021	0.208	3600	50000	0.015	1	0.007	15	0.0094	
			0.5	2	597	1194			0.1													
	1-3	2	0.5	1	617	1234	1240	2480	0.1	210285	1.18	2376	0.0023	0.208	3600	50000	0.015	1	0.007	15	0.0101	
			0.5	2	623	1246			0.1													
	2-1	2	0.5	1	1045	2090	2111	4222	0.1	210285	2.01	4118	0.0039	0.208	3600	50000	0.015	1	0.007	15	0.0174	
			0.5	2	1066	2132			0.1													
	2-2	2	0.5	1	913	1826	1839	3678	0.1	210285	1.75	3574	0.0034	0.208	3600	50000	0.015	1	0.007	15	0.0151	
			0.5	2	926	1852			0.1													
	2-3	2	0.5	1	883	1766	1789	3578	0.1	210285	1.70	3474	0.0033	0.208	3600	50000	0.015	1	0.007	15	0.0147	
			0.5	2	906	1812			0.1													
	3-1	2	0.5	1	1704	3408	3438	6876	0.1	210285	3.27	6772	0.0064	0.208	3600	50000	0.015	1	0.007	15	0.0286	
			0.5	2	1734	3468			0.1													
	3-2	2	0.5	1	1712	3424	3464	6928	0.1	210285	3.29	6824	0.0065	0.208	3600	50000	0.015	1	0.007	15	0.0289	
			0.5	2	1752	3504			0.1													

Table A-8. Placental Aromatase Assay Technician 3, Day 2 (Continued)

[illegible]

Table A-8. Placental Aromatase Assay Technician 3, Day 2 (Continued)

[illegible]

Table A-9. Placental Aromatase Assay Technician 3, Day 3

[illegible]

Table A-9. Placental Aromatase Assay Technician 3, Day 3 (Continued)

[illegible]

Table A-9. Placental Aromatase Assay Technician 3, Day 3 (Continued)

[illegible]

Table A-9. Placental Aromatase Assay Technician 3, Day 3 (Continued)

[illegible]

Table A-10. Placental Aromatase Assay Technician 3, Day 4

[illegible]

Table A-10. Placental Aromatase Assay Technician 3, Day 4 (Continued)

[illegible]

Table A-10. Placental Aromatase Assay Technician 3, Day 4 (Continued)

[illegible]

Table A-10. Placental Aromatase Assay Technician 3, Day 4 (Continued)

[illegible]

Table A-11. Recombinant Aromatase Assay Technician 1, Day 1

[illegible]

Table A-11. Recombinant Aromatase Assay Technician 1, Day 1 (Continued)

[illegible]

Table A-11. Recombinant Aromatase Assay Technician 1, Day 1 (Continued)

[illegible]

Table A-11. Recombinant Aromatase Assay Technician 1, Day 1 (Continued)

[illegible]

Table A-12. Recombinant Aromatase Assay Technician 1, Day 2

[illegible]

Table A-12. Recombinant Aromatase Assay Technician 1, Day 2 (Continued)

[illegible]

Table A-12. Recombinant Aromatase Assay Technician 1, Day 2 (Continued)

[illegible]

Table A-12. Recombinant Aromatase Assay Technician 1, Day 2 (Continued)

[illegible]

Table A-13. Recombinant Aromatase Assay Technician 1 Day 3

[illegible]

Table A-13. Recombinant Aromatase Assay Technician 1 Day 3 (Continued)

[illegible]

Table A-13. Recombinant Aromatase Assay Technician 1 Day 3 (Continued)

[illegible]

Table A-13. Recombinant Aromatase Assay Technician 1 Day 3 (Continued)

[illegible]

Table A-14. Recombinant Aromatase Assay Technician 2, Day 1

[illegible]

Table A-14. Recombinant Aromatase Assay Technician 2, Day 1 (Continued)

[illegible]

Table A-14. Recombinant Aromatase Assay Technician 2, Day 1 (Continued)

[illegible]

Table A-14. Recombinant Aromatase Assay Technician 2, Day 1 (Continued)

[illegible]

Table A-15. Recombinant Aromatase Assay Technician 2, Day 2

[illegible]

Table A-15. Recombinant Aromatase Assay Technician 2, Day 2 (Continued)

[illegible]

Table A-15. Recombinant Aromatase Assay Technician 2, Day 2 (Continued)

[illegible]

Table A-16. Recombinant Aromatase Assay Technician 2, Day 3

[illegible]

Table A-16. Recombinant Aromatase Assay Technician 2, Day 3 (Continued)

[illegible]

Table A-16. Recombinant Aromatase Assay Technician 2, Day 3 (Continued)

[illegible]

Table A-16. Recombinant Aromatase Assay Technician 2, Day 3 (Continued)

[illegible]

Table A-17. Recombinant Aromatase Assay Technician 3, Day 1

[illegible]

Table A-17. Recombinant Aromatase Assay Technician 3, Day 1 (Continued)

[illegible]

Table A-17. Recombinant Aromatase Assay Technician 3, Day 1 (Continued)

[illegible]

Table A-17. Recombinant Aromatase Assay Technician 3, Day 1 (Continued)

[illegible]

Table A-18. Recombinant Aromatase Assay Technician 3, Day 2

[illegible]

Table A-18. Recombinant Aromatase Assay Technician 3, Day 2 (Continued)

[illegible]

Table A-18. Recombinant Aromatase Assay Technician 3, Day 2 (Continued)

[illegible]

Table A-19. Recombinant Aromatase Assay Technician 3, Day 3

[illegible]

Table A-19. Recombinant Aromatase Assay Technician 3, Day 3 (Continued)

[illegible]

Table A-19. Recombinant Aromatase Assay Technician 3, Day 3 (Continued)

[illegible]

Table A-19. Recombinant Aromatase Assay Technician 3, Day 3 (Continued)

[illegible]