PLACENTAL AROMATASE ASSAY VALIDATION STUDY: POSITIVE CONTROL STUDY

WA 4-16, Task 4

OVERALL TASK DRAFT FINAL REPORT

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EXECUTIVE SUMMARY

The aromatase assay using human placental 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 placental microsomal aromatase assay when performed by the participating laboratories and to obtain intralaboratory and interlaboratory values for aromatase enzyme activity and aromatase inhibition (IC_{50}).

The human placental microsomal protein concentration (mean ± SEM, % CV) was determined to be 13.1 ± 1.5 (19.7 %), 13.4 ± 1.2 (15.1 %), and 11.4 ± 1.2 (20.2 %) mg/mL for Labs A, B, and C, respectively. The overall task mean ± SEM protein concentration was 12.6 ± 0.6 mg/mL (% CV = 8.2).

The human placental aromatase activity (mean ± SEM, % CV) was determined to be 0.0499 ± 0.005 (18.5 %), 0.0719 ± 0.007 (15.8 %), and 0.0520 ± 0.009 (33.2 %) nmol/mg protein/min for Labs A, B, and C, respectively. The overall task mean ± SEM aromatase activity was 0.0579 ± 0.007 nmol/mg protein/min (% CV = 20.9).

Increasing concentrations of 4-OH ASDN decreased the activity (as a percent of control) of the placental 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 5 to 8 percent. At 10^{-9} M 4-OH ASDN, there was little to no aromatase inhibition; the laboratory percent of control values ranged from 95 to 100 percent. Overall task mean ± SEM percent of control values at 10^{-6} and 10^{-9} M 4-OH ASDN were 6.52 ± 0.92 and 97.95 ± 1.48 percent, respectively. The overall percent CV values over all six concentrations by laboratory were less than 13, 6, and 9 percent for laboratories A, B, and C, respectively, except for laboratory A at a 4-OH ASDN concentration of 10^{-6} M, which had overall percent CV value of 44 percent. The overall task percent CV values ranged from 2 to 25 percent.

The 4-OH ASDN IC_{50} values (mean ± SEM, % CV) were determined to be 57.9 ± 8.6 (17.7 %), 47.3 ± 2.6 (9.6 %), and 81.1 ± 5.5 (13.4 %) for Laboratories A, B, and C, respectively. The overall task mean ± SEM IC_{50} value was 62.1 ± 10.0 (% CV = 27.8).

The principal results of the inter-laboratory analysis are summarized below.

a) Laboratory C had the highest estimated $\log_{10}IC_{50}$ among the three laboratories; Laboratory B had the lowest. The variance among laboratories was at least 6 times higher than the unweighted average within laboratory variance for $\log_{10}IC_{50}$. The coefficient of variation among laboratories was 10% when replicate 1 in Laboratory C was included and 11% when replicate 1 in Laboratory C was excluded.

b) Results for the slope were consistent for three laboratories. The estimated variance among the laboratories was zero or near zero. The coefficient of variation among laboratories was 3.7% when replicate 1 in Laboratory C was included and 3.2% when replicate 1 in Laboratory C was excluded.

c) No significant differences existed between background activity control at the end and at the beginning for each laboratory or across three laboratories. The estimated variance among the laboratories was zero or near zero.

d) No significant differences existed between full enzyme activity control at the end and at the beginning of the replicates, across the three laboratories or for Laboratories B and C. Laboratory A had a significantly higher value at the beginning when an outlier was excluded but not a significant difference when the outlier was included. The estimated variances among the laboratories were smaller than the unweighted average within laboratory variance whether the outlying value in Laboratory C was included or excluded. The unweighted average within laboratory variance was inflated by the within laboratory variance in Laboratory B.

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

1.2 Task Description and Objectives

In this task, 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 placental microsomes were prepared 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 placental microsomal aromatase assay when performed by three participating laboratories that had the capabilities but only limited experience with this assay, to obtain intralaboratory and

interlaboratory values for aromatase enzyme activity and aromatase inhibition (IC50) 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 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 95% ethanol. The participating laboratories prepared fresh dilutions of the stock formulation using 95% 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	1×10^{-6}
Working Stock #2	0.1	100	900	2	0.01	1×10^{-7}
		50	950	3	0.005	5×10^{-8}
		25	975	4	0.0025	2.5×10^{-8}
Working Stock #3	0.01	100	900	5	0.001	1×10^{-8}
Working Stock #4	0.001	100	900	6	0.0001	1×10^{-9}

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

2.2 Human Placental Microsomes

Human placental microsomes were provided by RTI (Lot No. 11343-7) and 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 RTI-reported protein concentration was approximately 14.0 mg/mL. For use in the assay, the microsomes were diluted in the assay buffer in two serial dilutions. A 50-fold dilution was made to achieve a concentration of approximately 0.28 mg/mL. Another 10-fold dilution was made to achieve the desired final working stock concentration of approximately 0.025 mg/mL. The final target protein concentration in the incubation mixture was approximately 0.0125 mg/mL.

2.3 Other Assay Components

Information about the other assay components is provided in Table 3. The Chemical Repository obtained the NADPH (β-Nicotinamide Adenine Dinucleotide Phosphate, reduced form) and ethanol and distributed it to the participating laboratories so that it would be from the same supplier and lot.

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	Fisher
Sodium phosphate dibasic (buffer)	Sigma-Aldrich	J. T. Baker	J. T. Baker
Sodium phosphate monobasic (buffer)	Sigma-Aldrich	J. T. Baker	J. T. Baker
Methylene chloride	Burdick and Jackson	Not provided	Not provided

a. Supplied by the EDSP Chemical Repository at Battelle.

2.4 Protein Determination

The microsomal protein concentration was determined using a DC Protein Assay kit from Bio-Rad (Hercules, CA). The 6-point standard curve was prepared using bovine serum albumin (BSA) reconstituted in Milli-Q water. The standard curve range was from 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 Placental Microsomes

Assay Components	Component Volume Added to the Assay	Final Concentration in the Assay
Microsomal Protein	1.0 mL	0.0125 mg/mL
NADPH	100 µL	0.3 mM
[^{3}H]ASDN	100 µL	100 nM
Propylene glycol	100 µL	5% (v/v)
4-OH ASDN	20 µL	10^{-6} to 10^{-9} M

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	1×10^{-6}
4-OH ASDN Concentration 2	3	Complete assay with 4-OH ASDN added	1×10^{-7}
4-OH ASDN Concentration 3	3	Complete assay with 4-OH ASDN added	5×10^{-8}
4-OH ASDN Concentration 4	3	Complete assay with 4-OH ASDN added	2.5×10^{-8}
4-OH ASDN Concentration 5	3	Complete assay with 4-OH ASDN added	1×10^{-8}
4-OH ASDN Concentration 6	3	Complete assay with 4-OH ASDN added	1×10^{-9}

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

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:

$$Y=100/(1+10^{((\text{Log}_{10}\text{IC}_{50}-X)*\text{HillSlope})})$$

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 μ (log IC_{50}) and slope were analyzed using a one-way random effects analysis of variance model. For each replicate the estimated $\log_{10}IC_{50}$ (μ), within replicate standard error of μ, IC_{50}, slope (β), within replicate standard error of β, 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 appendix.

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 appendix. 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 for each intralaboratory analysis.

1. Concentration response 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.

Results for Laboratories A and B were reported based on three replicates, while Laboratory C provided results based on replicates 1 to 4, as well as results based on replicates 2 to 4. There was an outlying value among the full enzyme activity controls for Laboratory A. The results for Laboratory A were reported both including and excluding this data point. The reported standard error of the average results across replicates for Laboratories A and C incorporated the among replicate component of variation, while that 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 intra-laboratory analysis to assess relationships among the laboratory results, 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 the test laboratories in the intra-laboratory analyses. The inter-laboratory analysis also compares among laboratories the average differences of the full enzyme activity 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 variabilities among laboratories for the parameters mentioned above.
- Determine the coefficients of variation among laboratories for the $\log_{10}IC_{50}$ and slope parameters.
- Estimate the ratios of the among laboratory variation to the 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 for Laboratory C and excluding an outlier for full enzyme activity in Laboratory A.

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 (i.e. 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 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 variations were based on the squares of the standard errors associated with the endpoint estimates, as determined by each intralaboratory analysis. The analysis of variance provided an estimated weighted average across all laboratories and its associated standard error as well as an estimate of the laboratory-to-laboratory component of variation. The weights entering into the weighted averages incorporated both laboratory-to-laboratory variations and within laboratory variations. The degrees of freedom associated with the overall average effect was calculated as

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

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 within-laboratory variation and laboratory to laboratory variation) and degrees of freedom were used to construct a 95% confidence interval. The individual effect and associated 95% confidence interval (based on the within laboratory standard error) for each laboratory 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 mean $\log_{10}IC_{50}$ and slope and associated standard errors 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 and associated standard errors when comparing the full enzyme activity and background activity controls obtained at the beginning and those obtained at the end of each replicate, while Laboratory B reported only beginning and end values. The sums of the beginning and end values must be equal to 0 for background activity control and 200 for full enzyme activity control. Therefore, for Laboratory B the differences were calculated as −2 x (End value) for background activity control and 200 − 2 x (End value) for full enzyme activity controls. The associated standard errors for these differences are 2 x (standard error associated with the end values).

To describe the variability among the laboratories relative to the average value, coefficients of variation (CV) and their associated 95% confidence intervals (95% CI) were calculated for the $\log_{10}IC_{50}$ and 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 distributions of the endpoint parameter.

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

$$CV=[\exp(S^2) -1])^{½} \times 100\%$$

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% CI is based on the chi square distribution and is calculated as

$$[(\exp(df*S^2/(\chi^2_{df,\ 0.975}))- 1)^{½},\ (\exp(df*S^2/(\chi^2_{df,\ 0.025}))- 1)^{½}]$$

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

For slope (β), 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 $S \equiv \sqrt{S^2}$. The endpoints of the confidence interval for CV are based on the non-central t distribution (Lehmann, 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 is calculated as

$$R=S_{lab}^2/[⅓(s_1^2 + s_2^2 + s_3^2)]$$

where S_{lab}^2 is the random component of 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 (ν_{lab}, ν_{wi}) degrees of freedom,

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

where $\nu_{lab}=2$ and ν_{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 reports tables by one or a small number of trailing digits in the last decimal place. This is due to differences in rounding in intermediate calculations between the intralaboratory analyses and the interlaboratory analysis.

2.8 Good Laboratory Practices

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

2.9 Personnel

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

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 83 days (12 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 12.6 ± 0.6 mg/mL with a percent CV of 8.2 percent.

According to the lead laboratory, where the human placental microsomes were prepared, the protein concentration was approximately 14 mg/mL. The overall mean from the laboratories was compared to the lead laboratory value (assumed to be the standard) resulting in a percent relative error (%RE) of -10.0 percent.

Table 6. Human Placental Microsomal Protein Concentration Determinations by Replicate and Laboratory[a]

Replicate	Protein Concentration (mg/mL)		
	Lab A	Lab B	Lab C
1	14.4	12.1	12.4
2	--	12.3	14.1
3	14.7	15.7	10.5
4	10.1	--	8.78
Average	13.1	13.4	11.4
Sd	2.6	2.0	2.3
SEM	1.5	1.2	1.2
% CV	19.7	15.1	20.2

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

3.4 Human Placental 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 0.0579 ± 0.007 nmol/mg protein/min with a percent CV of 20.9 percent.

Table 7. Human Placental Aromatase Activity Control Determinations by Replicate and Laboratory[a]

	Aromatase Activity (nmol/mg protein/min)		
Replicate	Lab A	Lab B	Lab C
1	0.0555	0.0797	0.0410
2	--	0.0771	0.0365
3	0.0392	0.0588	0.0558
4	0.0549	--	0.0748
Average	0.0499	0.0719	0.0520
Sd	0.009	0.011	0.017
SEM	0.005	0.007	0.009
% CV	18.5	15.8	33.2

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, when the full enzyme activity controls were averaged across replicates there were no significant differences between the beginning and the end portions. The variation among replicates was constrained to be zero and the variation of the portion (end vs. beginning) effects among replicates was estimated to be zero. However, it is important to note that one of the full enzyme activity control values at the beginning of replicate 3 (56.9%) appeared to possibly be an outlier on the low side. This inflated the standard error and the repetition variance component of the full enzyme activity controls. If this extreme value was excluded, the repetition variance was reduced from 305.41 to 22.87 and the full enzyme activity control values at the beginning were significantly higher than those at the end.

For Laboratories B and C, there were no significant differences between the beginning and end full enzyme activity control values. The complete statistical analysis narrative is presented in the appendix.

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.

No significant differences (beginning minus end) existed between full enzyme activity controls across the three laboratories or for Laboratories B and C. Laboratory A had a significantly higher full enzyme activity control at the beginning when an outlying value was excluded but not a significant difference when the outlying value was included (Table 11). The estimated variance among the laboratories for the background activity controls was near 0 (Table 12). The estimated variance among the laboratories for the full enzyme activity controls was near 0 when the outlying value was included and was less than the unweighted average within laboratory variance (which is inflated by the within laboratory variance in Laboratory B) when the outlying value 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.

Intra- and interlaboratory statistical analysis results indicated that there were no statistically significant differences between the end and beginning background activity control values for each laboratory and across the three laboratories. The interlaboratory statistical analysis results are shown in Tables 11 and 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) by Laboratory

Laboratory	Log 4-OH ASDN Conc (M)	Overall Percent of Control by Laboratory			
		Mean	Sd	SEM	%CV
Laboratory A	-6.00	6.76	2.95	1.70	43.63
	-7.00	37.61	4.72	2.72	12.55
	-7.30	53.00	4.39	2.54	8.29
	-7.60	73.44	5.43	3.13	7.39
	-8.00	86.55	8.45	4.88	9.76
	-9.00	98.20	7.23	4.18	7.37
Laboratory B	-6.00	4.81	0.16	0.09	3.27
	-7.00	31.76	0.65	0.37	2.04
	-7.30	47.48	2.03	1.17	4.28
	-7.60	65.73	3.71	2.14	5.64
	-8.00	85.49	4.05	2.34	4.74
	-9.00	100.38	5.62	3.24	5.60
Laboratory C	-6.00	7.98	0.49	0.24	6.09
	-7.00	44.98	1.81	0.91	4.03
	-7.30	61.92	2.17	1.08	3.50
	-7.60	76.05	6.46	3.23	8.49
	-8.00	88.54	5.85	2.93	6.61
	-9.00	95.28	1.48	0.74	1.56
Overall Task	**Log 4-OH ASDN Conc (M)**	**Overall Mean**	**Overall sd**	**Overall SEM**	**Overall %CV**
	-6.00	6.52	1.60	0.92	24.5
	-7.00	38.12	6.62	3.82	17.4
	-7.30	54.13	7.29	4.21	13.5
	-7.60	71.74	5.37	3.10	7.5
	-8.00	86.86	1.55	0.89	1.8
	-9.00	97.95	2.56	1.48	2.6

The individual 4-OH ASDN inhibition response curves by replicate for each laboratory are reported in the appendix. 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 placental 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 5 to 8 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 95 to 100 percent.

Overall task mean ± SEM percent of control values at 10^{-6} and 10^{-9} M were 6.52 ± 0.92 and 97.95 ± 1.48 percent, respectively.

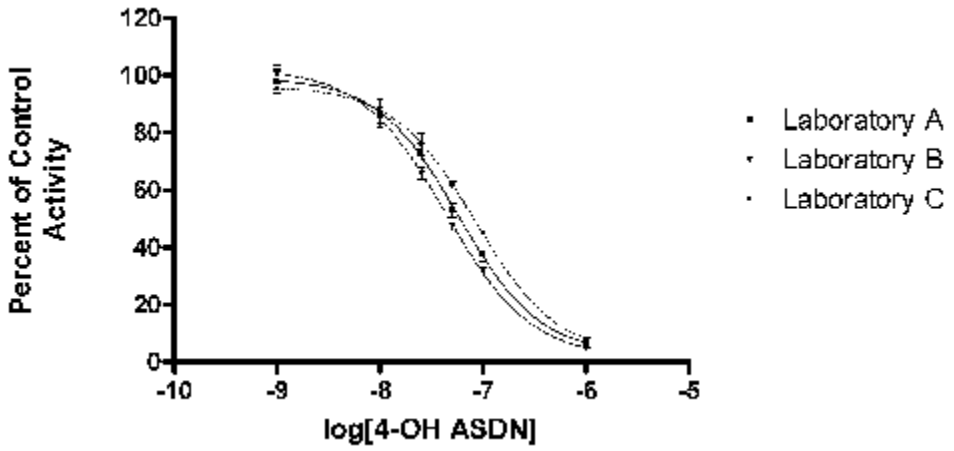

Figure 1. Overall 4-OH ASDN Inhibition Response Curve by Laboratory

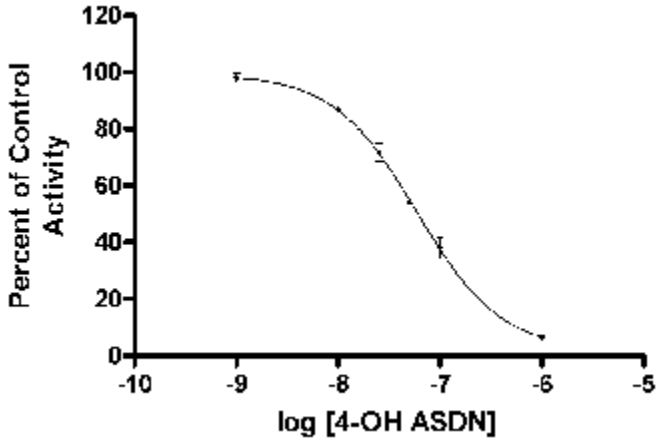

Figure 2. Overall Task 4-OH ASDN Inhibition Response Curve

The CV in percent of control for each replicate and laboratory are reported in the appendix. The overall percent CV values by laboratory (Table 8) were less than 13, 6, and 9 percent for laboratories A, B, and C, respectively, except for laboratory A at a 4-OH ASDN concentration of 10^{-6} M, which had an overall percent CV value of 44 percent. The overall task percent CV values ranged from 2 to 25 percent and showed a trend to decrease with decreasing concentrations of the inhibitor.

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 57.9 ± 5.9, 47.3 ± 2.6, and 81.1 ± 5.5; the percent CV values were 17.7, 9.6, and 13.4 percent, respectively. The overall task mean ± SEM IC_{50} value was 62.1 ± 10.0 and the percent CV was 27.8 percent.

Table 9. IC_{50} Values by Replicate and Laboratory[a]

	IC_{50} Values		
Replicate	Lab A	Lab B	Lab C
1	46.8	51.8	68.3
2	--	47.5	93.8
3	60.0	42.7	77.3
4	67.0	--	85.3
Average	57.9	47.3	81.1
Sd	10.3	4.6	10.9
SEM	5.9	2.6	5.5
% CV	17.7	9.6	13.4

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 -0.9751 ± 0.0671, -1.0070 ± 0.0364, and -0.9706 ± 0.0307; the percent CV values were 11.9, 6.3, and 6.3 percent, respectively. The overall task mean ± SEM IC_{50} value was -0.9842 ± 0.0115 and the percent CV was 2.0 percent.

Table 10. Slope Values by Replicate and Laboratory[a]

	Slope Values		
Replicate	Lab A	Lab B	Lab C
1	-1.1030	-1.0478	-0.8969
2	--	-1.0389	-1.041
3	-0.9464	-0.9343	-0.9511
4	-0.8759	--	-0.9933
Average	-0.9751	-1.0070	-0.9706
Sd	0.1162	0.0631	0.0613
SEM	0.0671	0.0364	0.0307
% CV	11.9	6.3	6.3

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, replicate 3 had a higher IC_{50} value than replicates 1 and 4. Replicate 1 had a more negative slope than the other replicates. The complete statistical analysis narrative is presented in the appendix.

For Laboratory B, there were no significant differences between the IC50 or slope values. The complete statistical analysis narrative is presented in the appendix.

For Laboratory C, since replicate 1 had a lower IC_{50} and more slowly decreasing slope, the average across replicates 2 to 4 had higher IC_{50} and more rapidly decreasing slope than average across four replicates. However, the differences were slight. The variance components across replicates 1 to 4 are greater than those across replicates 2 to 4. For $\log_{10}IC_{50}$, replicate to replicate variation was more than five times the individual replicate within replicate variances, when all four replicates were considered, and more than two times the individual replicate within-replicates variances when just replicates 2 to 4 were considered.

3.7.2 Interlaboratory Statistical Analysis

Table 11 displays the estimated parameter values and 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 and with and without the full enzyme activity control outlying value for laboratory A. 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 overall means for $\log_{10}IC_{50}$ and for the slope are also presented in Table 11.

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

Laboratory C had a higher value for $\log_{10}IC_{50}$ than Laboratories A and B (Table 11), which contributed to a relatively high random laboratory variation (more than 6 times higher than the unweighted average within laboratory variation), regardless of whether replicate 1 in laboratory C was included or excluded (Table 2). The coefficient of variation for $\log_{10}IC_{50}$ was 10% when replicate 1 in Laboratory C was included and 11% when replicate 1 in Laboratory C was excluded.

The results for the slope estimates were consistent among the three laboratories Table 1). The estimated variance among the laboratories was zero or near zero (Table 2). The coefficients of variation among laboratories were 3.7% when replicate 1 in Laboratory C was included and 3.2% when replicate 1 in Laboratory C was excluded.

Table 11. Parameter Estimate and the 95% Confidence Interval for the Percent of Control Responses for Placental Aromate Assay

Parameter	Estimate and 95% Confidence Interval[1]				CV(%)and 95% CI[4]
	Lab A	Lab B	Lab C	Average[2,3]	
Rep 1-4 for Lab C					
$Log_{10}IC_{50}$	-7.2190 (-7.4543, -6.9837)	-7.3260 (-7.4293, -7.2227)	-7.0940 (-7.1885, -6.9995)	-7.2136 (-7.3881, -7.0392)	10.1621 (5.8912, 34.3980)
Slope	-0.9830 (-1.2685, -0.6975)	-1.0070 (-1.1619, -0.8521)	-0.9662 (-1.0616, -0.8708)	-0.9816 (-1.0403, -0.9228)	3.7072 (2.2125, 10.8498)
Difference Between End and Beginning for Background Activity Control	-0.1416 (-1.8038, 1.5206)	-0.0040 (-0.1933, 0.1853)	0.1340 (-0.0715, 0.3395)	0.0253 (-0.0611, 0.1116)	
Difference Between End and Beginning for Full Enzme Activity Control	0.6019 (-21.8796, 23.0834)	-1.9780 (-37.4060, 33.4500)	2.5365 (-1.8436, 6.9166)	2.2127 (-2.1833, 6.6087)	
Rep 2-4 for Lab C Outlier Deleted for Lab A					
$Log_{10}IC_{50}$	-7.2190 (-7.4543, -6.9837)	-7.3260 (-7.4293, -7.2227)	-7.0720 (-7.1783, -6.9657)	-7.2047 (-7.3959, -7.0135)	11.0910 (6.4149, 38.1054)
Slope	-0.9830 (-1.2685, -0.6975)	-1.0070 (-1.1619, -0.8521)	-0.9852 (-1.0791, -0.8913)	-0.9907 (-1.0432, -0.9381)	3.1878 (1.8770, 9.8890)
Difference (Beginning Minus End) for Background Activity Control	-0.1472 (-1.8078, 1.5134)	-0.0040 (-0.1933, 0.1853)	0.1787 (-0.1270, 0.4844)	0.0207 (-0.0697, 0.1110)	
Difference (Beginning Minus End) for Full Enzme Activity Control	10.5925 (4.0417, 17.1433)	-1.9780 (-37.4060, 33.4500)	0.3623 (-4.3839, 5.1085)	4.2022 (-4.9895, 13.3939)	

1. The estimates and 95% CI were as reported in the intralaboratory analyses based on the data tested by the three participating laboratories. Laboratory C provided results separately for replicates 1 to 4 and for replicates 2 to 4. Laboratory A had results with and without an outlier for full enzyme activity controls.
2. The overall effects and standard errors were estimated using a one-way ANOVA mixed model assuming the variances differed among the three laboratories, where the variances for each laboratory were fixed to be the reported variances.
3. The averages were calculated as the following:
   - including all three replicates for Laboratories A and B and all four replicates for Laboratory C;
   - including all three replicates for Laboratory B, all three replicates for Laboratory A but excluding an outlier for full enzyme activity control, and replicates 2 to 4 for Laboratory C.
4. CV is calculated for the average results for $Log_{10}IC_{50}$ and slope parameters.

Table 12. Variance Component, and Ratio of Variance between Between Laboratories and Within Laboratoriesfor the Percent of Control Responses for Placental Aromate Assay

Parameter	Within Lab Variance[1]				Random Laboratory Variance and (p-value) (df=2)[3]	Mean Variance[4,5]	Ratio and 95% CI of Random Lab-to-Lab Variation to Average Within Lab Variation[6]
	Lab A	Lab B	Lab C	Pooled Unweighted Simple Average Results[2]			
Rep 1-4 for Lab C							
$Log_{10}IC_{50}$	0.003045 /df=2.019	0.000575 /df=2	0.00082 /df=2.823	0.00148/df=3.95	0.008904 (p=0.1297)	0.00342/df=3.40	6.0149 (0.5560, 236.055)
Slope	0.005089 /df=2.166	0.001296 /df=2	0.000771 /df=2.646	0.002385/df=3.93	0 (p=1.000)	0.000441/df=3.93	0 (-)
Difference (Beginning Minus End) for Background Activity Control	0.5565 /df=10	0.0019 /df=2	0.0071 /df=6	0.1885 /df=10.322	$3.33x10^{-22}$ (p=1.000)	0.001515/df=10.32	$1.7684x10^{-21}$ ($3.2831x10^{-22}$, $6.9678x10^{-20}$)
Difference (Beginning Minus End) for Full Enzme Activity Control	101.80 /df=10	67.7988 /df=2	4.1706 /df=14	57.9245 /df=9.052	$3.4x10^{-22}$ (p=1.000)	3.78291/df=9.05	$5.8692x10^{-24}$ ($1.0298x10^{-24}$, $2.3117x10^{-22}$)

Parameter	Within Lab Variance[1]				Random Laboratory Variance and (p-value) (df=2)[3]	Mean Variance[4,5]	Ratio and 95% CI of Random Lab-to-Lab Variation to Average Within Lab Variation[6]
	Lab A	Lab B	Lab C	Pooled Unweighted Simple Average Results[2]			
	Rep 2-4 for Lab C Outlier Deleted for Lab A						
$Log_{10}IC_{50}$	0.003045 /df=2.019	0.000575 /df=2	0.000548 /df=1.894	0.00139/df=3.535	0.01094 (p=0.1234)	0.00408/df=3.37	7.8730 (0.6308, 308.744)
Slope	0.005089 /df=2.166	0.001296 /df=2	0.00049 /df=2.03	0.002292 /df=3.66	$4.14x10^{-22}$ (p=1.000)	0.000332/df=3.66	$1.8x10^{-19}$ ($1.5x10^{-20}$, $7.1x10^{-18}$)
Difference (Beginning Minus End) for Background Activity Control	0.5556 /df=10	0.0019 /df=2	0.0121 /df=4	0.1898 /df=10.499	$5x10^{-22}$ (p=1.000)	0.001664/df=10.50	$2.6361x10^{-21}$ ($4.927x10^{-22}$, $1.0387x10^{-19}$)
Difference (Beginning Minus End) for Full Enzme Activity Control	8.3857/ df=9	67.7988 /df=2	4.5373 /df=10	26.9072 /df=2.8230	17.7142 (p=0.2214)	10.5319/df=3.81	0.6583 (0.0369, 25.7706)

1. The within laboratory variance for a given laboratory is the square of the standard error associated with the parameter estimate, which was reported in the intra-laboratory analyses based on the data tested by the three participant laboratories. Laboratory C provided results separately for replicates 1 to 4 and for replicates 2 to 4. Laboratory A had results with and without an outlier for the full enzyme activity controls
2. Pooled unweighted average results for within laboratory are the simple averages of the within laboratory variances among the three laboratories, and the associated degree of freedom was calculated using Satterthwaite's approximation.
3. A one-way ANOVA mixed model assuming the variances differed among the three labs, where the within laboratory variance for each laboratory was fixed to be the reported variance, was fitted to estimate the random laboratory variance.
4. Mean Variance is the square of the standard error of the pooled weighted mean, including among laboratory variation.
5. Degrees of freedom for the Mean Variance was estimated as $2*((1/K)*\sum(SL2 + Si2))2/(var(SL2)+(2/K2)* \sum(Si4/dfi))$, where SL2 is random lab variance, Si2 and dfi are reported variance and degree of freedom for a given laboratory, (var(SL2) is the variance associated with the estimation of SL2, and K is the number of laboratories (Hartung and Makambi, 2001).
6. Ratio of random among laboratory variance and unweighted simple average of within laboratory variances

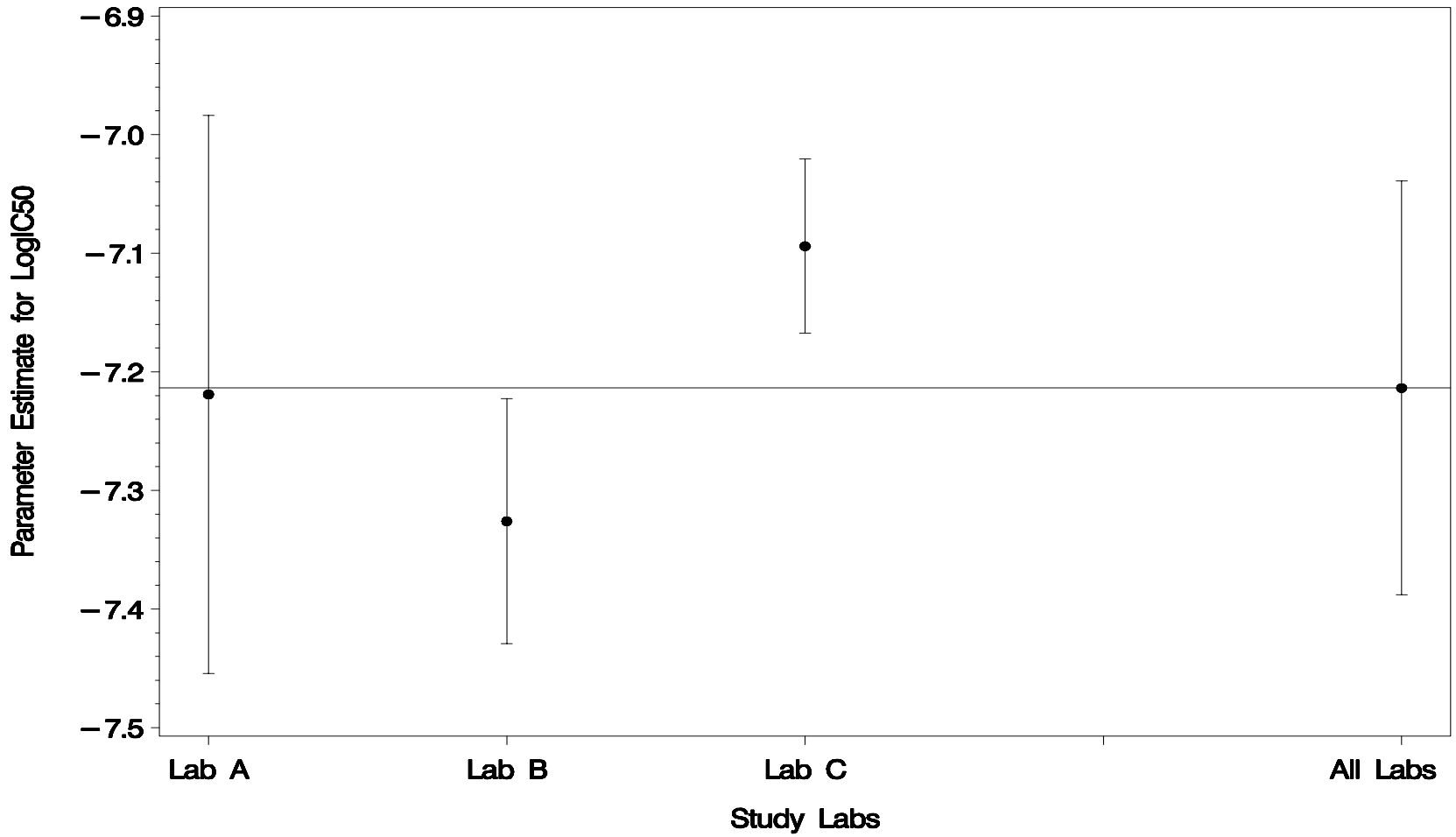

Figure 3. **Parameter Estimates and Their Associated 95% Confidence Intervals for $Log_{10}IC_{50}$ in Placental Aromatase Assay, Across Laboratories and by Each Laboratory. The Reference Line Corresponds to the Average Across Laboratories. Laboratory C Includes Replicates 1-4.**

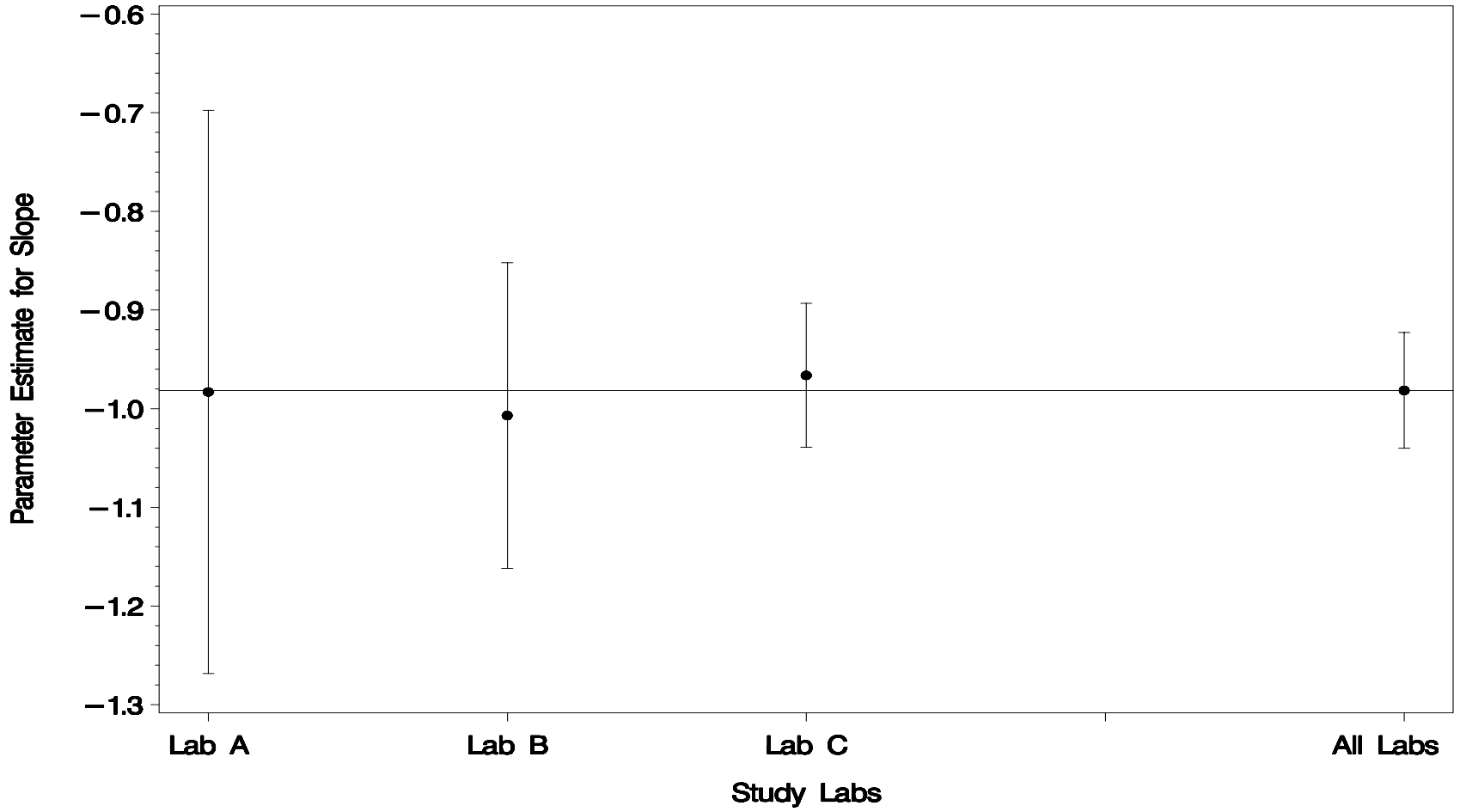

Figure 4. Parameter Estimates and Their Associated 95% Confidence Intervals for Slope in Placental Aromatase Assay, Across Laboratories and by Each Laboratory. The Reference Line Corresponds to the Average Across Laboratories. Laboratory C Includes Replicates 1-4.

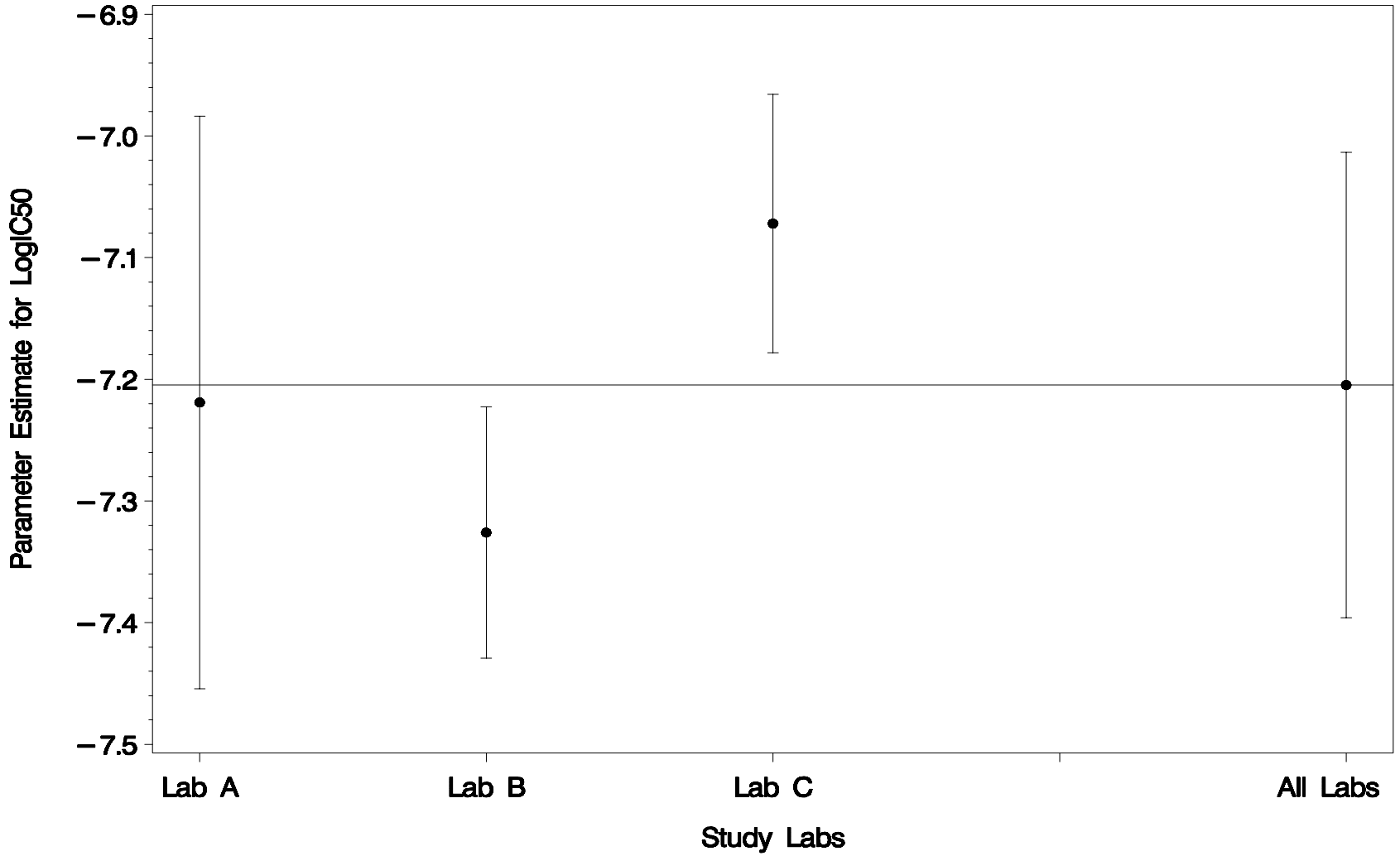

Figure 5. Parameter Estimates and Their Associated 95% Confidence Intervals for $Log_{10}IC_{50}$ in Placental Aromatase Assay, Across Laboratories and by Each Laboratory. The Reference Line Corresponds to the Average Across Laboratories. Laboratory C Includes Replicates 2-4.

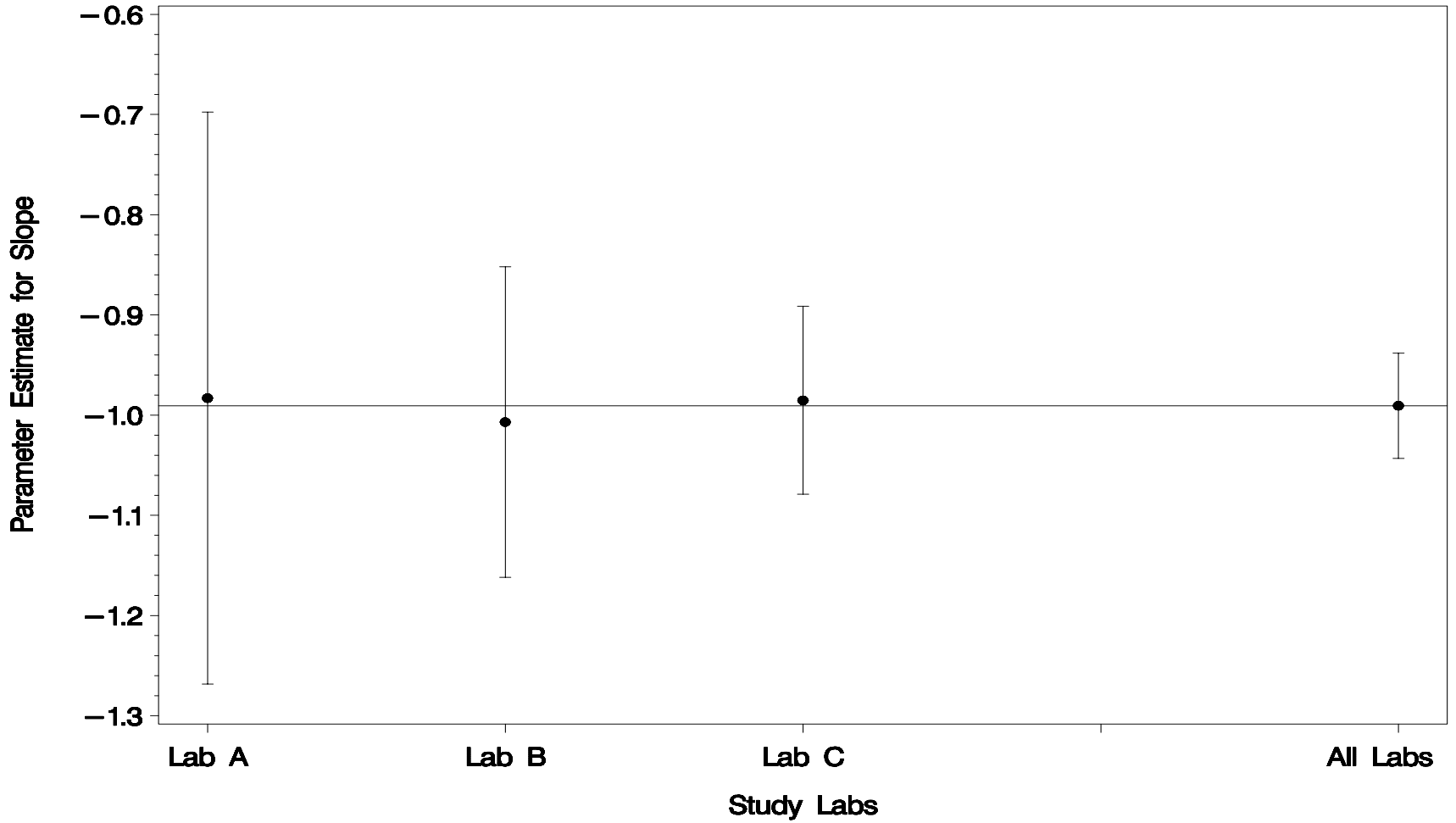

Figure 6. Parameter Estimates and Their Associated 95% Confidence Intervals for Slope in Placental Aromatase Assay, Across Laboratories and by Each Laboratory. The Reference Line Corresponds to the Average Across Laboratories. Laboratory C Includes Replicates 2-4.

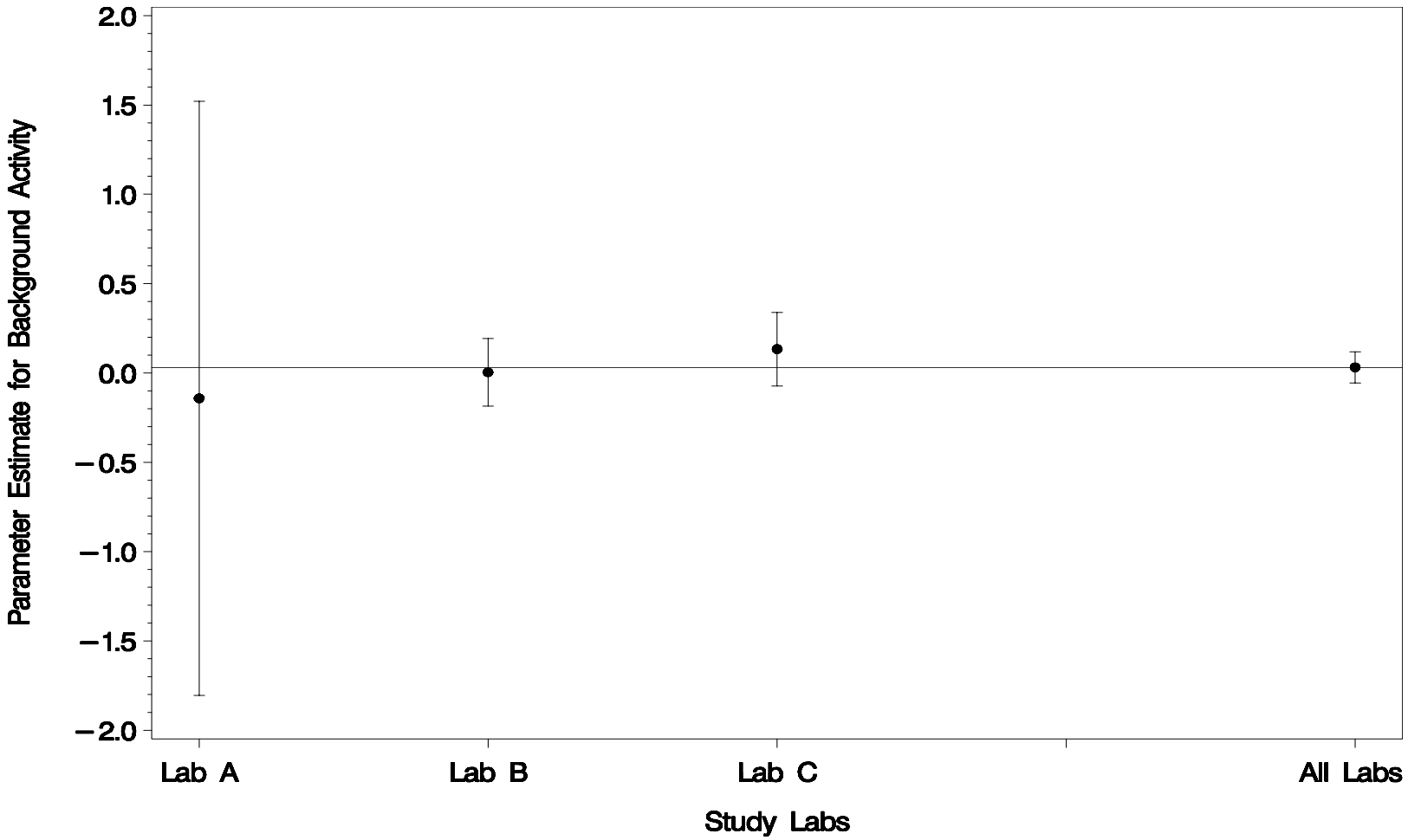

Figure 7. Parameter Estimates and Their Associated 95% Confidence Intervals for Difference Between Beginning and End for Background Activity Control in Placental Aromatase Assay, Across Laboratories and for Each Laboratory. The Reference Line Corresponds to the Average Across Laboratories. Laboratory C Includes Replicates 1-4.

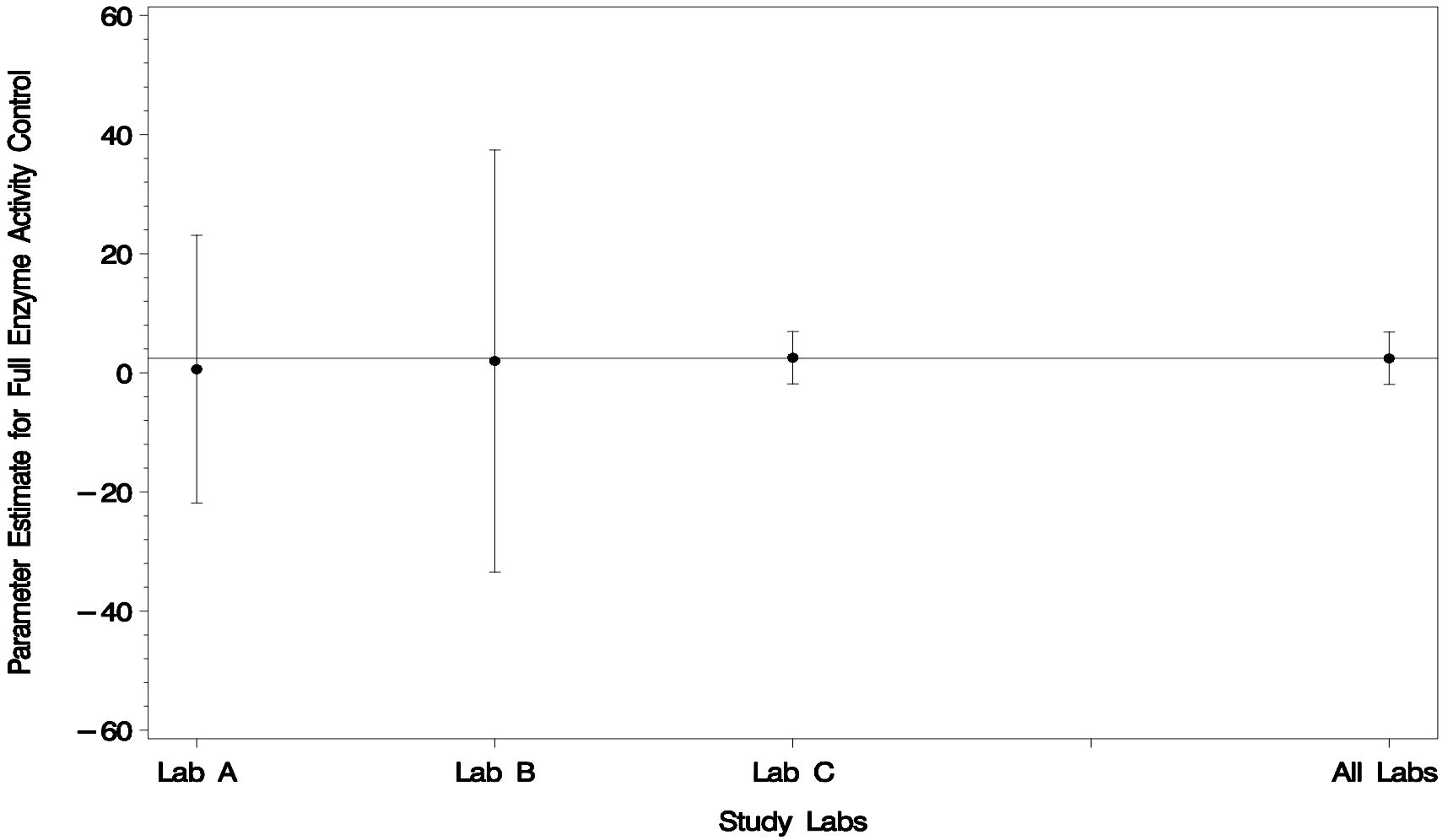

Figure 8. **Parameter Estimates and Their Associated 95% Confidence Intervals for Difference Between Beginning and End for Full Enzyme Activity Control in Placental Aromatase Assay, Across Laboratories and for Each Laboratory. The Reference Line Corresponds to the Average Across Laboratories. Laboratory C Includes Replicates 1-4.**

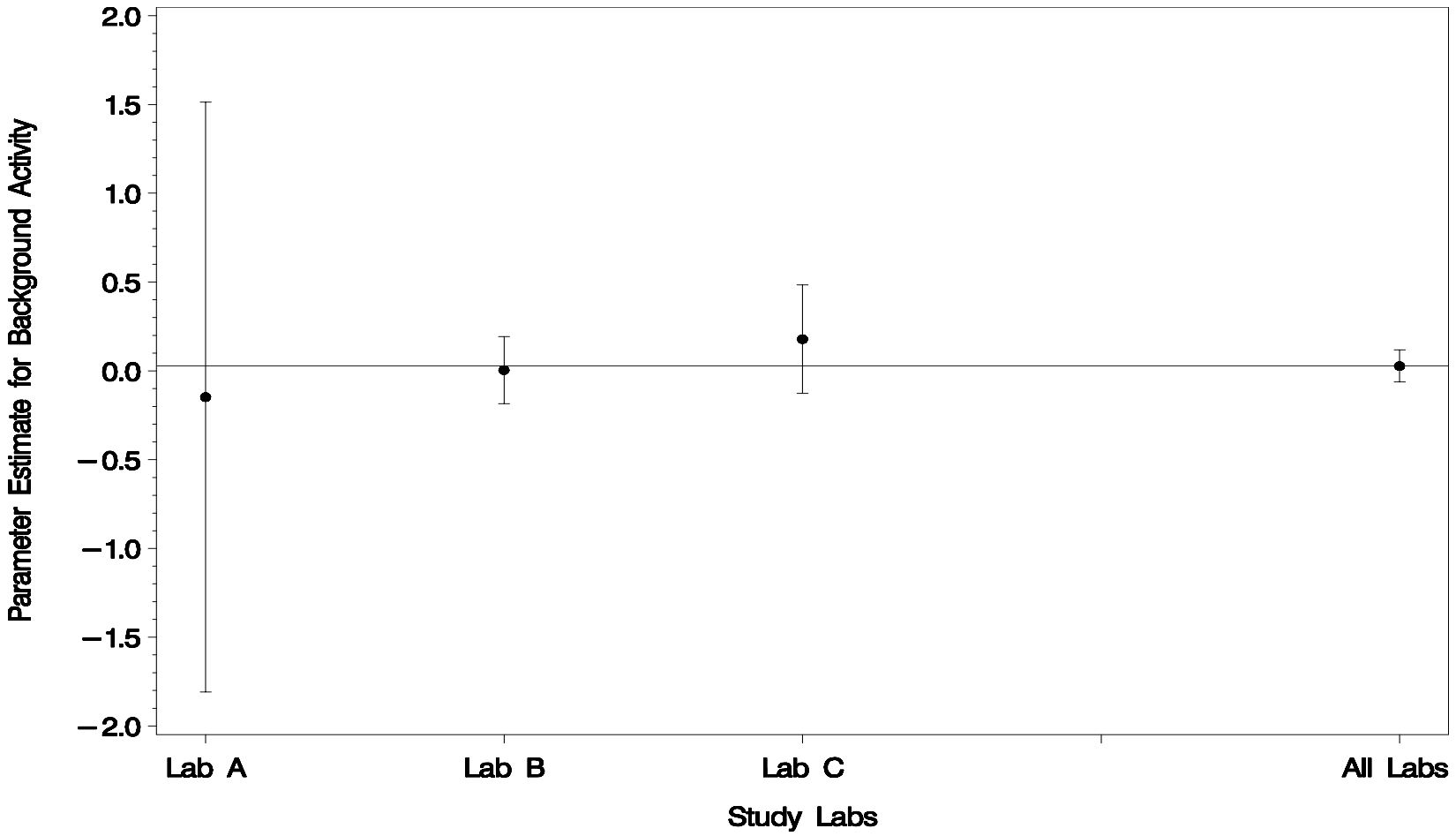

Figure 9 **Parameter Estimates and Their Associated 95% Confidence Intervals for Difference Between Beginning and End for Background Activity Control in Placental Aromatase Assay, Across Laboratories and for Each Laboratory. The Reference Line Corresponds to the Average Across Laboratories. Laboratory C Includes Replicates 2-4 and Laboratory A Excludes an Outlying Value for Full Enzyme Activity.**

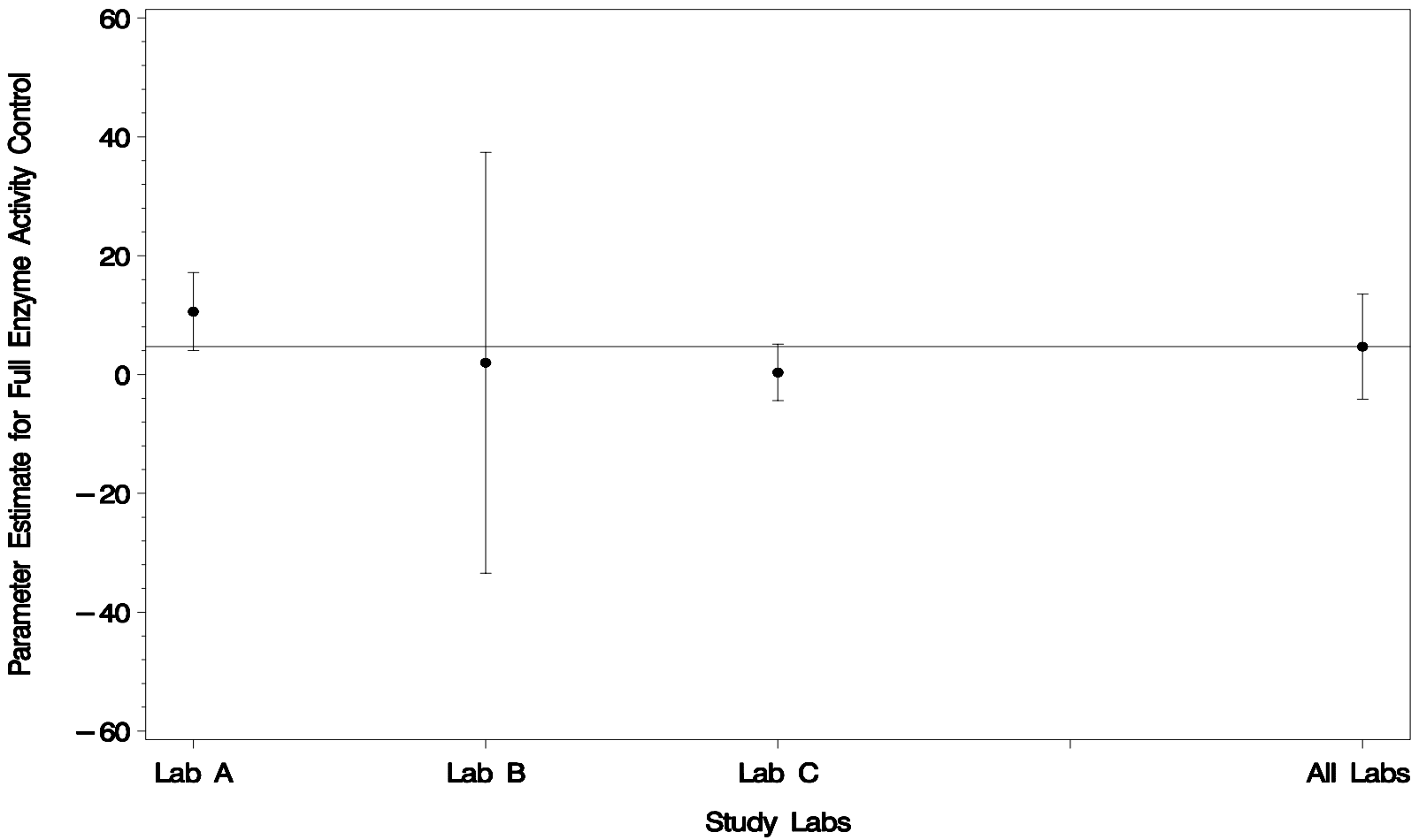

Figure 10. Parameter Estimates and Their Associated 95% Confidence Intervals for Difference Between Beginning and End for Full Enzyme Activity Control in Placental Aromatase Assay, Across Laboratories and for Each Laboratory. The Reference Line Corresponds to the Average Across Laboratories. Laboratory C Includes Replicates 2-4 and Laboratory A Excludes an Outlying Value for Full Enzyme Activity.

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 obtained by the lead laboratory. However, this task also demonstrated that the laboratories had difficulties that could be attributed to their degree of experience, as well as procedures that were not known to affect the assay. In regard to this latter point, one laboratory discovered that using a repeat pipettor to dispense the diluted microsomes apparently caused the microsomes to become compromised, possibly due to shear forces or unpredictable settling of the microsomes suspension. Upon investigation, it was discovered that the cause of the very low control enzyme activity could be attributed to using this particular type of pipettor. By switching to a normal air-displacement pipet, this problem was resolved. As for difficulties that could be attributed to degree of experience, one laboratory initially thought that it had made a procedural error but was uncertain where the error occurred. After repeating a replicate, the lab discovered that no procedural error had occurred; rather, the error was in processing the results of the replicate. Another problem that was corrected through additional experience was the production of high background values, which was attributed to a laboratory contaminating the background tubes with NADPH. This issue was rectified by changing pipette tips and learning how important it was to keep NADPH away from the background tubes. Thus, the results of this task provide a measure of the variability obtained by multiple laboratories that conducted the aromatase assay with a minimal degree of training and when the assay procedure was followed as planned (replicates that were found not to be conducted according to the procedure as planned were not used).

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 47.3 to 81.1 with an overall average (± SEM) IC_{50} value of 62.1 ± 10.0 nM. In WA 4-10, Task 3, RTI reported an average (± sd) IC_{50} value for 4-OH ASDN to be 56.0 ± 10.3 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).

5.0 CONCLUSIONS

In conclusion, the results from this task indicated that the human placental 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.

6.0 REFERENCES

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