# INTEGRATED SUMMARY REPORT

**on**

# AROMATASE

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# 1.0 Executive Summary

The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) was charged by the EPA to provide recommendations on the implementation of an Endocrine Disruptor Screening Program (EDSP). EDSTAC recommended a series of *in vitro* and *in vivo* assays for inclusion in Tier I screening battery to detect chemicals that may affect the estrogen, androgen, and thyroid hormone systems through any one of the known modes of action, such as interruption of hormone production or metabolism, binding of the hormone with its receptor, or interference with hormone transport. This Integrated Summary Report on aromatase incorporates information from the scientific literature and EPA's validation program that supports the conclusion that the microsomal aromatase assays are valid for the purpose of identifying chemicals that have the potential to inhibit aromatase.

**BACKGROUND**

Aromatase is the cytochrome P450 enzyme complex responsible for the conversion of androgens to estrogens during steroidogenesis. Alterations in the amount of aromatase present or in the catalytic activity of the enzyme will alter the levels of estrogens in tissues and dramatically disrupt estrogen hormone action. Inhibition of aromatase alters the catalytic activity of the enzyme and results in a rapid decrease in the levels of estrogens. Suppression or induction of the aromatase protein levels also can dramatically influence the subsequent levels of estrogens in tissues and affect hormone action. Environmental agents, toxicants, and various natural products can act via aromatase inhibition and/or alteration in aromatase protein levels to result in altered levels of estrogen and function as endocrine disruptors. The scientific basis of the aromatase assay is presented in further details in Chapter 3.

EPA selected microsomal aromatase assays for validation. The human placental microsomal aromatase assay is one of the most common *in vitro* assays used for measuring aromatase and aromatase inhibition because of its reliability, reproducibility, and ease of use. This assay method has been utilized routinely for the determination of the presence of aromatase in tissues, and the overall protocol is within the ability of individuals with undergraduate laboratory experience in biology, biochemistry, or molecular biology. The source of the aromatase is a microsomal preparation isolated from human term placenta, consisting of the endoplasmic reticulum membrane of the cell and contains the membrane-bound cytochrome $P450_{arom}$ and the NADPH (β-Nicotinamide Adenine Dinucleotide Phosphate) cytochrome P450 reductase. Complete enzyme activity requires the addition of either NADPH or an NADPH-generating system, and the activity can be measured using either the product isolation method or the radiometric method. The human placental microsomal aromatase assay has been used extensively in academic labs and pharmaceutical firms as the initial biological evaluation for potential steroidal and nonsteroidal aromatase inhibitors. Alternatively, aromatase present in microsomal preparations from a variety of aromatase expression systems can be obtained and may be used instead of microsomes harvested from placenta tissues. Both the human placental microsomal assay and the recombinant assay using human recombinant microsomes from Gentest ([Human CYP19 + P450 Reductase SUPERSOMES], Woburn, MA) were carried through the validation stages to demonstrate their equivalence.

## TEST METHODS

Chapter 4 is a general description of the test methods examined, with detailed protocols of the placental and recombinant assays provided in Appendix A. The two procedures are similar, except that the activity of the protein is higher in the recombinant assay system. For use in the assay, the placental microsomes are diluted in the assay buffer to approximately 0.025 mg/ml, with the final target protein concentration in the incubation mixture of approximately 0.0125 mg/ml. The recombinant microsomes are diluted in the assay buffer to approximately 0.008 mg/ml, with the final target protein concentration in the incubation mixture should be approximately 0.004 mg/ml. The substrate for the assays is androstenedione (ASDN), which is converted by aromatase to estrone. The progress of the reaction can be followed by measuring the formation of either of the two reaction products. Formation of estrone can be measured directly as the indicator of aromatase activity. The formation of $^3H_2O$ can be measured by liquid scintillation counter if the radiolabeled substrate, [1β-$^3$H]-androstenedione, is used. In these assays, tritiated water formation is measured and a mixture of non-radiolabeled and radiolabeled ASDN is used as the substrate. The aromatase inhibitor 4-hydroxyandrostenedione (4-OH ASDN) is the positive control, and this compound is a mechanistic or suicide inhibitor and binds irreversibly to aromatase causing enzyme inactivation.

## DATA ANALYSIS

The general methods for data modeling, analysis, and interpretation are described in Chapter 5. The two different kinds of inhibitors are competitive inhibitors and non-competitive inhibitors. In competitive inhibition, the inhibitor competes with the substrate for the enzyme's active sites, diminishing the amount of enzyme available to catalyze the conversion of substrate to product thus slowing down the rate of product formed. At high levels of substrate, this effect is diminished as the overwhelming concentration of substrate out-competes the inhibitor. In contrast, non-competitive inhibitors bind reversibly to one or more sites on the enzyme other than the active site, either with the enzyme itself or the enzyme-substrate complex. Both types of inhibitors have been tested in the validation program.

The usual approach to measuring the inhibitory effect of chemicals on enzyme activity is to hold the enzyme and substrate concentrations constant and vary the concentration of the inhibitor. The $IC_{50}$ value of a test chemical is the concentration of the compound that results in a 50-percent reduction of enzyme activity. For each assay, the estimated baseline response (B), the estimated maximum response (T), the estimated $\log_{10}IC_{50}$ (μ), the IC50, and the slope (ß) are determined. In addition, since the test chemical competes with the substrate, the model for this approach is completely analogous to that of receptor binding. The $\log_{10}$ of the inhibitor concentration is plotted on the x-axis and enzyme activity from 100 percent (the full enzyme activity level) to 0 percent on the y-axis, and increasing concentrations of inhibitor produce a sigmoid dose-response curve determined by the law of mass action with a slope of approximately -1.

## REFERENCE AND SUPPLEMENTARY CHEMCIALS

Eleven reference chemicals were selected by EPA on the basis of data in the scientific literature and tested in the lead laboratory as part of the assay optimization process. Two of these chemicals were dropped and three others added to form the reference library used in the interlaboratory study noted in Chapter 6. The aromatase inhibitor 4-hydroxyandrostenedione is

the positive control, and lindane was selected as the negative control.. The reference compounds used in the interlaboratory validation study included nonsteroidal aromatase inhibitors (aminoglutethimide), pesticides (lindane, atrazine, dicofol), a flavonoid (chrysin), a polycyclic aromatic hydrocarbon (dibenzanthracene), imidazole fungicides (econazole, fenarimole, ketoconazole, prochloraz), and an alkylphenol (4-nonylphenol). For the interlaboratory validation studies, EPA used chemicals that had been tested for aromatase in several different assay systems in multiple laboratories. Before using these substances in the interlaboratory validation studies, these substances were tested multiple times in the lead laboratory to confirm the results cited in the scientific literature. Sixteen supplementary reference chemicals were also identified.

## PROTOCOL OPTIMIZATION

The lead laboratory, Research Triangle Institute (RTI), conducted assay development and optimization experiments to identify the optimal factors and conditions for the assay (Chapter 7). Three sets of preoptimization exploratory experiments were conducted with human, bovine, and porcine placental microsomes and human recombinant microsomes. Each microsomal preparation (human, bovine, and porcine placental microsomes and the human recombinant microsomes) was analyzed for protein concentration, cytochrome P450 content, and aromatase activity. The P450 content measurement was used to ensure that the enzyme was present (and in what concentration/preparation type) prior to beginning the more elaborate aromatase activity assay. A single aromatase activity assay run using each type of microsomal preparation was included as a pre-optimization experiment in order to determine whether the preparations were of sufficient activity to conduct the optimization experiments.

The bovine and porcine placenta were unsuitable for use because they gave unsatisfactorily low levels of aromatase activity. In addition, procurement of bovine and porcine placentas was problematic because of the seasonality of livestock breeding and the impractical collection of fresh placentas. Both the human placental and recombinant microsomal assays were optimized and met expectations, and each assay demonstrated linearity for protein concentration and time and maintenance of initial rate conditions with less than 10 to 15 percent of substrate consumed. Furthermore, the dose-response curves and $IC_{50}$ data for 4-hydroxy-androstenedione in the human placental and human recombinant assays fell within the 20 to 50 nM historical range reported in the protocol. Day-to-day variation within the human placental assay was very low, while a somewhat higher variation was found in the recombinant assay.

## PRELIMINARY INTERLABORATORY VALIDATION

RTI, the lead laboratory, tested the11 test substances listed above in both assays in order to determine the response of the assay to these known inhibitors and non-inhibitors (Chapter 8) and determine their suitability for the interlaboratory studies. Each of 11 test substances at up to eight different concentrations (ranging from no less than $10^{-9}$ M to no higher than $10^{-3}$ M) were tested using each of the two microsomal preparations for which optimal conditions were determined. The dose-response curves for placental and recombinant microsomal assays indicate that genistein, atrazine, bis(2-ethylhexyl)phthalate, nonylphenol, lindane and dibenz*(a,h)*anthracene do not significantly inhibit aromatase activity over the range of concentrations tested. Therefore, no meaningful $IC_{50}$ values could be calculated for those compounds. Econazole, aminoglutethimide, chrysin, ketoconazole, and 4-OH ASDN did inhibit

aromatase activity over the concentration ranges tested. Generally, similar $IC_{50}$ values were obtained in both the placental and recombinant assays for each test chemical. The experimentally determined $IC_{50}$ values fall within the literature ranges cited in the protocol for all test chemicals except for econazole and ketoconazole, where both of the measured $IC_{50}$ values were lower than the literature value ranges cited in the protocol. Differences among technicians and day-to-day variability within technicians for enzymatic activity at various inhibitor concentrations were estimated, and no significant variance of activity was found among technicians. There were significant day-to-day variances within technicians for both types of microsomal preparations. These effects may be due to slight differences in microsome dilution preparation. However, the similarity in responses across labs, chemicals, and test protocols indicates that the two test methods are reproducible and generally consistent with the literature values.

**PRELIMINARY INTERLABORATORY TESTING**

Battelle, the prime contractor to EPA for the validation of the aromatase assay, selected three contract laboratories (Battelle, In Vitro Technologies, and WIL Research Laboratories) to participate in the interlaboratory validation study. RTI, which conducted the prevalidation studies, served as the lead laboratory for the aromatase assay validation studies. Study directors and key technical staff of the participating laboratories were trained in the RTI laboratory. After training, each of the participating laboratories conducted at least three independent runs of the basic aromatase assay to demonstrate the conversion of substrate. This was following a positive control study using microsomes prepared by RTI. Statistical analyses were carried out on the background activity control (a measure of the baseline activity), and portion effect for full enzyme activity control (a measure of any changes in the full activity control at the beginning and end of the assay set-up used to indicate when the number of tubes in a run is unreasonably large, significantly altering the activity measures between the beginning and end of the run).

With the placental microsomal aromatase assay, increasing concentrations of the positive control, 4-OH ASDN, decreased the aromatase activity, and the decrease was dose-dependent for all three laboratories. The shape of the enzyme activity versus 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. Based on the curve-fit of the percent of control aromatase activity values across six concentrations of 4-OH ASDN, the average $IC_{50}$ values ± SEM $IC_{50}$ values for Laboratories A, B, and C and the overall task means are shown in Table 1.0-1.

**Table 1.0-1. Positive Control Values**

| Lab | $IC_{50}$ | CV |
|---|---|---|
| **A** | 57.9 ± 5.9 | 17.7% |
| **B** | 47.3 ± 2.6 | 9.6% |
| **C** | 81.1 ± 5.5 | 13.4% |
| **Overall task mean $IC_{50}$ ± SEM $IC_{50}$** | 62.1 ± 10.0 | |
| **Overall CV (%)** | | 27.8% |

The results for the slope estimates were consistent among the three laboratories. The estimated variance among the laboratories was zero or near zero. The coefficients of variation for the slope among laboratories were less than 4.0 percent. The results of the positive controls in the various laboratories compared favorably as indicated in Table 1.0-2.

**Table 1.0-2. 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 |

Following successful completion of the positive control studies, four chemicals were tested with the RTI supplied microsomes. The $IC_{50}$ values measured for the four reference chemicals (econazole, aminoglutethimide, chrysin, ketoconazole) in this study compared well with those found in previous studies conducted in the validation program. Laboratories reported significant differences between the beginning and the end portions for all control types when comparing measurements taken at the beginning with those taken at the end of a run. This implies a reduction in aromatase activity between the beginning and the end of a run. This finding suggests that the time required to analyze the samples decreased the enzyme activity and may have implications on the number of samples that can be analyzed at a given time. Virtually identical interlaboratory studies were also carried out with recombinant microsomes with the 10 reference chemicals to enable a comparison of their performance. The effect of increasing concentrations of the reference chemicals on aromatase activity was determined, and the results were expressed as a percent of the control aromatase activity.

Two recommendations flow from this work, which were adopted and incorporated in the validation studies described in chapter 9: (1) The assay should use a standard curve for protein determinations that encompasses the protein concentration of the microsomal dilution rather than extrapolate as was sometimes done here. This may reduce the variability that was seen with some of the laboratory results. (2) A four-parameter data analysis model (top, bottom, slope, and $IC_{50}$ are all variables used to fit the curve) rather than a two-parameter model (top and bottom of the curve are fixed at 100% and 0% respectively and only the slope and $IC_{50}$ are allowed to vary to fit the data) should be used for future analyzes.

Two laboratories were selected to prepare placental microsomes for the interlaboratory validation study, which were shared with the other two laboratories. These preparations were characterized with respect to activity and positive control studies were conducted as above by each of the laboratories.

**INTERLABORATORY VALIDATION**

Chapter 9 provides a summary description of the interlaboratory validation studies conducted by four laboratories (RTI, Battelle, In Vitro, and WIL) for the validation of the placental and recombinant aromatase assays. Individual laboratory reports provide all of the details including the statistical analysis of within run and run to run data. All laboratories followed Good Laboratory Practices (GLPs), standardized requirements for planning, performing, monitoring documenting and recording laboratory experiments), and the same model protocol; however, laboratories were free to use judgment to vary some concentrations when confronted with issues such as insolubility at higher concentrations. These deviations from the model protocol reflect real-world conditions and are noted. Overviews of the placental and recombinant assays are presented, followed by chemical-specific results. Within each chemical, the placental and recombinant results are given, including respective results on $IC_{50}$ and slope determinations. Based on the curve-fit of the percent of control aromatase activity values across the dose response curve for each reference chemical, the calculated $IC_{50}$ values are summarized by run and laboratory. Aminoglutethimide, chrysin, dicofol, econazole, fenarimol, ketoconazole, 4-nonylphenol, and prochloraz inhibited aromatase activity over the concentration ranges tested.

**Table 1.0-3. Average $IC_{50}$ +/- SEM for aromatase inhibitors tested across labs**

| LAB<br>CHEMICAL | RTI<br>Average $IC_{50}$ +/- SEM | BATTELLE<br>Average $IC_{50}$ +/- SEM | IN VITRO<br>Average $IC_{50}$ +/- SEM | WIL<br>Average $IC_{50}$ +/- SEM | Overall task group mean +/- /SEM $IC_{50}$ |
|---|---|---|---|---|---|
| ***Aminoglutethimide*** | | | | | |
| Placental | 4.02 +/-0.15 μM | 4.98 +/- 0.51 μM | 4.09 +/-0.4 μM | 4.11 +/- 0.27 μM | 4.30 +/- 0.23 μM |
| Recombinant | 5.23 +/- 0.45 μM | 5.71 +/- 0.63 μM | 3.89 +/- 0.28 μM | 4.22 +/- 0.85 μM | 4.76 +/- 0.43 μM |
| | | | | | |
| ***Chrysin*** | | | | | |
| Placental | 1.72 +/- 0.13 μM | 2.21 +/- 0.13 μM | 3.02 +/- 0.48 μM | 3.06 +/- 0.33 μM | 2.50 +/- 0.33 μM |
| Recombinant | 2.18 +/- 0.17 μM | 1.48 +/- 0.36 μM | 3.02 +/- 0.46 μM | 3.41 +/- 0.20 μM | 2.52 +/- 0.43 μM |
| | | | | | |
| ***Dicofol*** | | | | | |
| Placental | 62.91 +/- 35.86 μM | 24.14 +/- 3.72 μM | 501 +/-489 μM | 53.13 +/-16.56 μM | 29.13 +/- 8.62 μM |
| Recombinant | 35.77 +/- 0.79 μM | 17.54 +/- 2.13 μM | 6.71 +/- 1.95 μM | 39.96 +/- 6.55 μM | 25.0 +/- 7.80 μM |
| | | | | | |
| ***Econazole*** | | | | | |
| Placental | 1.5 +/- 0.06 nM | 2.43 +/- 0.27 nM | 0.818 +/- 0.127 nM | 1.69 +/- 0.04 nM | 1.61 +/- 0.33 nM |
| Recombinant | 2.47 +/- 0.05 nM | 2.39 +/- 0.34 nM | 1.63 +/- 0.03 nM | 2.38 +/- 0.09 nM | 2.22 +/- 0.20 nM |
| | | | | | |
| ***Fenarimol*** | | | | | |
| Placental | 6.01 +/- 0.31 μM | 6.05 +/- 0.04 μM | 4.25 +/- 0.65 μM | 7.05 +/- 0.40 μM | 5.84 +/- 0.58 μM |
| Recombinant | 6.83 +/- 0.33 μM | 5.42 +/- 0.13 μM | 3.79 +/- 1.01 μM | 6.95 +/- 0.77 μM | 5.63 +/- 0.69 μM |
| | | | | | |
| ***Ketoconazole*** | | | | | |
| Placental | 6.72 +/- 0.11 μM | 8.10 +/- 1.26 μM | 4.58 +/- 1.77 μM | 6.48 +/- 0.15 μM | 6.47 +/- 0.72 μM |
| Recombinant | 9.38 +/- 0.73 μM | 11.07 +/- 3.03 μM | 3.34 +/- 0.67 μM | 6.73 +/- 1.05 μM | 7.63 +/- 1.69 μM |
| | | | | | |
| ***4-Nonylphenol*** | | | | | |
| Placental | 25.37 +/- 0.65 μM | 37.98 +/- 10.57 μM | 26.74 +/- 0.23 μM | 26.31 +/- 2.97 μM | 29.10 +/- 2.97 μM |
| Recombinant | 20.98 +/- 0.77 μM | 20.21 +/- 0.84 μM | 18.7 +/- 2.06 μM | 15.59 +/- 1.33 μM | 18.87 +/- 1.19 μM |
| | | | | | |
| ***Prochloraz*** | | | | | |
| Placental | 20.2 +/- 0.001.8 nM | 026.9 +/- 0.003.1 nM | 40.8 +/- 2.2 nM | 25.8 +/- 1.3 nM | 28.4 +/- 4.4 nM |
| Recombinant | 33.4 +/- 0.7 nM | 32.5 +/- 4.7 nM | 19.8 +/- 0.9 nM | 28.4 +/- 2.1 nM | 28.5 +/- 3.1 nM |

Initial results summarized above clearly indicate that the data were consistent across labs demonstrating that both methods are reliably reproducible across the labs. This was especially evident with 4-nonylphenol, which gave an unusually-shaped curve, but is consistent across the labs.

**DATA ASSESSMENT OF INTERLABORATORY VALIDATION RESULTS**

Performance assessment (Chapter 10) and the interlaboratory analyses of the concentration response curve parameters were performed for only the eight positive chemicals. The two non-inhibitor chemicals, dibenz(a,h)anthracene (DBA) and atrazine (ATZ), were correctly identified as non-inhibitors in all four laboratories, and, as anticipated, the data for these substances did not fit the model. The mixed model analysis of variance comparisons among the laboratories accounted for potential systematic differences between the two sources of microsomes. The estimates for $\log_{10}IC_{50}$ were similar among the four laboratories for most chemicals except for econazole. Also, the estimates for slope were similar among the four laboratories for most chemicals except for econazole. The estimates for the top were similar for the four laboratories. Some laboratories had very large within-laboratory variances relative to the others for some chemicals, however there were no consistent patterns for any laboratory. For the background activity controls, there were no significant differences between the beginnings and the ends of runs. For the full enzyme activity control, the end portion was statistically significantly lower than the beginning portion for each individual laboratory and on average across laboratories.

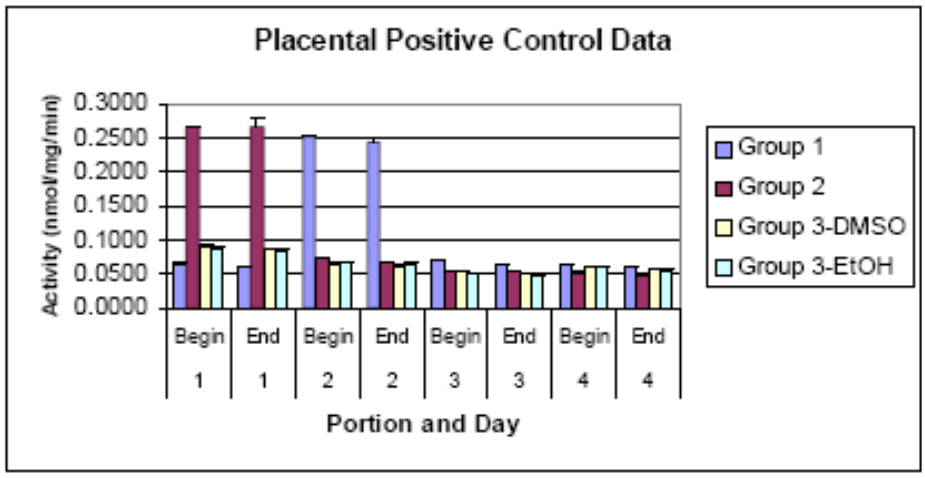


**Figure 1.0-1. Mean Placental Positive Control Activities by Day and Portion**
n = 2 for each bar

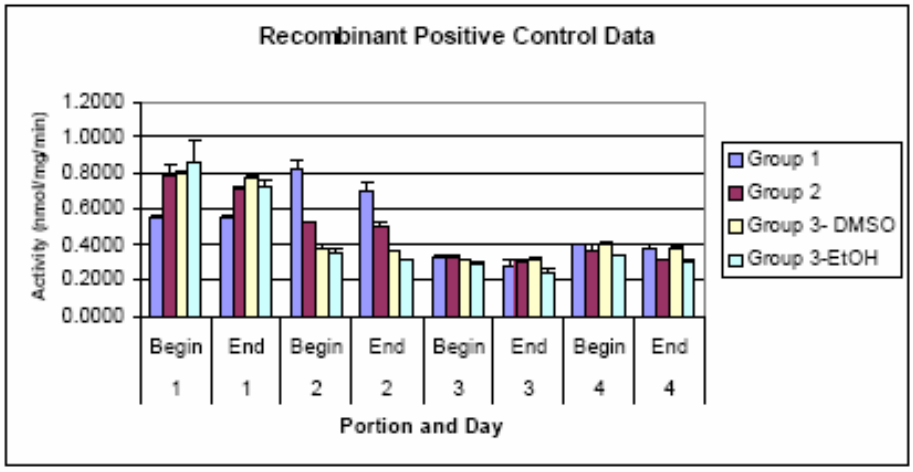


**Figure 1.0-2. Mean Recombinant Positive Control Activities by Day and Portion**
n = 2 for each bar

Nonylphenol was tested in early pre-validation studies in the range of $10^{-9}$ to $10^{-5}$ M. At this concentration range, nonylphenol did not inhibit aromatase. During the later interlaboratory studies, the concentration range was extended to $10^{-3}$ M. At these higher concentrations, nonylphenol appeared to be an inhibitor but exhibited a steeper than normal slope. Because of these unexpected results, EPA decided to investigate whether or not nonylphenol was a true competitive inhibitor or denatured the enzyme at the higher concentrations used in the interlaboratory studies. The investigative approach was to conduct Ki determinations with recombinant aromatase in which the concentration of the substrate is varied in each of a series of tests. The data obtained indicate that nonylphenol acts primarily as a competitive inhibitor of recombinant aromatase, although the relationship between the slopes of the Lineweaver-Burk plot and the inhibitor concentration (shown graphically on the secondary plots) may not be strictly linear. This may be indicative of a small contribution of another inhibition type to the interaction of nonylphenol and aromatase

Overall the EPA felt that both of the aromatase assays were reproducible, giving consistent results for runs within and between labs. The data were comparable to historical data that exist in the literature for similar procedures, with the exception of some data run with different assay systems (e.g., cell lines versus recombinant systems or human placenta). In these cases, the reason for discrepancies was generally justifiable due to differences in the assay systems or methods. Finally EPA felt that the number of chemicals tested interlaboratory represented a reasonable range of chemicals with historical information on what results to expect, especially given the consistency of results and the development (based on these data) of performance criteria to be used to ensure quality data in future aromatase assays.

## PERFORMANCE CRITERIA

The development of performance criteria is one of the key outputs of a validation program. Performance criteria ensure that the assay is being performed correctly and producing reliable results. It thus assures the testing laboratory, the client, and the regulatory authority that the data can be used with confidence. EPA established performance criteria (minimum and maximum aromatase activity respectively) for the full activity control and the background controls and for

the top, bottom, slope, and log $IC_{50}$ for the positive control, 4-hydroxyandrostenedione (see Tables 1.0.4 and 1.0.5 below).

**Table 1.0-4. Full Activity and Background Control Criteria***

| | Parameter | Value |
|---|---|---|
| **Full Activity Control** | **Minimum Activity** | **0.100 nmol/mg/min** |
| **Background Control** | **Maximum Activity** | **1% of full activity control** |

*The full activity control for the placental assay is 0.03 nmol/mg/min

**Table 1.0-5. Performance Criteria (Tolerance Intervals) for the Positive Control**

| | Parameter | Lower limit | Upper Limit |
|---|---|---|---|
| **Positive Control** | **Slope** | **-1.2** | **-0.8** |
| | **Top (%)** | **90** | **110** |
| | **Bottom (%)** | **-5** | **+6** |
| | **Log $IC_{50}$** | **-7.3** | **-7.0** |

**DATA INTERPRETATION**

EPA adopted a data interpretation procedure to classify test compounds as inhibitors or non-inhibitors, based on whether an inhibition curve was generated or not. The procedure requires that the mean of the activity level at the highest concentration be used to determine whether a chemical is classified as an inhibitor, a non-inhibitor, or equivocal. If there is less than 50% activity, the chemical is classified as an inhibitor; between 50 and 75% activity, it is equivocal; and if the activity level is greater than 75%, the chemical is classified as a non-inhibitor.

**TESTING THE METHODS—SUPPLEMENTARY TESTING**

A study of an additional, more diverse set of 16 chemicals was then conducted in the lead laboratory to test the protocol (Chapter 12).In the study of these additional 16 chemicals, six compounds are clearly inhibitors. These six compounds were bisphenol A, flavone, triadimefon, imazalil, apigenin, and nitrofen. In no case was the most inhibited level between 75 percent and 50 percent of control, which would lead to the classification of equivocal. Five of the six inhibitors exhibited maximal inhibition of less than 20 percent of control; the other inhibitor (nitrofen) had a maximal inhibition of about 32 percent of control. These are all well within the 'inhibitor' classification range. In all cases, the 95 percent confidence bands were well below 50 percent of control at the highest concentration. Ten chemicals are clearly noninhibitors, and no concentration-dependent response was observed for any of the noninhibitors These are Vinclozolin, Bisphenol A, Tributyltin, Diethylhexyl phthalate, Methoxychlor, Aldicarb, Flavone, Triadimefon, Imazalil, Apigenin, Ronidazole, Ronidazole, Genestein, p,p'-DDE, Alachlor, Nitrofen, and Trifluralin.

In these additional studies as with the validation studies, the dose-response curves in the positive control (4-OH ASDN) assay were generally very narrow, indicating a good curve fit and low variance across all concentrations. Overall, the control data show that, in most cases, even the bounds of the 95 percent confidence intervals fall within the performance criteria ranges, indicating a high degree of certainty that the correct estimate falls within that range and that the performance criteria ranges are appropriate. For each reference chemical, the aromatase activity

found in each assay tube was normalized to percent of control by dividing by the average full enzyme activity for the run. The percent of control values for each reference chemical run and tube, along with the mean, SD, SEM, and CV of the percent of control across tubes within a run. Generally, there was less than 10 percent coefficient of variation from tube-to-tube within a run. For some high reference chemical concentrations, where the percent of control values were near zero, the CV values were higher, in part due to increased variance in the measurement of disintegrations per minute (DPM) at these near background levels. Run-to-run reproducibility was good, with most CV values less than 10 percent. Again, some higher CVs were observed when the percent of control was near zero.

**FINAL CONCLUSIONS**

Data interpretation criteria will be used to decide upon the proper classification of a test chemical as either an inhibitor, a noninhibitor, or as a chemical for which the data are equivocal. Proposed performance criteria for the four model fit parameters (Top, Bottom, LogIC50, and HillSlope) for the positive control assay were met in 39 of 48 assays, and there was some indication that failures in four of the assays were due to a solution preparation problem. Most of the failures involved only one of the four parameters, and the values were usually just outside the criteria ranges. Data and curve fits for the assays that had performance criteria failures were examined and did not indicate any serious flaw in the functioning of the aromatase assay. EPA concludes that both versions of this assay were successfully validated and their equivalence demonstrated.

# 2.0 Introduction

## 2.1 The Endocrine Disruptor Screening Program

Section 408(p) of the Federal Food Drug and Cosmetic Act (FFDCA 1996) requires EPA to:

> *develop a screening program, using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen, or other such endocrine effect as the Administrator may designate [21 U.S.C. 346a(p)].*

Subsequent to passage of the Act, EPA formed the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), a committee of scientists and stakeholders that EPA charged to provide it with recommendations on how to implement its Endocrine Disruptor Screening Program (EDSP). Upon recommendations from EDSTAC, the EDSP was expanded using the Administrator's discretionary authority to include the androgen and thyroid hormone systems and wildlife effects.

## 2.2 Definition of an Environmental Endocrine Disruptor

An EPA Risk Assessment Forum was established to promote scientific consensus on risk assessment issues and to ensure that this consensus is incorporated into appropriate risk assessment guidance. The Forum released a report that addressed the hypothesis that certain chemicals may disrupt the endocrine system (EPA 1997). In the report, an environmental endocrine disruptor was defined as:

> *an exogenous agent that interferes with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior.*

## 2.3 Tiered Approach

EPA accepted the EDSTAC's recommendations for a two-tier screening program (EPA 1998). The purpose of Tier I is to identify the potential of chemicals to interact with the estrogen, androgen, or thyroid hormone systems. A negative result in Tier I would be sufficient to put a chemical aside as having low to no potential to cause endocrine disruption. Chemicals testing positive in Tier I would be further evaluated in Tier II. The purpose of Tier II is to identify and characterize the adverse effects resulting from that interaction and the exposures required to produce them. Tier II is comprised of multigeneration tests in species representative of the following taxa: mammals, birds, fish, amphibians, and invertebrates.

## 2.4 The Tier I Battery

The EDSTAC concluded that Tier I should comprise a battery of complementary assays having the following characteristics:

- The Tier I screening battery should maximize sensitivity to minimize false negatives while permitting an as yet undetermined, but acceptable, level of false positives.
- The Tier I battery should include a range of organisms representing known or anticipated differences in metabolic activity. The battery should include assays from representative vertebrate classes to reduce the likelihood that important pathways for metabolic activation or detoxification of parent substances or mixtures are overlooked.
- The Tier I battery should be designed to detect all known modes of action for the endocrine endpoints of concern. All chemicals known to affect the action of estrogen, androgen, and thyroid hormones should be detected.
- The Tier I battery should include a sufficient range of taxonomic groups among the test organisms. There are known differences in endogenous ligands, receptors, and response elements among taxa that may affect endocrine activity of chemical substances or mixtures.
- The Tier I battery should incorporate sufficient diversity among the endpoints and assays to reach conclusions based on "weight-of evidence" considerations. Decisions based on the battery results will require weighing the data from several assays.

To detect chemicals that may affect the estrogen, androgen, and thyroid hormone systems through any one of the known modes of action—interruption of hormone production or metabolism, binding of the hormone with its receptor, interference with hormone transport, etc.—EDSTAC recommended a series of *in vitro* and *in vivo* assays for inclusion in Tier I screening battery, as shown in Table 2.4-1.

In addition, EDSTAC recognized other combinations of assays that might substitute for some components of the recommended battery and also recommended that EPA validate the assays listed in Table 2.4-2 as alternatives.

The EDSP is described in detail on the following website: http://www.epa.gov/scipoly/oscpendo/

**Table 2.4-1. Assays recommended for consideration for the Tier I screening battery**

| Assay | Nature of Assay and Reasons for Inclusion |
|---|---|
| Estrogen receptor (ER) binding or transcriptional activation assay | A sensitive *in vitro* test to detect chemicals that may affect the endocrine system by binding to the estrogen receptor. |
| Androgen receptor (AR) binding or transcriptional activation assay | A sensitive *in vitro* test to detect chemicals that may affect the endocrine system by binding to the androgen receptor. |
| *In vitro* steroidogenesis assay | A sensitive *in vitro* test to detect chemicals that interfere with the synthesis of the sex steroid hormones |
| Uterotrophic Assay | An *in vivo* assay to detect estrogenic chemicals by measuring a chemical's effect on uterine weight. (It can also be run as an assay to detect antiestrogens.) It offers the advantage over the binding assay of incorporating absorption, distribution, metabolism, and excretion (ADME). |
| Hershberger Assay | An *in vivo* assay to detect androgenic and antiandrogenic chemicals by measuring a chemical's effect on the weight of five tissues that require androgen for growth. It offers the advantage over the binding assay of incorporating ADME and differentiating between AR agonists and antagonists. |
| Pubertal female assay | An assay in which female weanling rats are dosed through puberty. The assay detects chemicals that act on estrogen or through the hypothalamic-pituitary-gonadal (HPG) axis that controls the estrogen and androgen hormone systems. It is also enhanced to detect chemicals that interfere with the thyroid system. |
| Frog metamorphosis assay | An assay in which frogs are dosed in the early stages of metamorphosis. Because metamorphosis is a thyroid dependent process, this assay is a sensitive assay for detection of chemicals that interfere with the thyroid hormone system. |
| Fish screening assay | Fish are the furthest removed from mammalians among vertebrates both from the standpoint of evolution—their receptors and metabolism are different from mammals—and exposure/habitat, since they would be subject to exposure through the gills, whole body, and diet. Thus, the fish assay would augment information found in the mammalian assays and would be more relevant than the mammalian assays in triggering concerns for fish. |

**Table 2.4-2. Alternative Assays for Tier I**

| Assay | Nature of Assay and Reasons for Inclusion |
|---|---|
| Placental Aromatase Assay | The aromatase assay detects chemicals that inhibit aromatase, the enzyme that metabolizes androgens such as testosterone to estrogens. It would be needed if either of the two following assays using males were substituted for the female pubertal assays. This is because the male is not believed to be as sensitive to alterations in aromatase as the female and would not therefore be sufficient to detect interference with aromatase in the screening battery. |
| Pubertal Male | An assay in which male weanling rats are dosed through puberty. The assay detects chemicals that act on androgen or through the HPG axis that controls the estrogen and androgen hormone systems. It is also enhanced to detect chemicals that interfere with the thyroid system. This assay could in part substitute for the female pubertal assay. |
| Adult Male | An assay in which adult male rats are dosed for 15 days. The assay is also designed to detect chemicals that act on androgen or through the HPG axis that controls the estrogen and androgen hormone systems. It is also enhanced to detect chemicals that interfere with the thyroid system. This assay could in part substitute for the female pubertal assay. |

## 2.5 Validation

As noted in Section 2.1 above, Section 408(p) of the FFDCA requires EPA to use validated test systems. Validation has been defined as "the process by which the reliability and relevance of a test method are evaluated for a particular use" (OECD 1996; NIEHS 1997).

- *Reliability* is defined as the reproducibility of results from an assay within and between laboratories.

- *Relevance* describes whether a test is meaningful and useful for a particular purpose (OECD 1996). For Tier I EDSP assays, relevance can be defined as the ability of an assay to detect chemicals with the potential to interact with the endocrine system.

Federal agencies are also instructed by the Interagency Coordinating Committee for the Validation of Alternative Methods Authorization Act (ICCVAM 2000) to ensure that new and revised test methods are valid prior to their use.

In general, EPA is following the five-part or stage validation process outlined by the ICCVAM (NIEHS 1997). EPA believes that it is essential to recognize that this process was specifically developed for *in vitro* assays that were intended to replace *in vivo* assays. The fundamental problem confronting the EPA is how to adapt and work with this process for a far wider range of rodent and ecological *in vivo* assays ranging from simple, lower tier screens to higher tier, multigenerational reproductive and developmental tests for Tier II.

The first stage of the process outlined by ICCVAM is *test development*, an applied research function culminating in an initial protocol. As part of this phase, EPA prepares a Detailed Review Paper (DRP) to explain the purpose of the assay, the context in which it will be used, and the scientific basis upon which the assay's protocol, endpoints, and relevance rest. The DRP reviews the scientific literature for candidate protocols and evaluates them with respect to a number of considerations, such as whether the candidate protocols meet the assay's intended purpose, the costs, and other practical considerations. The DRP also identifies the developmental status and questions related to each protocol; defines the information needed to answer the questions; and, when possible, recommends an initial protocol for the initiation of the second stage of validation, *standardization and optimization,* in which the protocol is refined, optimized, standardized, and initially assessed for transferability and performance. Several different types of studies are conducted during this second phase, depending upon the state of development of the method and the nature of the questions that the protocol raises. The initial assessment of transferability is generally a trial in a second laboratory to determine that another laboratory besides the lead laboratory can follow the protocol and execute the study. In the third phase, *interlaboratory validation* studies are conducted in independent laboratories with the optimized protocol. The results of these studies are used to determine interlaboratory variability and to set or cross-check performance criteria. Interlaboratory validation is followed by *peer review*, an independent scientific review by qualified experts, and by *regulatory acceptance*, adoption for regulatory use by an agency. EPA has developed extensive guidance on the conduct of peer reviews because the Agency believes that peer review is an important step in ensuring the quality of science that underlies its regulatory decisions (EPA, 2006).

Criteria for the validation of alternative test methods *(in vitro* methods designed to replace animal tests in whole or in part) have generally been agreed upon in the U.S. by ICCVAM, in Europe by the European Centre for the Validation of Alternative Methods (ECVAM), and internationally by the Organisation for Economic Co-Operation and Development (OECD). These criteria, as stated by ICCVAM (NIEHS 1997), are as follows:

1. The scientific and regulatory rationale for the test method, including a clear statement of its proposed use, should be available.
2. The relationship of the endpoints determined by the test method to the *in vivo* biologic effect and toxicity of interest must be addressed.
3. A formal detailed protocol must be provided and must be available in the public domain. It should be sufficiently detailed to enable the user to adhere to it and should include data analysis and decision criteria.
4. Within-test, intralaboratory and interlaboratory variability and how these parameters vary with time should have been evaluated.
5. The test method's performance must have been demonstrated using a series of reference chemicals preferably coded to exclude bias.
6. Sufficient data should be provided to permit a comparison of the performance of a proposed substitute test to that of the test it is designed to replace.
7. The limitations of the test method must be described (e.g., metabolic capability).
8. The data should be obtained in accordance with Good Laboratory Practices (GLPs).
9. All data supporting the assessment of the validity of the test methods including the full data set collected during the validation studies must be publicly available and, preferably, published in an independent, peer-reviewed publication.

For technical guidance in developing and validating the various Tier I screens and Tier II tests, EPA chartered two federal advisory committees: the Endocrine Disruptor Methods Validation Subcommittee (from 2001 to 2003) and the Endocrine Disruptor Methods Validation Advisory Committee (from 2003 to 2006). These committees, composed of scientists from government, academia, industry, and various interest groups, were charged to provide expert advice to the EPA on protocol development and validation. EPA also cooperates with member countries of the OECD to develop and validate assays of mutual interest to screen and test for endocrine effects.

Even though assays are being validated and peer reviewed individually (i.e., their strengths and limitations are being evaluated as stand-alone assays), the Tier I assays will, in fact, be used in a complementary battery of screens. An individual assay may serve to strengthen the weight of evidence in a determination (e.g., positive results in an ER binding assay in conjunction with positive results in the uterotrophic and pubertal female assays would provide a consistent signal for estrogenicity) or to provide coverage of a mode of action not addressed by other assays in the battery. Peer review of the information supporting the validation of an individual assay will be followed at a later date by a review by the FIFRA Scientific Advisory Panel (SAP) of EPA's recommendations for the Tier I battery. The battery peer review will focus on the issue of coverage of the known modes of endocrine disruption and how well the assays work in concert.

Although attempts have been made to thoroughly comply with all validation criteria, these assays are not replacement assays; consequently, large data bases do not exist as a reference to establish their predictive capacity (e.g., determination of false positive and false negative rates). Review of results from the testing of the first group of 50 to 100 chemicals that was recommended by the SAP (EPA, 1999) is expected to allow a more thorough assessment of the performance of the Tier I screening battery.

### 2.6 Purpose and Organization of the Integrated Summary Report

The purpose of this Integrated Summary Report is to summarize in a single coherent document all of the information from the scientific literature and EPA's validation program that supports the conclusion that the microsomal aromatase assays are valid for the purpose of identifying chemicals that have the potential to inhibit aromatase. The documents supporting the validation of aromatase track the various stages of the validation process. They are listed in the reference section of this document. In addition, for each of the multiple-laboratory studies, separate laboratory reports exist for the work performed at RTI, Battelle, WIL, and In Vitro.

Subsequent chapters will describe the scientific basis of the aromatase assay (Chapter 3); the test method (Chapter 4); how data are modeled and analyzed (Chapter 5); the chemicals used to test the protocol (Chapter 6); development and optimization of the assay (Chapter 7); preliminary interlaboratory studies with placental microsomes prepared in the lead laboratory (Chapter 8); interlaboratory studies with ten reference chemicals in placental and recombinant microsomes (Chapter 9); assessment of the interlaboratory validation studies (Chapter 10); performance criteria and data interpretation procedure (Chapter 11); the study of an additional, more diverse set of 16 chemicals to test the protocol (Chapter 12); general conclusions and compliance with validation criteria (Chapter 13). Reference lists and appendices with protocols and supporting data follow the report.

Sources for this integrated summary report are listed in two groups: reports supporting the validation of the aromatase assay (listed numerically in Section 14.1), and other references (listed alphabetically in Section 14.2). For convenience in tracking these sources, the validation reports are cited in text using numbers, for example, (Reference 1). The other sources are cited in text using parenthetical author-date citations, for example, (Lombardi 2001).

# 3.0 Scientific Basis of the Aromatase Assay

## 3.1 Endocrinology of Estrogens

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, primarily in the ovary in mature, premenopausal women (Strauss and Hseuh 2001) 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.

The main biochemical mechanism of estrogen action is the regulation of gene expression and subsequent induction of protein biosynthesis via specific, high-affinity estrogen receptors, (Weigel and Rowan, 2001) as illustrated in Figure 3.1-1. These receptors are soluble intracellular proteins that can both bind steroid ligands with high affinity and act as ligand-dependent transcriptional factors via interaction with specific deoxyribonucleic acid (DNA) sites and other proteins associated with the chromatin. Receptor binding sites for estradiol are located in the nucleus of target cells and exhibit both high affinity ($K_D = 10^{-11}$ to $10^{-10}$ M) and high specificity.

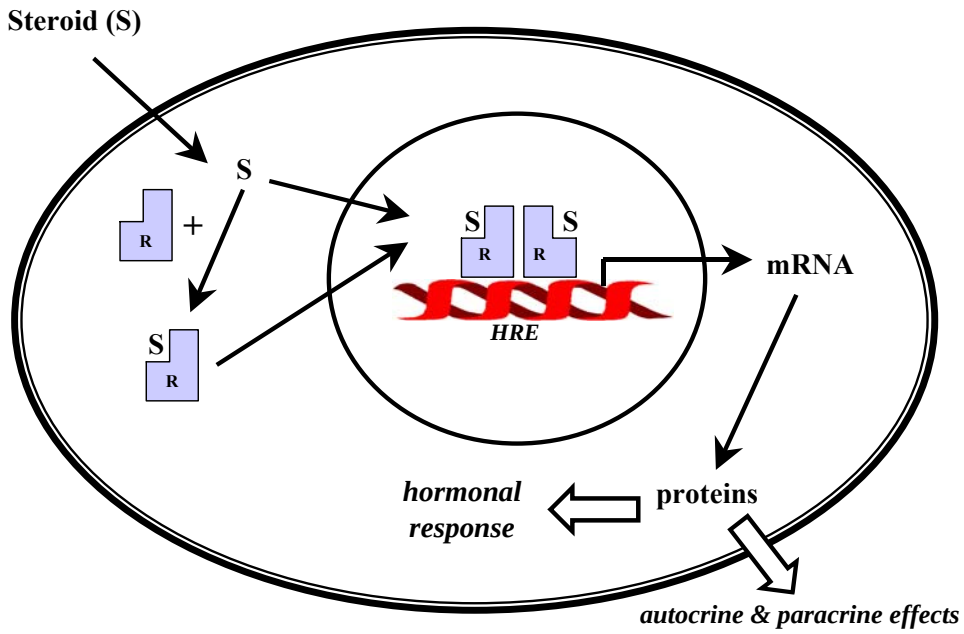


**Figure 3.1-1. General Mechanism of Steroid Hormone Action**

Estrogens act on many tissues in the female, such as those of the reproductive tract, breast, and CNS. In the female, the primary physiological action of estrogens is to stimulate the development of secondary sex tissues. The growth and development of tissues in the

reproductive tract of animals, in terms of actual weight gained, are not seen for as long as 16 hours after administration of the estrogen, although some biochemical processes in the cell are affected immediately. The growth response produced in the uterus by estrogens is temporary, and the maintenance of such growth requires the hormone to be available almost continuously. The initial growth induced by the estrogen is therefore of limited duration, and atrophy of the uterus occurs if the hormone is withdrawn. Another physiologic effect of estrogens, observed 1 hour after their administration, is edema in the uterus. During this period, vasodilation of the uterine pre- and postcapillary arterioles occurs, and there is an increase in permeability to plasma proteins. These effects appear to occur predominantly in the endometrium and not to any great extent in the myometrium. Another target of estrogens is breast tissue. Estrogens can stimulate the proliferation of breast cells and promote the growth of hormone-dependent mammary carcinoma. Because the breast is the primary site for cancer in women, considerable research has been focused on understanding breast cancer and the factors that influence its growth. Estradiol will stimulate gene expression and the production of several proteins in breast cancer cells via the estrogen receptor mechanism. These proteins include both intracellular proteins important for breast cell function and growth, and secreted proteins that can influence tumor growth and metastasis.

Estrogens are also important for male sexual development and reproductive function (Simpson et al. 2000; Lombardi et al. 2001). Estrogens, produced locally by aromatase in the Sertoli cells, are important for spermatogenesis in rats, and aromatase has been detected in human testis (Brodie et al. 2001). Estrogen receptors are present in male reproductive tissues such as Sertoli cells, Leydig cells, epididymis, and accessory sex organs (Simpson et al. 2000). During gestational development of the rodent, the local aromatization of testosterone to estrogen within the brain plays a key role in the structural organization of the hypothalamus, which can effect sexual differentiation the male brain (Lephart 1996, 1997). Although there is currently no definitive evidence to demonstrate analogous effects of estrogen on masculinization of the human brain during gestational development, it is likely that estrogen and androgens play a critical role in producing the sexual dimophism of the primate (Cooke et al. 1998).

### 3.2 The Nature and Role of Aromatase

Estradiol, the most potent endogenous estrogen in humans, is biosynthesized from androgens by the cytochrome P450 enzyme complex called aromatase. This enzyme was first reported in human placental tissues by Ryan (1959). In humans, aromatase is present in the ovaries of premenopausal women, in the placenta of pregnant women, and in the peripheral adipose tissues of women and of men, and these tissues are the major sources for estrogens circulating in the blood stream (Simpson et al. 1989). Aromatase is also important for the local tissue production of estrogens. Aromatase activity has been demonstrated in breast tissue *in vitro* (James et al. 1987; Miller and O'Neill 1987; Reed et al. 1989; Reed 1994). Furthermore, expression of aromatase is highest in or near breast tumor sites (Miller and O'Neill 1987; Bulun et al. 1993); various regions in the brains of both men and women contain the enzyme aromatase (Lephart 1996; Naftolin 1994), as do the male testis, and bone in men and women (Simpson et al. 1999, 1997a).

Aromatase is the enzyme complex responsible for the conversion of androgens to estrogens during steroidogenesis (Simpson et al. 1994). The enzyme complex is bound in the endoplasmic reticulum of the cell and is comprised of two major proteins (Simpson et al. 1994; Kellis and Vickery 1987). One protein is cytochrome $P450_{arom}$, a hemoprotein that converts $C_{19}$ steroids (androgens) into $C_{18}$ steroids (estrogens) containing a phenolic A ring. The second protein is NADPH-cytochrome P450 reductase, which transfers reducing equivalents to cytochrome $P450_{arom}$. Three moles of NADPH and three moles of oxygen are utilized in the conversion of one mole of androgen substrate into one mole of estrogen product (Figure 3.2-1). Aromatization of androstenedione, the preferred substrate, proceeds via three successive oxidation steps, with the first two being hydroxylations of the angular C-19 methyl group. The final oxidation step, whose mechanism remains for complete elucidation, proceeds with the aromatization of the A ring and loss of the C-19 carbon atom as formic acid.

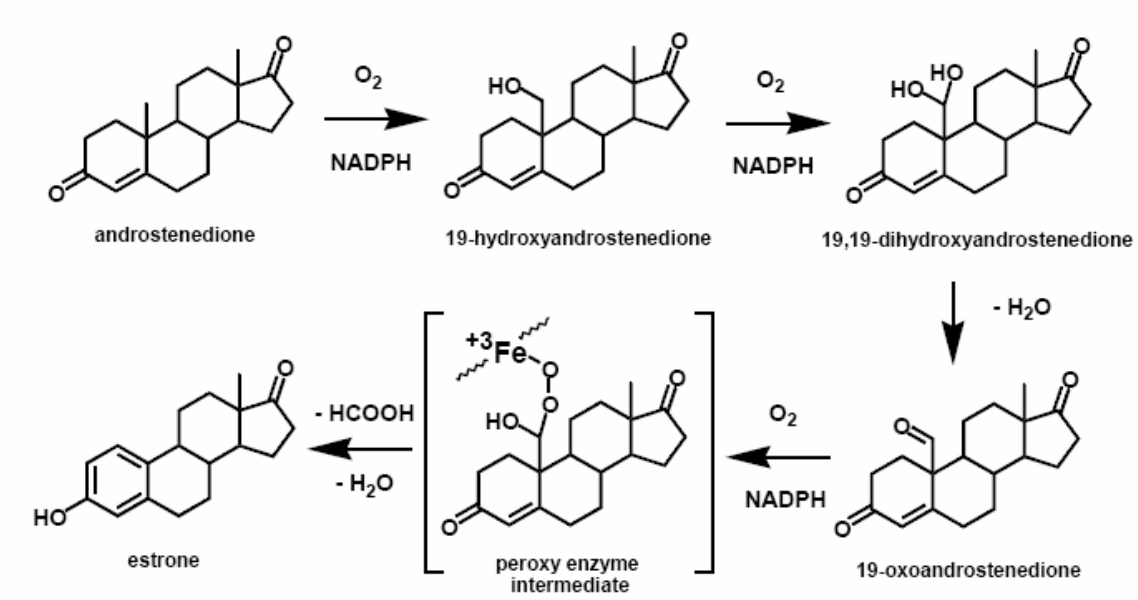


**Figure 3.2-1. The aromatase pathway**

Over the past two decades, knowledge of the biochemistry, molecular biology, and regulation of aromatase has increased greatly.

Effective aromatase inhibitors have been developed as therapeutic agents for estrogen-dependent breast cancer to reduce the growth stimulatory effects of estrogens in breast cancer. Investigations on the development of aromatase inhibitors began in the 1970s and have expanded greatly in the past three decades. Research summaries of aromatase inhibitors have been presented at international aromatase conferences (Simpson 2000; Harvey et al, 1982; Santen 1987; Brodie et al. 1993; Simpson et al. 1997b; Simpson and Pasqualini 2001; Sasano et al. 2003; Miller and Pasqualini 2005) and several reviews have also been published (Johnston and Metcalf 1984; Banting et al. 1988, 1989; Covey 1988; Bruggemeier 1990, 1994; Cole and Robinson 1990; Brodie and Njar 1996; Brodie et al. 1999; Santen and Harvey, 1999; Brueggemeier et al., 2005).

In addition to the extensive investigations on the importance of human aromatase in normal physiology and in cancer, this enzyme complex has also been well studied in other mammalian species (rodents, cows, pigs, horses) and in nonmammalian vertebrates as well. In general, these investigations have focused on identifying the presence of aromatase in various tissues, comparative endocrinology of aromatase, and examination of the importance of aromatase in the physiology of various species.

Alterations in the amount of aromatase present or in the catalytic activity of the enzyme will alter the levels of estrogens in tissues and dramatically disrupt estrogen hormone action. Inhibition of aromatase alters the catalytic activity of the enzyme and results in a rapid decrease in the levels of estrogens. This mechanism of enzyme inhibition is the reason for the therapeutic effectiveness of aromatase inhibitors in the treatment of estrogen-dependent breast cancer and illustrates the importance of estrogen levels to estrogen action. Suppression or induction of the aromatase protein levels also can dramatically influence the subsequent levels of estrogens in tissues and affect hormone action. Environmental agents, toxicants, and various natural products can act via aromatase inhibition and/or alteration in aromatase protein levels to result in altered levels of estrogen and function as endocrine disruptors.

Aromatase deficiencies in humans and in aromatase knockout (ArKO) mice dramatically illustrate the importance of aromatase (Simpson et al. 2002). In human females with aromatase deficiency (currently a total of eight patients worldwide), newborns have psuedohermaphroditism and, at puberty, these patients have primary amenorrhea, lack of breast development, hypogonadism, and cystic ovaries. Two males with aromatase deficiency have been reported, and these individuals exhibit tall stature due to failure of epiphyseal fusion, osteopenia, and infertility. Female ArKO mice exhibit infertility and underdeveloped uteri and mammary glands, and male ArKO mice have impaired spermatogenesis and enlarged prostates (Fisher et al. 1998; Robertson et al. 1999). Both male and female ArKO mice have excessive intra-abdominal fat and elevated serum lipid levels (Jones et al. 2000), and both have excessive long bone growth and osteopenia (Oz et al. 2000).

Additional information regarding the significance of aromatase and the effects of aromatase overexpression is obtained from studies of transgenic mice. Tekmal and colleagues generated transgenic mice that overexpress int-5/aromatase under the control of mouse mammary tumor virus enhancer/promoter (Tekmal et al. 1996; Kirma et al., 2001). In this transgenic mouse model, overexpression of aromatase is observed in mammary glands, and this overexpression of the enzyme results in biosynthesis of estrogen locally in the tissues (*in situ* production). The early and continued exposure of mammary epithelial cells to *in situ* estrogen production results in the enlargement of ducts, with hyperplastic, dysplastic, or fibroadenoma lesions.

### 3.3 Selection of the Placental and Recombinant Microsomal Assays

The Detailed Review Paper for Aromatase (Reference 1) covers a variety of *in vitro* and *in vivo* methods. EPA selected microsomal aromatase assays for validation over cell-based assays due to the Agency's assessment that cell-based assays, being a much younger technology, would need more development prior to validation.

The human placental microsomal aromatase assay is one of the most common *in vitro* assays used for measuring aromatase and aromatase inhibition because of its reliability, reproducibility, and ease of use. The overall protocol is within the ability of individuals with undergraduate laboratory experience in biology, biochemistry, or molecular biology. This assay method has been utilized routinely for the determination of the presence of aromatase in tissues since the first report of aromatase by Ryan (1959). The source of the aromatase is a microsomal preparation isolated from human term placenta, a rich and inexpensive source of the enzyme. This microsomal preparation consists of the endoplasmic reticulum membrane of the cell and contains the membrane-bound cytochrome $P450_{arom}$ and the NADPH cytochrome P450 reductase. Complete enzyme activity requires the addition of either NADPH or an NADPH-generating system, and the activity can be measured using either the product isolation method or the radiometric method.

The procedure selected by EPA involves use of liquid scintillation counting, which provides excellent efficiency (50 percent) for the detection of $^3H$ radioisotope used as the endpoint in this assay. Since $^3H$ is produced by the aromatization of the radiolabeled substrate, the measurement of the endpoint directly correlates with the amount of aromatase activity present. The assay is sensitive: it can detect 0.20 pmol of estrogen formed per mg microsomal protein per minute. The only false positive for aromatase inhibition is the decrease in enzyme activity as a result of enzyme degradation. Consequently, the human placental microsomal aromatase assay has been used extensively in academic labs and pharmaceutical firms as the initial biological evaluation for potential steroidal and nonsteroidal aromatase inhibitors.

As an alternative to using microsomes harvested from placenta, CYP19 has been obtained using a variety of expression systems, including insect cells (Hi5 and Sf9), mammalian cells, yeast and *E. coli* (Sigle et al. 1994). The highest expression levels have been achieved in insect cells as compared to the other expression systems (Lahde et al. 1993; Sigle et al. 1994; Chen et al. 1993). Kinetic analysis of the conversion of testosterone to estradiol by reconstituted aromatase has been reported to have K*m* values in the range of 100 to 200 nM, while K*m* values in tissue microsomes were lower, in the 10 to 60 nM range (Kellis and Vickery 1987; Chen et al. 1993; Sethumadhaven and Bellino 1991).

Both the human placental microsomal assay and the recombinant assay were carried through the validation stages to demonstrate their equivalence, although the convenience and safety of the recombinant microsomal assay will undoubtedly make it the assay of choice.

### 3.4 Limitations of the Microsomal Assays

In selecting the microsomal assays, EPA was aware of certain limitations to the assays. Unlike cell-based assays such as the JEG, KGN, or H295R assays, the microsomal assays cannot detect chemicals that induce aromatase because aromatase is already preformed in the microsome rather than synthesized *in situ* during exposure to the chemical. Since the signal is the amount of one of the reaction products produced, denaturation of the enzyme, which would slow or curtail the formation of reaction products, would be expected to give false positive results. In addition, the simple assay, as conducted, cannot distinguish between competitive, non-competitive, and un-competitive inhibition. A more complex method, in which the concentration of enzyme as well

as substrate is varied, can be used to make this distinction. (See Section 10.6 on the investigation of nonylphenol.) Furthermore, because the microsome does not have a full complement of metabolizing enzymes, the assay has limited ability to metabolize xenobiotics.

# 4.0 Description of the Test Method

The following is a general description of the test method; detailed protocols of the placental and recombinant assays are provided in Appendix A. The two procedures are similar, except that the activity of the protein is higher in the recombinant assay system. Other differences between the two test systems are noted below.

## 4.1 Substrate – Androstenedione (ASDN)

The substrate for the assays is androstenedione (ASDN), which is converted by aromatase to estrone. The progress of the reaction can be followed by measuring the formation of either of the two reaction products. Formation of estrone can be measured directly as the indicator of aromatase activity. The formation of $^{3}H_2O$ can be measured by liquid scintillation counter if the radiolabeled substrate, [1β-$^{3}$H]-androstenedione, is used. In these assays, tritiated water formation is measured and a mixture of non-radiolabeled and radiolabeled ASDN is used as the substrate. The non-radiolabeled ASDN used in these assays had a reported purity of 100 percent. The radiolabeled ASDN had a reported specific activity of 25.3 Ci/mmol. Radiochemical purity was reported by the supplier to be > 97 percent. 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.

## 4.2 Positive Control – 4-Hydroxyandrostenedione (4-OH ASDN)

4-Hydroxyandrostenedione is the positive control. Originally thought to be a competitive inhibitor with a Ki of approximately 170 nM, it is now known as a mechanistic or suicide inhibitor and binds irreversibly to aromatase causing enzyme inactivation. A mechanism-based inhibitor mimics the substrate, is converted by the enzyme to a reactive intermediate, which binds irreversibly to—and results in the inactivation of—the enzyme. Such inhibitors are recognized by the enzyme as substrate during the normal catalytic process and produce a time-dependent inactivation of the enzyme (Brueggemeier et al. 2005). 4-Hydroxyandrostenedione is a second-generation breast cancer therapy and has been used to treat advanced cancer after treatments with tamoxifen fail to arrest tumor growth. Table 4.2-1 summarizes the salient information for 4-OH ASDN.

**Table 4.2-1. 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 is prepared as a 0.01 M solution in 95 percent ethanol and is diluted using 95 percent ethanol to make the needed concentrations of positive control according to the procedure described in Table 4.2-2.

**Table 4.2-2. Preparation of 4-OH ASDN dilutions for the positive control study**

| 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 | 10 | 100 | 900 | A | 1.0 | N/A |
| Working Stock #1 | 1.0 | 100 | 900 | 1 | 0.1 | $1 \times 10^{-6}$ |
| Working Stock #2 | 0.1 | 100 | 900 | 2 | 0.01 | $1 \times 10^{-7}$ |
| | | 50 | 950 | 3 | 0.005 | $5 \times 10^{-8}$ |
| | | 25 | 975 | 4 | 0.0025 | $2.5 \times 10^{-8}$ |
| Working Stock #3 | 0.01 | 100 | 900 | 5 | 0.001 | $1 \times 10^{-8}$ |
| Working Stock #4 | 0.001 | 100 | 900 | 6 | 0.0001 | $1 \times 10^{-9}$ |

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## 4.3 Preparation of Microsomes

### 4.3.1 Human Placental Microsomes

Placenta are obtained from a local hospital and kept on ice during dissection to keep the tissue chilled. The membrane and fibrous material is dissected, removed, and discarded. The spongy tissue is cut into small pieces, placed in a beaker containing ice-cold Buffer A (0.25 M sucrose; 0.04 M nicotinamide, 0.05 M sodium phosphate, pH 7.0), and homogenized. The homogenate is transferred to centrifuge tubes and centrifuged at the setting of 10,000 g for 30 minutes at 4°C. The supernatant is transferred to ultracentrifuge tubes and centrifuged at 100,000 g for 1 hour at 4°C to obtain a crude microsomal pellet. The supernatant is decanted and the microsomal pellet dislodged with a few mL of Buffer B (0.1 M sodium phosphate, pH 7.4). The clear pellet on the bottom is left in the tube and disposed of. The microsomal pellet is poured into Potter-Elvejhem homogenizer and resuspended in Buffer B. The suspension is transferred to ultracentrifuge tubes and is centrifuged at 100,000 g for 1 hour at 4°C to wash the microsomes. This washing procedure (supernatant decanting, pellet resuspension, and centrifugation) is repeated one additional time. The supernatant is decanted and the twice-washed microsomal pellet is dislodged from the bottom wall of the tube by gentle swirling in a few mL of ice cold Buffer C (0.1 M sodium phosphate, 0.25 M sucrose, 20 percent glycerol, 0.05 mM dithiothreitol, pH 7.4). All microsomal pellets are combined into a single lot and resuspended in Buffer C. The microsomal suspension is aliquoted (ca. 200 µL tube) into labeled cryotubes, flash frozen in liquid nitrogen, and stored at ca. -70°C until removed for use.

On the day of use, the microsomes are thawed rapidly in a 37 ± 1°C water bath, rehomogenized using a Potter-Elvejhem homogenizer, and then kept on ice until used. For use in the assay, the microsomes are diluted in the assay buffer to approximately 0.025 mg/ml. The final target protein concentration in the incubation mixture is approximately 0.0125 mg/ml.

### 4.3.2 Recombinant Microsomes

Human recombinant microsomes (Gentest [Human CYP19 + P450 Reductase SUPERSOMES], Woburn, MA) are stored at approximately -70°C until the time of the assay. On the day of use,

the microsomes are thawed rapidly in a 37 ± 1°C water bath, rehomogenized using a Potter-Elvejhem homogenizer, and then kept on ice until used. The reported protein concentration should be approximately 4.9 mg/mL. For use in the assay, the microsomes are diluted in the assay buffer in two serial dilutions in order to achieve the desired final working stock concentration of approximately 0.008 mg/mL. The final target protein concentration in the incubation mixture should be approximately 0.004 mg/mL.

### 4.4 Other Assay Components

As a point of reference, in the validation tests the following components and suppliers were used (Table 4.4-1). The NADPH (β-Nicotinamide Adenine Dinucleotide Phosphate) is obtained in reduced form.

**Table 4.4-1. Other assay components**

| Component | Supplier | | |
|---|---|---|---|
| | Battelle | In Vitro | WIL |
| NADPH (co-factor) | 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 |

### 4.5 Protein Determination

The microsomal protein concentration must be determined prior to conducting the aromatase assay. Microsomal protein concentration is determined using a commercial protein assay kit (DC Protein Assay kit from Bio-Rad, Hercules, CA). A 6-point standard curve is prepared using bovine serum albumin (BSA) reconstituted in Milli-Q water. The standard curve should range from approximately 0.10 to 1.5 mg protein/mL. The absorbance at a wavelength of 750 nm is measured using a spectrophotometer. The protein concentration of the microsomal sample is determined from the absorbance value using linear regression to the absorbance of the protein standards.

### 4.6 Aromatase Assay Procedure

The assays are performed in test tubes maintained at 37 ± 1°C in a shaking water bath. Propylene glycol, [$^3$H]ASDN, NADPH, and assay buffer are combined in the test tubes with or without inhibitor to the total volume of 1.0 mL. The final concentrations for the major components of the assay are presented in Table 4.6-1. The tubes and the microsomal suspension are 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 4.6-1. Aromatase Assay Conditions**

| Assay Components | Component Volume Added to the Assay | Final Concentration in the Placental Assay | Final Concentration in the Recombinant Assay |
|---|---|---|---|
| Microsomal Protein | 1.0 mL | 0.0125 mg/mL | 0.004 mg/mL |
| NADPH | 100 µL | 0.3 mM | 0.3 mM |
| [$^3$H]ASDN | 100 µL | 100 nM | 100 mM |
| Propylene glycol | 100 µL | 5% (v/v) | 5%(v/v) |
| 4-OH ASDN | 20 µL | $5 \times 10^{-8}$ M | $5 \times 10^{-8}$ M |
| Test Chemical | 20 µL | $10^{-3}$ to $10^{-10}$ M | $10^{-3}$ to $10^{-10}$ M |
| Buffer | 680 µL | --- | --- |

The total assay volume is 2.0 mL. The tubes are incubated for 15 minutes at 37 ± 1°C. The incubations are stopped by the addition of methylene chloride (2 mL); the tubes are vortex-mixed for ca. 5 seconds and placed on ice. The tubes are then vortex-mixed an additional 20 to 25 seconds to extract unreacted ASDN, then centrifuged for 10 minutes to facilitate separation of the organic and aqueous layers. The methylene chloride layer is removed and discarded; the aqueous layers is extracted two more times, each time with 2 mL of methylene chloride. The aqueous layers are transferred to vials and duplicate aliquots (0.5 mL) are transferred to 20 mL liquid scintillation counting vials. Liquid scintillation cocktail is added to each counting vial and the vials shaken to mix.

Analysis of the samples is 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 is determined by dividing the total amount of $^3H_2O$ formed by the specific activity of the [$^3$H]ASDN substrate (expressed in dpm/nmol). Results are presented as the activity (velocity) of the enzyme reaction and expressed in nmol (mg protein)$^{-1}$min$^{-1}$.

Each chemical is tested in three independent runs and, for a given run, each inhibitor concentration is performed in triplicate. In each run, full enzyme activity and background activity control samples are 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 are included with each run of the assay run and were processed in the same manner as the other samples. The controls sets are split, so that two tubes (for each full and background activity control set) are run at the beginning, and two at the end of each assay. The study design used for the interlaboratory validation studies is summarized in Table 4.6-2. The protocol has since been modified to include a positive control dose-response curve rather than a single concentration.

**Table 4.6-2. Test chemical study design**

| Sample Type | Replicates (Test Tubes) | Description | Final Test Chemical 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 |
| Positive Control | 4 | Complete assay with 4-OH ASDN | $5 \times 10^{-8}$ |
| Test Chemical Concentration 1 | 3 | Complete assay with Test Chemical added | $1 \times 10^{-3}$ |
| Test Chemical Concentration 2 | 3 | Complete assay with Test Chemical added | $1 \times 10^{-4}$ |
| Test Chemical Concentration 3 | 3 | Complete assay with Test Chemical added | $1 \times 10^{-5}$ |
| Test Chemical Concentration 4 | 3 | Complete assay with Test Chemical added | $1 \times 10^{-6}$ |
| Test Chemical Concentration 5 | 3 | Complete assay with Test Chemical added | $1 \times 10^{-7}$ |
| Test Chemical Concentration 6 | 3 | Complete assay with Test Chemical added | $1 \times 10^{-8}$ |
| Test Chemical Concentration 7 | 3 | Complete assay with Test Chemical added | $1 \times 10^{-9}$ |
| Test Chemical Concentration 8 | 3 | Complete assay with Test Chemical added | $1 \times 10^{-10}$ |

a. The complete assay contains buffer, propylene glycol, microsomal protein, [$^{3}$H]ASDN and NADPH.
b. The scheme described above was used in the validation, but does not reflect the final protocol.

# 5.0 Data Modeling and Analysis

## 5.1 Introduction

Enzyme kinetics is well studied and its mathematical treatment, which dates from the work of Leonor Michaelis and Maude Menten in 1913, can be found in any introductory textbook on biochemistry.

Figure 5.1-1 shows what happens to the velocity of an equation in the presence of two different kinds of inhibitors: competitive inhibitors and non-competitive inhibitors. In competitive inhibition, the inhibitor competes with the substrate for the enzyme's active sites, diminishing the amount of enzyme available to catalyze the conversion of substrate to product thus slowing down the rate of product formed. At high levels of substrate, this effect is diminished as the overwhelming concentration of substrate out-competes the inhibitor.

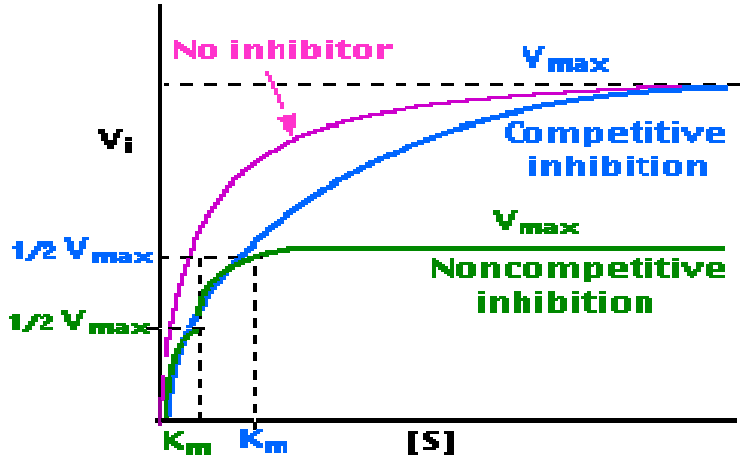



**Figure 5.1-1 Reaction velocity as a function of substrate concentration**

In contrast, non-competitive inhibitors bind irreversibly to one or more sites on the enzyme other than the active site, either with the enzyme itself or the enzyme-substrate complex, and render the enzyme inactive, i.e., it prevents the formation of products. As shown above, Vmax itself is reduced but Km remains unchanged because the active site of the enzyme that has not been inhibited is unchanged. Both types of inhibitors have been tested in the validation program.

## 5.2 The Hill Equation

The usual approach to measuring inhibitory effect of chemicals on enzyme activity is to hold the enzyme and substrate concentrations constant and vary the concentration of the inhibitor. As explained in Chapter 4, this is the approach taken in the aromatase assay. Because the test chemical competes with the substrate, the model for this approach is completely analogous to that of receptor binding. With the $\log_{10}$ of the inhibitor concentration plotted on the x-axis and enzyme activity running from 100 percent (the full enzyme activity level) to 0 percent on the y-

axis, increasing concentrations of inhibitor produce a sigmoid dose-response curve determined by the law of mass action with a slope of approximately -1. Figure 5.2-1 shows the inhibition curve obtained with 4-hydroxyandrostenedione, the positive control used in the aromatase assay.

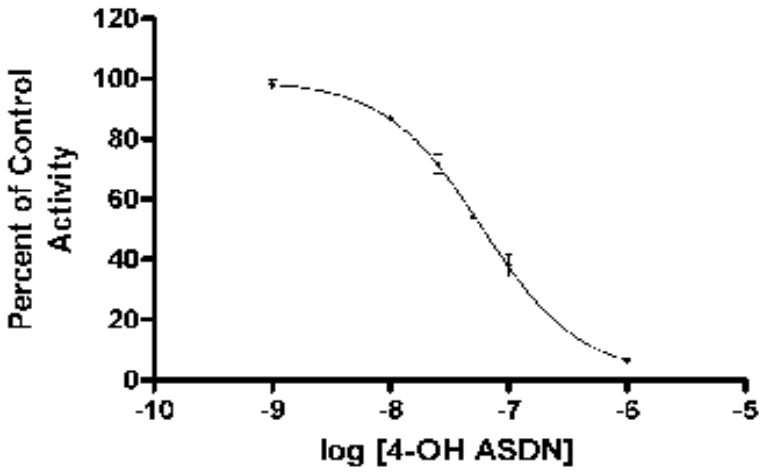



**Figure 5.2-1 Concentration response curve for 4-hydroxyandrostenedione**

This sigmoid curve is described by an equation known as the Hill Equation (Hill 1910; Motulsky and Christopoulos 2005):

$$Y = B + \frac{(T-B)}{1 + 10^{(\text{Log } EC_{50} - X)\,\beta}} + \varepsilon$$

Where:

X = the logarithm of the concentration of the inhibitor (test chemical)
Y = percent activity
B = Bottom = the lower plateau (minimum response)
T = Top = the upper plateau (maximum response)
β = Hillslope, the slope of the curve, normally -1
$EC_{50}$ = the concentration of the inhibitor halfway between the top and the bottom
ε = the random variation about the concentration response relation, with mean 0 and variance a function of the expected value of Y (often modeled as a constant, $\sigma^2$).

Note that the above equation employing the $EC_{50}$ estimates the $IC_{50}$, the concentration at 50 percent activity, only if the curve spans the full range of 100 percent to 0 percent. For all other cases, the $EC_{50}$ is half of the value between the top and bottom of the inhibition curve. A general expression incorporating the true $IC_{50}$ can be derived from the equation above by recognizing that Y = 50 when X = log $IC_{50}$. This general equation then becomes

$$Y = B + \frac{(T-B)}{1+10^{(\text{Log } IC_{50}-X)\beta+\log[(T-B/50-B)-1]}} + \varepsilon$$

The percent of control activities and their respective inhibitor concentrations were fitted by non-linear regression to a four-parameter model (Top, Bottom, slope, $IC_{50}$) Hill equation using Graphpad Prism Version 4 or 5 software (Motulsky 2003, 2007) software or general purpose statistical systems such as SAS (2003). In some cases, noted later in the ISR, data were fit to a two-parameter model in which top and bottom are fixed at 100 percent and 0 percent. In general the four-parameter model is preferred.

### 5.3 Statistical Analysis

The model fits result in parameter estimates and associated standard errors as well as estimates of residual variability. These are used for inferences about the concentration response model parameters and for statistical comparisons between the test chemical and the standard within a run, among runs within test laboratory, and across test laboratories.

For each test chemical at least three runs were carried out. For each run, estimates of:

- B, the bottom plateau
- T, the top plateau
- β, the "hill slope" (β is necessarily negative).
- $Log_{10}IC_{50}$, the logarithmic concentration at which E(Y) = 50 percent.

and their standard errors are obtained. For each parameter a one-way random effects analysis of variance model with heterogeneous variances among runs is fitted to determine an overall estimate and its associated standard error and confidence intervals. The R (often R=3) runs for a test chemical are treated as a random effect, with R-1 degrees of freedom. The within run variances are the squares of the parameter standard errors within each run, with residual degrees of freedom based on the nonlinear regression fit. The pooled parameter estimate is a weighted average of the estimates across runs, with standard error incorporating both the within run and the between run components of variance, and degrees of freedom a weighted combination of the degrees of freedom for each variance component, with the larger variances having greater weight[1] (Hartung and Makambi 2001). The estimated $IC_{50}$ and lower and upper confidence bounds for each chemical are estimated by the model.

The weighted combination of parameter estimates across runs can be determined either by random effects analysis of variance, as mentioned above, or by a method of moments calculation

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

suggested by DerSimonian and Laird (1986). The random effects analysis of variance method results in maximum likelihood estimates of parameters with more desirable statistical properties. The statistical analysis applied to individual study types is described in the chapter discussing the study.

# 6.0 Reference Chemicals

## 6.1 Reference Chemicals Selected for the Interlaboratory Studies

Twelve chemicals, shown in Table 6.1-1, were selected by EPA on the basis of data in the scientific literature for use in prevalidation. One, 4-hydroxyandrostenedione, was selected as a positive control for the validation studies, and lindane was initially selected to be a negative control; however, it was felt that the negative control was not necessary given that the background activity control served this purpose. The remaining 10 chemicals were used as coded reference chemicals for the interlaboratory validation studies.

For the interlaboratory validation studies EPA used chemicals that had been tested for aromatase in several different assay systems in multiple laboratories. Before using these substances in the interlaboratory validation studies, these substances were tested multiple times in the lead laboratory (Section 7.4) to confirm the results cited in the scientific literature. One limitation in selecting chemicals for aromatase validation is that only a few categories of chemicals have been tested.

## 6.2 Supplementary Chemicals Selected for Testing in the Lead Lab

As noted in Chapter 12, EPA tested an additional 16 chemicals, for which results were less well documented, in order to broaden the types of chemicals represented. These chemicals and the reasons for their selection are given in Table 6.2-1.

Appendix B contains supplementary literature results on aromatase testing for numerous compounds and test systems.

**Table 6.1-1. Reference chemicals used in the validation of the aromatase assay**

| Chemical | Supplier | Mfgr. Purity (%) | CAS No. | Mol. Formula | Mol. Wt. (g/mol) | Basis for Selection | Test System | $IC_{50}$ (µM) | Ref |
|---|---|---|---|---|---|---|---|---|---|
| 4-Hydroxy androstenedione | Sigma-Aldrich | 99 | 566-48-3 | $C_{19}H_{26}O_3$ | 302.4 | Steroidal aromatase inhibitor (positive control) | HT | 0.031 | Stresser |
| | | | | | | | KGN | 0.00115 | Ohno |
| | | | | | | | RT-BM | 0.009 | Hinfray 2006 |
| | | | | | | | RT-OM | 0.00015 | Hinfray 2006 |
| Lindane | Sigma-Aldrich | 99.6 | 58-89-9 | $C_6H_6Cl_6$ | 290.8 | Organochlorine pesticide known not to inhibit aromatase (negative control) | JEG-3 cells | 56.2 ± 2.7 | Nativelle-Serpentini et al. |
| Amino-glutethimide | Sigma- Aldrich | >99 | 125-84-8 | $C_{13}H_{16}N_2O_2$ | 232.3 | Non-steroidal aromatase inhibitor | KGN | 2.25 | Ohno |
| | | | | | | | HT | 0.77 | Stresser |
| | | | | | | | H adip | 7.4 | Campbell |
| | | | | | | | RT-BM | 197 | Hinfray 2006 |
| | | | | | | | RT-OM | 36.8 | Hinfray 2006 |
| Atrazine | Supelco/Chem Service | 98 | 1912-24-9 | $C_8H_{14}ClN_5$ | 215.7 | Pesticide affecting aromatase gene, no aromatase inhibition | H295R | Neg. | Sanderson 02 |
| Chrysin | Sigma-Aldrich | 98.2 | 480-40-0 | $C_{15}H_{10}O_4$ | 254.2 | Potent flavanoid aromatase inhibitor | KGN | 1.89 | Ohno |
| | | | | | | | H adip | 4.6 | Campbell |
| | | | | | | | HT | 0.70 | Stresser |
| | | | | | | | H295R | 7 | Sanderson 04 |
| Dibenz[a,h,]-anthracene | Sigma | 97 | 53-70-3 | $C_{22}H_{14}$ | 278.4 | Ah receptor agonist, not an aromatase inhibitor | None Found | None Found | None Found |
| Dicofol | Ultra Scientific | 96.5 | 115-32-2 | $C_{14}H_9Cl_5O$ | 370.5 | Organochlorine pesticide, aromatase inhibitor | KGN | Inhib | Morinaga |
| Econazole | Sigma-Aldrich | 98 | 24169-02-6 | $C_{18}H_{15}Cl_3N_2O{\cdot}HNO_3$ | 444.7 | Potent imidazole anti-fungal aromatase inhibitor | HT | 0.004 | Kragie |
| | | | | | | | PM | 0.023 | Ayub |
| Fenarimol | Supelco/Chem Service | 99 | 60168-88-9 | $C_{17}H_{12}Cl_2N_2O$ | 331.2 | Pyrimidine fungicide, aromatase inhibitor | H295R | 80 | Sanderson 02 |
| | | | | | | | PM | 10 | Vinggaard |
| | | | | | | | KGN | 2.0 | Ohno |
| | | | | | | | RT-BM | 6 | Hinfray 2006 |
| | | | | | | | RT-OM | 18 | Hinfray 2006 |
| | | | | | | | JEG-3 | 10 | Laville 2006 |
| Ketoconazole | Sigma-Aldrich | >99 | 65277-42-1 | $C_{26}H_{28}Cl_2N_4O_4$ | 531.4 | Weak imidazole anti-fungal aromatase inhibitor | Super | 281 | Trosken |
| | | | | | | | HT | 0.9 | Stresser |
| | | | | | | | HT | 2.0 | Kragie |
| 4-Nonylphenol | Acros Organics | >98.5 | 104-40-5 | $C_{15}H_{24}O$ | 220.4 | Alkylphenol affecting AR/ER binding, not an aromatase inhibitor | KGN | Neg | Morinaga |

| Chemical | Supplier | Mfgr. Purity (%) | CAS No. | Mol. Formula | Mol. Wt. (g/mol) | Basis for Selection | Test System | $IC_{50}$ (µM) | Ref |
|---|---|---|---|---|---|---|---|---|---|
| Prochloraz | Sigma- Reidel | 99.5 | 67747-09-5 | $C_{15}H_{16}Cl_3N_3O_2$ | 376.7 | Imidazole fungicide, known aromatase inhibitor | Super | 0.44 | Trosken |
| | | | | | | | H295R | 0.1 | Sanderson 02 |
| | | | | | | | PM | 0.04 | Vinggaard |
| | | | | | | | RT-BM | 1.3 | Hinfray 2006 |
| | | | | | | | RT-OM | 1.0 | Hinfray 2006 |
| | | | | | | | JEG-3 | < 1 | Laville 2006 |

Supersomes = BD Gentest supersomes, HT = Supersomes high throughput assay, PM= placental microsomes, KGN=a human ovarian granulose-like tumor cell line, H295R=a human adrenocortical carcinoma cell line, RT-BM=rainbow trout brain microsomes, RT-OM=rainbow trout ovarian microsomes, JEG-3=choriocarcinoma cell line

**Table 6.2-1. Supplementary reference chemicals**

| Chemical | Supplier | Mfgr. Purity (%) | Chem. ID (Code) | CAS No. | Molec. Formula | Mol. Wt. (g/mol) | Basis for Section | Test System | $IC_{50}$ (µM) | Ref |
|---|---|---|---|---|---|---|---|---|---|---|
| Vinclozolin | Chem Service | 99.5 | CR11601 | 50471-44-8 | $C_{12}H_9Cl_2NO_3$ | 286.12 | Used in other validation studies and has been reported to be negative in both H295R and KGN cell-based assays. | H295R | Neg. | Sanderson 02 |
| | | | | | | | | KGN | Neg. | Morinaga |
| Bisphenol A | Aldrich | 99.9 | CR11602 | 80-05-7 | $C_{15}H_{16}O_2$ | 228.29 | A challenging chemical used extensively in ED testing. Structurally different from the pesticides test and reported to be an inhibitor in the KGN assay. | KGN | Inhib | Morinaga |
| | | | | | | | | JEG-3 cells | 24.7 ± 4.3 | Nativelle-Serpentini et al. |
| Tributyltin | Aldrich | 97.9 | CR11603 | 688-73-3 | $[CH_3(CH_2)_3]_3SnCl$ | 291.06 | Structurally different from all other chemicals tested and reported to be an aromatase inhibitor in the literature. | KGN | Inhib | Morinaga |
| Diethylhexyl phthalate | Aldrich | 99.6 | CR11604 | 117-81-7 | $C_{24}H_{38}O_4$ | 390.56 | Used in other validation studies. Reported to be an inhibitor in the KGN assay. | KGN | Inhib | Morinaga |
| Methoxychlor | Chem Service | 98 | CR11605 | 72-43-5 | $C_{16}H_{15}Cl_3O_2$ | 345.66 | Used in other validation studies. Known to be metabolized to a strong estrogen and to have antiandrogenic properties. Reported to be negative in the KGN assay. | KGN | Neg | Morinaga |
| Aldicarb | Chem Service | 99 | CR11606 | 116-06-3 | $C_7H_{14}N_2O_2S$ | 190.3 | Aldicarb is a carbamate and therefore structurally different from other pesticides tested in the validation program. It was reported to be an inhibitor in the KGN assay. | KGN | Inhib | Morinaga |
| Flavone | Sigma | 99.7 | CR11607 | 525-82-6 | $C_{15}H_{10}O_2$ | 222.24 | Flavone is the parent of a group of natural aromatase inhibitors. It is reported to be a weak inhibitor in the KGN assay and a moderate (10 uM) inhibitor in the PM assay. | KGN | Weak inhib | Ohno |
| | | | | | | | | H adip | 68 | Campbell |
| Triadimefon | Riedel- de Haën | 99.8 | CR11608 | 43121-43-3 | $C_{14}H_{16}ClN_3O_2$ | 293.75 | Is a weak to moderate azole fungicide inhibitor for which several literature values were found. | Super | 483 | Trosken |
| | | | | | | | | PM | 32 | Vinggaard |
| | | | | | | | | KGN | 3.59 | Ohno |
| | | | | | | | | RT-BM | 11 | Hinfray 2006 |
| | | | | | | | | RT-OM | 26 | Hinfray 2006 |
| Imazalil | Chem Service | 99 | CR11609 | 35554-44-0 | $C_{14}H_{14}Cl_2N_2O$ | 297.20 | An azole fungicide for which several literature values were | Super | 3.6 | Trosken |
| | | | | | | | | H295R | 0.1 | Sanderson 02 |

| Chemical | Supplier | Mfgr. Purity (%) | Chem. ID (Code) | CAS No. | Molec. Formula | Mol. Wt. (g/mol) | Basis for Section | Test System | $IC_{50}$ (µM) | Ref |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | found. | PM | 0.34 | Vinggaard |
| | | | | | | | | KGN | 0.0044 | Ohno |
| | | | | | | | | RT-BM | 0.43 | Hinfray 2006 |
| | | | | | | | | RT-OM | 0.32 | Hinfray 2006 |
| Apigenin | Aldrich | 98 | CR11610 | 520-36-6 | $C_{15}H_{16}O_5$ | 270.24 | A flavone with known aromatase activity. | KGN | 2.58 | Ohno |
| | | | | | | | | H295R | 20 | Sanderson 04 |
| Ronidazole | Sigma | 100.6 | CR11611 | 7681-76-7 | $C_6H_8N_4O_4$ | 200.15 | Reported to be a very weak azole fungicide inhibitor in the HT assay. | HT | >1000 | Kragie |
| Genestein | Sigma | 98.8 | CR11612 | 446-72-0 | $C_{15}H_{10}O_5$ | 270.24 | An isoflavone reported to be negative. Genestein has been used extensively in ER binding and other ED validation studies. | H295R | Neg. | Sanderson 04 |
| p,p'-DDE | Aldrich | 98.6 | CR11613 | 75-55-9 | $C_{14}H_8Cl_4$ | 318.03 | An organochlorine insecticide known to be estrogenic. It has been reported to be an inhibitor in the KGN assay. | KGN | Inhib | Morinaga |
| Alachlor | Chem Service | 99.2 | CR11614 | 15972-60-8 | $C_{14}H_{20}ClNO_2$ | 269.8 | A chloroacetonitrile reported to be negative in the KGN study. | KGN | Neg. | Morinaga |
| Nitrofen | Chem Service | 98 | CR11615 | 1836-75-5 | $C_{12}H_7Cl_2NO_3$ | 284.10 | A diphenyl ether reported to be negative in the KGN assay. | KGN | Neg. | Morinaga |
| Trifluralin | Chem Service-Supelco | 99.5 | CR11616 | 1582-09-8 | $C_{13}H_{16}F_3N_3O_4$ | 335.32 | A dinitroaniline reported to be negative in the KGN assay. | KGN | Neg. | Morinaga |

# 7.0 Assay Development and Optimization in the Lead Laboratory

The objective of these experiments was to identify the optimal factors and conditions for the assay. Three sets of preoptimization exploratory experiments were conducted with human, bovine, and porcine placental microsomes and human recombinant microsomes:

- Preoptimization Experiments
- Assay Optimization Experiments using a Factorial Design
- Optimized Assay Experiments using Selected Test Substances.

## 7.1 Preoptimization Experiments

The preoptimization experiments were designed to assess the chemical and biological properties of the critical components that are used in the aromatase assay. These experiments included characterizing the radiolabeled substrate and preparation of the placental microsomes. Placental tissue was obtained from three species (human, porcine, and bovine) and microsomes were prepared. Human recombinant CYP19 (expressed in Baculovirus-infected insect cells) microsomes were purchased from a commercial source. In addition, each of the four microsomal preparations (human, bovine, and porcine placental microsomes and the human recombinant microsomes) were analyzed for protein concentration, cytochrome P450 content, and aromatase activity. The P450 content measurement was used to ensure that the enzyme was present (and in what concentration/preparation type) prior to beginning the more elaborate aromatase activity assay. Finally, a single aromatase activity assay run using each type of microsomal preparation was included as a pre-optimization experiment in order to determine whether the preparations were of sufficient activity to conduct the optimization experiments. Experimental details can be found in the prevalidation study report (Reference 2).

It was discovered that bovine and porcine placenta were unsuitable for use because they gave unsatisfactorily low levels of aromatase activity. In addition, procurement of bovine and porcine placentas was problematic: there may not be a ready year-round supply of placental tissue because of the seasonality of livestock breeding and births are rarely attended by the farmers, making the collection of fresh placentas impractical. Because of these issues, further investigation of bovine and porcine placental microsomes was terminated by EPA (Reference 2).

**Human placental microsomes:** A single human placenta was obtained from a local hospital following a full-term Caesarean delivery. Microsomes were prepared and were found to have ample aromatase activity and protein content for use in the experiments. The human placenta was by far the easiest of the three placentas to process as it was only necessary to dissect the membranes away from the soft tissue prior to processing the soft tissue into microsomes; however, effective infection-control practices need to be followed when working with human tissue. A sample of the microsomes was thawed rapidly in a water bath and rehomogenized prior to assay for protein and P450 content and aromatase activity. The protein content of the human placental microsomes was determined to be ca. 44 mg/mL. The total protein yield for the preparation was calculated to be ca. 900 mg. This exceeds the 250 mg of protein criterion set in the protocol. P450 content of the human placental microsomes was determined to be ca. 0.048 nmol/mg protein, which exceeds the criterion of 0.005 nmol P450/mg protein set in the protocol.

The aromatase activity of the human placental microsomes was approximately 0.015 nmol estrogen formed/mg protein/min; this exceeds the 5 pmol estrogen formed/mg protein/min acceptance criterion for this parameter.

**Human recombinant microsomes:** Two batches of human recombinant microsomes (prepared from Baculovirus-infected insect cells) were purchased from a commercial supplier and were found to have sufficient P450 content and aromatase activity for use in these studies. One tube of Lot 2 was thawed rapidly at 37ºC and the contents were rehomogenized and analyzed for protein and P450 content and aromatase activity. The protein content was found to be 3.5 mg/mL, compared with the 4.2 mg/mL stated on the data sheet. The P450 content was calculated to be 0.38 nmol/mg protein. This value is similar to the 0.24 nmol/mg value calculated from the data sheet information. This microsomal preparation had aromatase activity of 0.022 nmol estrogen formed/mg protein/min under the conditions of the assay as described above. The data sheet reported an aromatase activity value (1.38 nmol/mg protein/min) obtained using a different substrate at a significantly higher concentration. It is unclear whether the activities determined under such different conditions should be similar. Besides ease of procurement, there is no need for special infection-control practices when working with the recombinant protein.

### 7.2 Factorial Optimization Experiments for Human Microsomes

A factorial design experiment designed to determine optimal assay conditions was conducted for both the human placental and recombinant microsomes. The original experimental design tested six different factors and five different levels of each factor for a total of 30 combinations. In order to assess the effects of each experimental factor on the aromatase activity, analysis of variance (ANOVA) models were fit to the data. Tests for interactions were conducted and where they were not detected as statistically significant (p=0.10), a reduced model was employed that retained the main effects. A more detailed description of the procedures to determine the factor combination that produced the maximum predicted value of logarithm of aromatase activity is described in the prevalidation report (Reference 2).

### 7.3 Supplemental Optimization Studies: Determination of protein concentration, linearity of product formation, and standard inhibition curve

Additional experiments were conducted to ensure that the conditions selected on the basis of the foregoing studies were in the linear range of the assay for protein concentration and time and would maintain initial rate conditions (with preferably no more than 10 to 15 percent of substrate consumed).

#### 7.3.1 Effect of Protein Concentration

The aromatase assay was conducted using human placental microsomes and repeated using recombinant microsomes. The placental aromatase assay was conducted at final protein concentrations of 0.0125, 0.025, and 0.05 mg/mL. Only the assay using 0.0125 mg/mL protein had a percent substrate conversion less than the target of 10 to 15 percent substrate consumption, and this concentration was selected for use in the placental assay. Similar experiments with the

recombinant microsomes led to the selection of a protein concentration of 0.005 mg/mL for the recombinant assay.

### 7.3.2 Linear Production of Product

The experiments (described in Reference 2) were designed to demonstrate whether production of product was linear over time (to 30 min) and to determine whether NADPH was limiting during the assay reaction time. For this work, the protein concentration was fixed at the standard conditions selected above: 0.0125 mg/mL (human placental microsomes) or 0.005 mg/mL (human recombinant microsomes); the [$_3$H]ASDN concentration was 100 nM, and NADPH concentration was 0.3 mM. Some tubes also contained 100 nM 4-OH ASDN as an inhibitor The assay was conducted with 5 different incubation times (5, 10, 15, 20, 30 min).

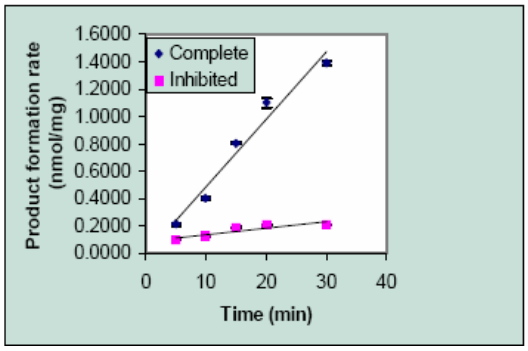
For each time point, the following conditions were used with all tubes in triplicate. The number of assay tubes was doubled (Total activity and Inhibition sets only) for the 30 min time point to use for NADPH experiment as outlined below. The number of assay tubes for the 15 min time point was doubled for Estrone/Estradiol Concentration analysis:

- Blank: (boiled microsomes, substrate but no NADPH)
- No NADPH: (microsomes, substrate but no NADPH)
- Total Activity: (microsomes, substrate, 0.3 mM NADPH)
- Inhibition: (microsomes, substrate, 0.3 mM NADPH, 0.1 μM 4-OH androstenedione)

The 4-OH androstenedione was added to the reaction mixture before the protein (enzyme) was added.

Whether or not NADPH became limiting during the course of the reaction was tested by the addition of a second aliquot (0.3 mM final concentration) of NADPH added midway through a 30 min incubation period of a second set of assay tubes. There was no increase in aromatase activity in either the presence or absence of inhibitor in the supplemented versus the unsupplemented case. Therefore, a concentration of 0.3 mM NADPH was determined to be sufficient to support full aromatase activity over at least a 30 min reaction time (Figures 7.3-1 and 7.3-2).

Human Placental




Recombinant

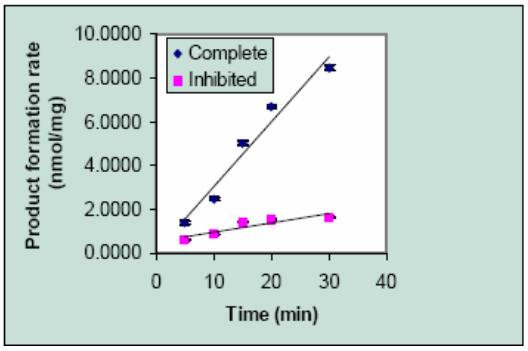
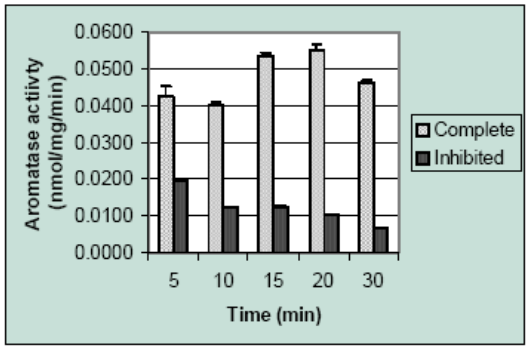



**Figure 7.3-1. Linearity of product formation with time**



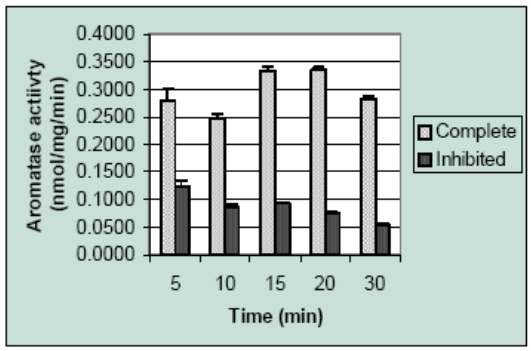




**Figure 7.3-2. Aromatase activity at various incubation times**

On the basis of the optimization experiments, the conditions shown in Table 7.3-1 were adopted for the placental and recombinant aromatase assays.

**Table 7.3-1. Optimized assay conditions for aromatase**

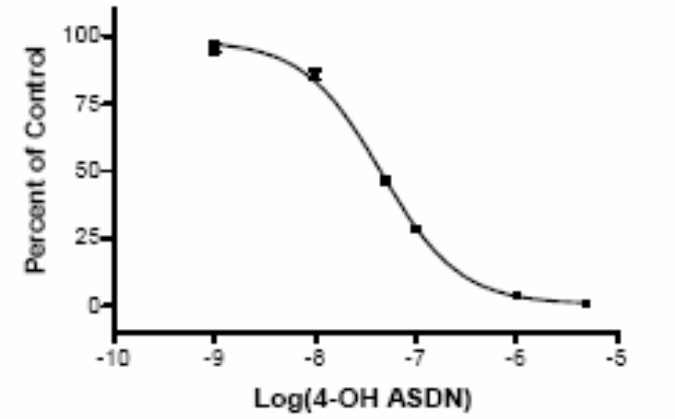
| Assay factor (units) | Assay Type | |
|---|---|---|
| | Human Placental | Human Recombinant |
| Microsomal Protein (mg/mL) | 0.0125 | 0.004 |
| NADPH (mM) | 0.3 | 0.3 |
| [$^3$H]ASDN (nM) | 100 | 100 |
| Incubation Time (min) | 15 | 15 |

### 7.3.3 Positive Control Study: Inhibition Curve with 4-Hydroxyandrostenedione

These experiments were designed to demonstrate the specificity of the assay through the generation of a competitive inhibition curve using 4-OH ASDN. Using the general assay method determined above and described in Chapter 4, the effect of varying concentrations of the inhibitor 4-OH ASDN (6 concentrations spanning the range 5 x $10^{-6}$ to 1 x $10^{-9}$ M ) on aromatase activity was determined. The aromatase activities in the presence of inhibitor were converted to percent of control activities. As noted in Chapter 5, these percent of control activities and their

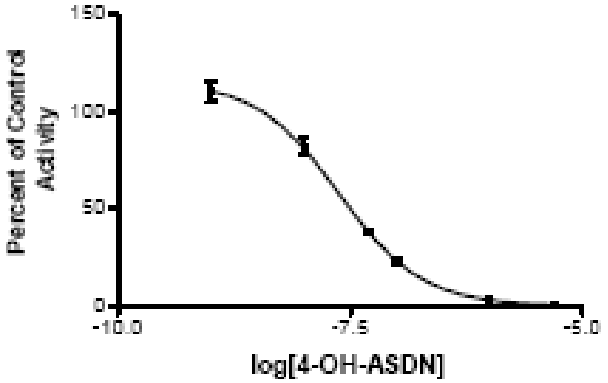
respective inhibitor concentrations were fitted to a nonlinear regression equation using Prism (Version 3.02) software.

The response curves and $IC_{50}$ data are presented in Figures 7.3-3 and 7.3-4 for the human placental and human recombinant assays, respectively. The calculated $IC_{50}$ for the human placental microsomes, 46 nM, falls within the 30 to 50 nM range reported in the protocol. The calculated $IC_{50}$ for the human recombinant microsomes, 24 nM, is near the reported range. Day-to-day variation within the human placental assay was very low. Somewhat higher variation was found in the recombinant assay.




| | |
|---|---|
| Equation 1 | |
| Best-fit values | |
| BOTTOM | 0.6545 |
| TOP | 98.49 |
| LOGEC50 | -7.340 |
| HILLSLOPE | -1.122 |
| EC50 | 4.5720e-008 |
| Std. Error | |
| BOTTOM | 0.2144 |
| TOP | 1.596 |
| LOGEC50 | 0.02030 |
| HILLSLOPE | 0.04674 |
| 95% Confidence Intervals | |
| BOTTOM | 0.1946 to 1.114 |
| TOP | 95.08 to 101.9 |
| LOGEC50 | -7.383 to -7.296 |
| HILLSLOPE | -1.222 to -1.022 |
| EC50 | 4.1360e-008 to 5.0540e-008 |
| Goodness of Fit | |
| Degrees of Freedom | 14 |
| $R^2$ (unweighted) | 0.9972 |
| Weighted Sum of Squares (1/Y) | 1.051 |
| Absolute Sum of Squares | 68.45 |
| Sy.x | 2.211 |
| Data | |
| Number of X values | 6 |
| Number of Y replicates | 3 |
| Total number of values | 18 |
| Number of missing values | 0 |

**Figure 7.3-3. Determination of $IC_{50}$ for 4-OH ASDN in the human placental aromatase assay**




| | |
|---|---|
| Equation 1 | |
| Best-fit values | |
| BOTTOM | 0.2224 |
| TOP | 115.7 |
| LOGEC50 | -7.622 |
| HILLSLOPE | -0.9707 |
| EC50 | 2.3880e-008 |
| Std. Error | |
| BOTTOM | 0.2409 |
| TOP | 2.783 |
| LOGEC50 | 0.03317 |
| HILLSLOPE | 0.04416 |
| 95% Confidence Intervals | |
| BOTTOM | -0.2944 to 0.7392 |
| TOP | 109.8 to 121.7 |
| LOGEC50 | -7.693 to -7.551 |
| HILLSLOPE | -1.065 to -0.8760 |
| EC50 | 2.0270e-008 to 2.8130e-008 |
| Goodness of Fit | |
| Degrees of Freedom | 14 |
| $R^2$ (unweighted) | 0.9963 |
| Weighted Sum of Squares (1/Y) | 1.637 |
| Absolute Sum of Squares | 110.3 |
| Sy.x | 2.807 |
| Data | |
| Number of X values | 6 |
| Number of Y replicates | 3 |
| Total number of values | 18 |
| Number of missing values | 0 |

**Figure 7.3-4. Determination of $IC_{50}$ for 4-OH ASDN in the human recombinant aromatase assay**

### 7.3.4 Assessment of Variability in the Optimized Assay

The optimized conditions for the human placental and recombinant assays were used in the variability assessment of the assays. (The activity results are presented in Tables B.7-1 and B.7-2 in Appendix B for the placental and recombinant assays, respectively.) Three technicians conducted each assay independently on three separate days. In addition, Technician 3 ran the assay using the diluted microsome samples prepared by Technicians 1 and 2 each day and using all other co-factors prepared by Technician 3. There is little variation in activities determined within a technician on a given day. Variability between technicians was marked but not significant (Tables B.7-3 and B.7-5 in Appendix B). Day to day variation was statistically significant ($p<0.1$, Tables B.7-4 and B.7-6 in Appendix B). Possible sources of this variation include 1) variation in protein concentration in final dilution of microsomes, 2) errors in substrate preparation and 3) technician technique. Variability between technicians using the same microsome dilution on a given day was usually less than 10 percent, so it appears that technician technique is uniform and that most of the variation seen between technicians and days may be related to variation in final protein concentrations.

## 7.4 Human Placental Aromatase and Recombinant Aromatase Studies with Reference Chemicals

After the generation of data demonstrating that both the human placental and microsomal assays were optimized and meeting expectations, the two assays were used in the testing of 11 different test substances in order to determine the response of the assay to these known inhibitors and non-inhibitors. Each of 11 test substances at up to 8 different concentrations (ranging from no less than $10^{-9}$ M to no higher than $10^{-3}$ M) were tested using each of the two microsomal preparations for which optimal conditions were determined. The vehicle controls were ethanol and DMSO, depending on the vehicle(s) used to formulate the test substances. The aromatase activity in the absence of any test substance was used as the benchmark (100 percent) activity (Reference 2).

Test substance groupings (Table 7.4.-1) were made based on solubility and whether the chemicals were expected to be inhibitors. Some inhibitors and some non-inhibitors were included in each group.

**Table 7.4-1. Test substance groupings and concentrations**

| Test Group | Test Substance | Levels | Target Concentrations (M) | Solvent |
|---|---|---|---|---|
| 1 | econazole | 8 | $10^{-6}$; $10^{-7}$; 2.5 and 5 x $10^{-7}$; $10^{-8}$; 2.5 and 5 x $10^{-8}$; $10^{-9}$ | DMSO |
| | genistein | 8 | $10^{-3}$; $10^{-4}$; 2.5 and 5 x $10^{-4}$; $10^{-5}$; 2.5 and 5 x $10^{-5}$; $10^{-6}$ | DMSO |
| | atrazine | 7 | $10^{-3}$ to $10^{-9}$ | DMSO |
| | bis-(2-ethylhexyl)phthlate | 7 | $10^{-3}$ to $10^{-9}$ | DMSO |
| 2 | aminoglutethimide | 8 | $10^{-3}$; $10^{-4}$; $10^{-5}$; 2.5 and 5 x $10^{-5}$; $10^{-6}$; $10^{-7}$; $10^{-9}$ | DMSO |
| | chrysin | 8 | $10^{-3}$; $10^{-4}$; $10^{-5}$; 2.5 and 5 x $10^{-5}$; $10^{-6}$; $10^{-7}$; $10^{-8}$ | DMSO |
| | nonylphenol | 7 | $10^{-3}$ to $10^{-9}$ | DMSO |
| | lindane | 7 | $10^{-3}$ to $10^{-9}$ | DMSO |
| 3 | 4-OH ASDN | 8 | $10^{-6}$; $10^{-7}$; 2.5 and 5 x $10^{-7}$; $10^{-8}$; 2.5 and 5 x $10^{-8}$; $10^{-9}$ | EtOH |
| | ketoconazole | 8 | 0.8 x $10^{-3}$; $10^{-4}$; 2.5 and 5 x $10^{-4}$; $10^{-5}$; 2.5 and 5 x $10^{-5}$; $10^{-6}$ | EtOH |
| | dibenz[*a*,*h*]anthracene | 6 | $10^{-4}$ to $10^{-9}$ | DMSO |

On a given day, a group of test substances was run in singlet over the entire test range (as defined in the protocol). Each group of test substances was run on each of four different days (Day 1,2,3,4). On each day of testing a full enzyme activity control (full assay with no test substance) and a background activity control (assay minus NADPH) were run in quadruplicate with each quadruplicate set, split so that half were conducted at the beginning of the test substance set and half at the end. Group 3 consisted of some chemicals that were soluble in EtOH and some that were soluble in DMSO. For this group only, a doubled set of full activity and background controls (quadruplicate each with DMSO and EtOH) were run. All placental assays were conducted by a single technician and all recombinant assays were conducted by a different technician. The assays were conducted on concurrent days so that, for example, Group 1, Day 1 of the placental and recombinant assays were conducted on the same day. The technicians shared a complete set of test substance dilutions on each day. Each technician prepared the ASDN substrate solution each day using a common stock, prepared fresh each day, of ASDN and [$^3$H]ASDN.

### 7.4.1 Control Analysis

Control activities were calculated and comparisons were made as described in the protocol (Appendix A). The average beginning (and end) full enzyme activity control activity was calculated for each Group/Day/Protein type. Significant variances were detected for all sources in the full enzyme activity control data. These variances may not have had a significant effect on calculation of $IC_{50}$ values since all data are analyzed as percent of control. (These average full enzyme activity control data are presented in Figures B.7-1 and B.7-2 in Appendix B for the placental and recombinant assay, respectively. Positive and negative control mean activities and standard deviations for both positive and negative controls are presented in Tables B.7-7 and B7-8 in Appendix B.) Whereas some of the negative control activities showed statistically significant differences between portion or in group and portion interactions, these differences are probably not of any practical importance since all of the negative control activities were essentially zero.

### 7.4.2 Test Substance Response Curves

Aromatase activities in the presence of the various test substances were converted to percent of full activity (full enzyme activity control). The aromatase activity and percent of full activity data are presented in Tables B.7-9 and B.7-10 in Appendix B for the placental and recombinant assay, respectively. In some instances, the test chemicals were not soluble at the stated concentrations in the assay mixture. Aromatase activities measured in those cases (noted in Tables B.7-9 and B.7-10) were excluded from the set of data from which response curves were generated. The data was fitted to the model presented in Chapter 5. The resulting response curves are presented in Figures 7.4-1 and 7.4-2 for the placental and recombinant assay, respectively.

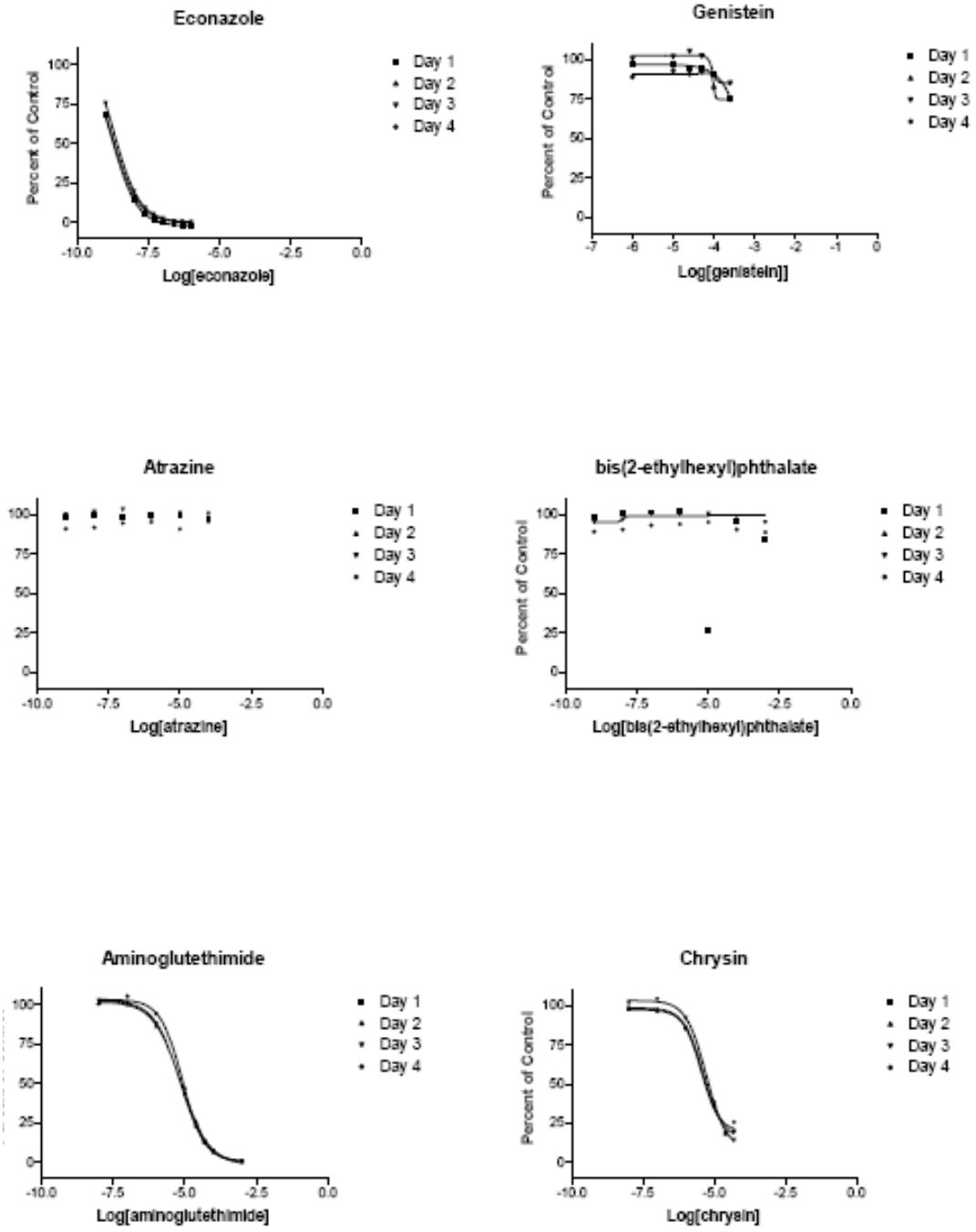



**Figure 7.4-1. Human placental aromatase assay inhibition response curves**

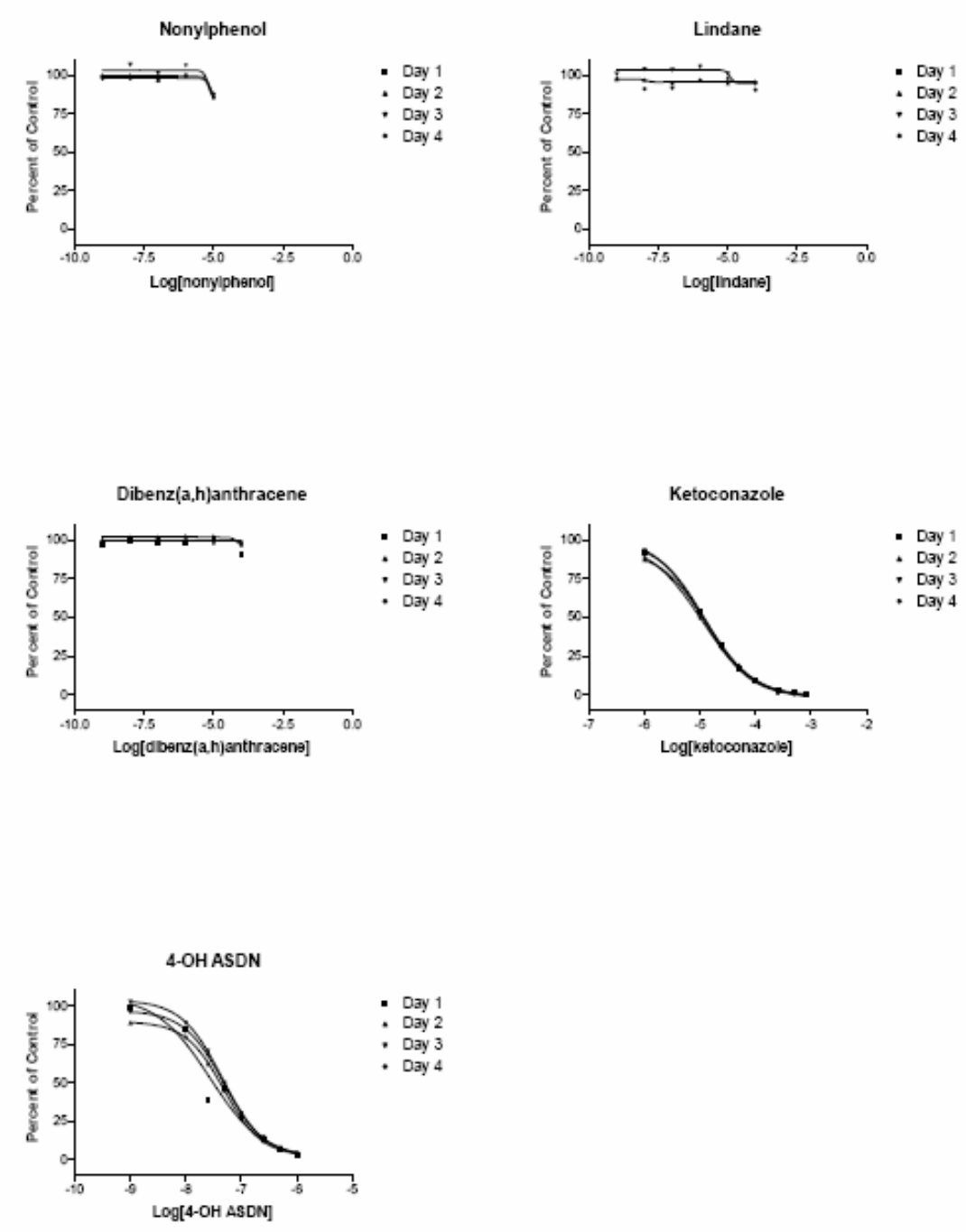



**Figure 7.4-1. Human placental aromatase assay inhibition response curves (cont)**

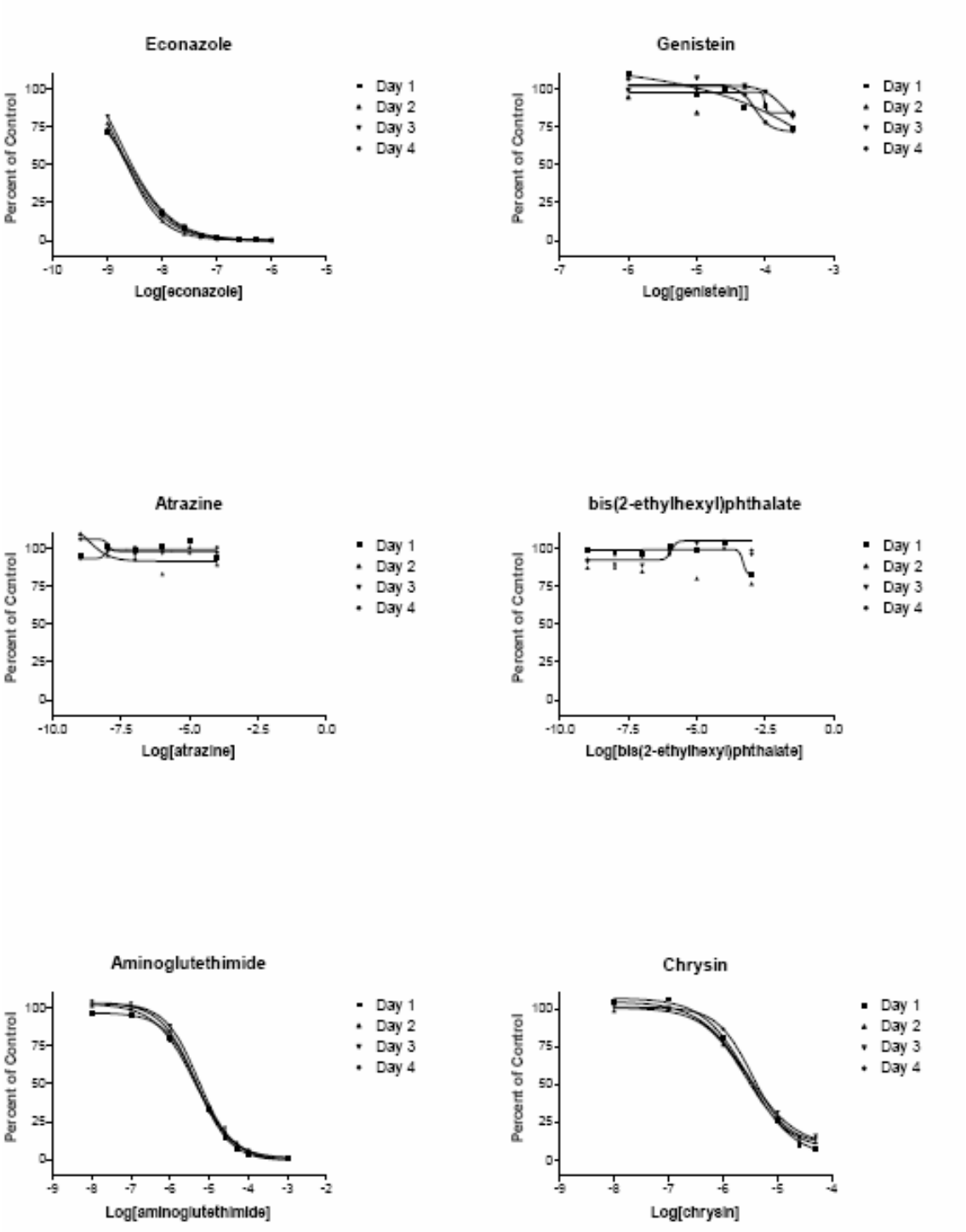



**Figure 7.4-2. Human recombinant aromatase assay inhibition response curves**

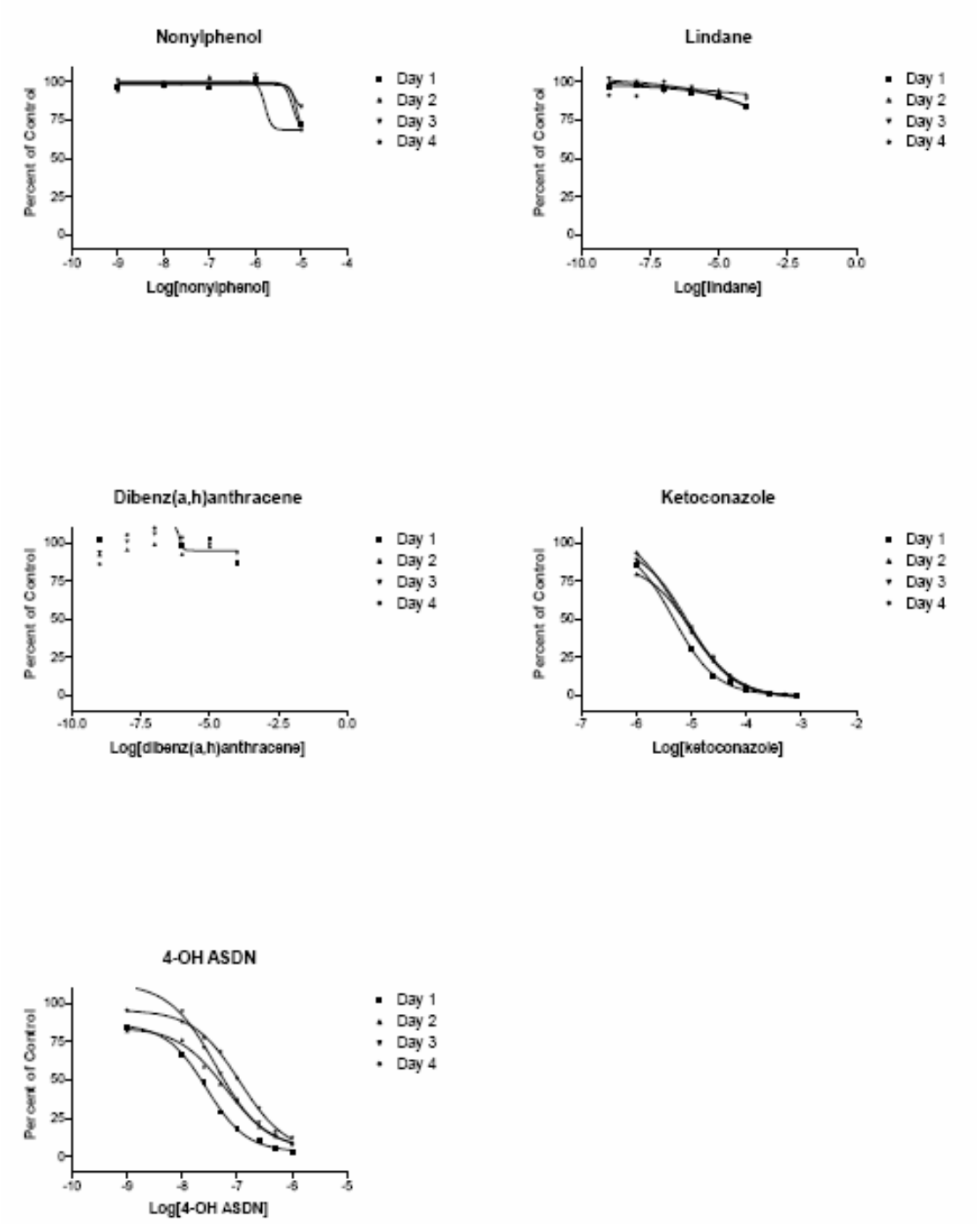



**Figure 7.4.2. Human recombinant aromatase assay inhibition response curves (cont)**

The response curves (Figures 7.4-1 and 7.4-2) for placental and recombinant, respectively) indicate that genistein, atrazine, bis(2-ethylhexyl)phthalate, nonylphenol, lindane and dibenz*(a,h)*anthracene do not significantly inhibit aromatase activity over the range of concentrations tested. Therefore, no meaningful $IC_{50}$ values could be calculated for those compounds. Econazole, aminoglutethimide, chrysin, ketoconazole and 4-OH ASDN did inhibit aromatase activity over the concentration ranges tested.

For those cases where there was a diagnosed problem with the substrate solutions used, econazole (Group 1/Day 2) and aminoglutethimide and chrysin (Group 2, Day 1) mean $IC_{50}$ values and slopes were calculated with the appropriate data excluded. When those data were excluded, no statistically significant ($p < 0.1$) day-to-day compared to within-day variance was found in the Log($IC_{50}$) or slopes for the placental assay. Day–to-day variance of Log($IC_{50}$) for 4-OH ASDN and ketoconazole was significantly greater than within-day variance for the recombinant assay. Summary statistics for Log($IC_{50}$), slope and their standard errors are presented in Table 7.4-2. Only Log($IC_{50}$) varied significantly ($p<0.1$) between assays and then only for aminoglutethimide, chrysin and ketoconazole. Generally, similar $IC_{50}$ values were obtained in both the placental and recombinant assays for each test chemical. The experimentally determined $IC_{50}$ values fall within the literature ranges cited in the protocol for all test chemicals except for econazole and ketoconazole where both of the measured $IC_{50}$ values were lower than the literature value ranges cited in the protocol. However, Ayub and Levell (1988) reported an $IC_{50}$ for ketoconazole of 6 μM and noted that literature values vary widely (7-60 μM). The response curve for genistein did not allow for estimation of a meaningful $IC_{50}$ although genistein has been identified in the literature as an inhibitor of aromatase with a reported $IC_{50}$ of 30-100 μM. White et al. (1999) found that genistein did not inhibit aromatase >20 percent even at concentrations up to 1 mM. Genistein was not soluble in the reaction mixture at the two highest concentrations tested (1 and 0.5 mM), so the activity data measured at those two concentrations were excluded from the response curve model. Even when those data were included, the response curve parameters were highly variant, so a good estimate of $IC_{50}$ was not possible (data not shown).

**Table 7.4-2 Summary statistics for Log ($IC_{50}$), slope and their standard errors**

| Chemical | Variable | Type | N | Mean | Std Error | Lower Limit of Mean | Upper Limit of Mean |
|---|---|---|---|---|---|---|---|
| Econazole | LogIC50 | Placental | 3 | -8.748 | 0.021 | -8.838 | -8.658 |
| Econazole | LogIC50 | Recombinant | 4 | -8.706 | 0.045 | -8.850 | -8.562 |
| Econazole | LogIC50 | Diff (1-2) | _ | -0.042 | 0.056 | -0.186 | 0.103 |
| Econazole | SE_LogIC50 | Placental | 3 | 0.043 | 0.021 | -0.045 | 0.131 |
| Econazole | SE_LogIC50 | Recombinant | 4 | 0.096 | 0.034 | -0.012 | 0.204 |
| Econazole | SE_LogIC50 | Diff (1-2) | _ | -0.053 | 0.044 | -0.165 | 0.060 |
| Econazole | Slope | Placental | 3 | -0.964 | 0.007 | -0.996 | -0.933 |
| Econazole | Slope | Recombinant | 4 | -1.080 | 0.059 | -1.269 | -0.891 |
| Econazole | Slope | Diff (1-2) | _ | 0.116 | 0.071 | -0.066 | 0.297 |
| Econazole | SE_Slope | Placental | 3 | 0.027 | 0.013 | -0.029 | 0.083 |
| Econazole | SE_Slope | Recombinant | 4 | 0.072 | 0.020 | 0.009 | 0.135 |
| Econazole | SE_Slope | Diff (1-2) | _ | -0.045 | 0.026 | -0.111 | 0.021 |
| Aminoglutethimide | LogIC50 | Placental | 3 | -5.118 | 0.022 | -5.211 | -5.025 |
| Aminoglutethimide | LogIC50 | Recombinant | 4 | -5.321 | 0.024 | -5.395 | -5.246 |
| Aminoglutethimide | LogIC50 | Diff (1-2) | _ | 0.202 | 0.033 | 0.117 | 0.288 |
| Aminoglutethimide | SE_LogIC50 | Placental | 3 | 0.025 | 0.007 | -0.004 | 0.055 |
| Aminoglutethimide | SE_LogIC50 | Recombinant | 4 | 0.032 | 0.003 | 0.022 | 0.042 |
| Aminoglutethimide | SE_LogIC50 | Diff (1-2) | _ | -0.006 | 0.007 | -0.024 | 0.011 |
| Aminoglutethimide | Slope | Placental | 3 | -0.993 | 0.071 | -1.299 | -0.687 |
| Aminoglutethimide | Slope | Recombinant | 4 | -0.965 | 0.041 | -1.095 | -0.835 |
| Aminoglutethimide | Slope | Diff (1-2) | _ | -0.028 | 0.077 | -0.225 | 0.170 |
| Aminoglutethimide | SE_Slope | Placental | 3 | 0.055 | 0.022 | -0.038 | 0.148 |
| Aminoglutethimide | SE_Slope | Recombinant | 4 | 0.053 | 0.008 | 0.029 | 0.077 |
| Aminoglutethimide | SE_Slope | Diff (1-2) | _ | 0.003 | 0.020 | -0.049 | 0.054 |
| Chrysin | LogIC50 | Placental | 3 | -5.373 | 0.053 | -5.600 | -5.146 |
| Chrysin | LogIC50 | Recombinant | 4 | -5.528 | 0.022 | -5.596 | -5.459 |
| Chrysin | LogIC50 | Diff (1-2) | _ | 0.155 | 0.051 | 0.024 | 0.286 |
| Chrysin | SE_LogIC50 | Placental | 3 | 0.099 | 0.037 | -0.061 | 0.260 |
| Chrysin | SE_LogIC50 | Recombinant | 4 | 0.076 | 0.012 | 0.039 | 0.113 |
| Chrysin | SE_LogIC50 | Diff (1-2) | _ | 0.023 | 0.034 | -0.064 | 0.111 |
| Chrysin | Slope | Placental | 3 | -1.296 | 0.037 | -1.455 | -1.136 |
| Chrysin | Slope | Recombinant | 4 | -1.105 | 0.087 | -1.382 | -0.827 |
| Chrysin | Slope | Diff (1-2) | _ | -0.191 | 0.108 | -0.468 | 0.085 |
| Chrysin | SE_Slope | Placental | 3 | 0.254 | 0.093 | -0.147 | 0.656 |
| Chrysin | SE_Slope | Recombinant | 4 | 0.144 | 0.012 | 0.106 | 0.183 |
| Chrysin | SE_Slope | Diff (1-2) | | 0.110 | 0.079 | -0.094 | 0.314 |

| Chemical | Variable | Type | N | Mean | Std Error | Lower Limit of Mean | Upper Limit of Mean |
|---|---|---|---|---|---|---|---|
| 4-OH androstenedione | LogIC50 | Placental | 4 | -7.385 | 0.061 | -7.581 | -7.189 |
| 4-OH androstenedione | LogIC50 | Recombinant | 4 | -7.273 | 0.129 | -7.685 | -6.861 |
| 4-OH androstenedione | LogIC50 | Diff (1-2) | _ | -0.112 | 0.143 | -0.462 | 0.239 |
| 4-OH androstenedione | SE_LogIC50 | Placental | 4 | 0.063 | 0.050 | -0.097 | 0.223 |
| 4-OH androstenedione | SE_LogIC50 | Recombinant | 4 | 0.043 | 0.009 | 0.016 | 0.070 |
| 4-OH androstenedione | SE_LogIC50 | Diff (1-2) | _ | 0.020 | 0.051 | -0.105 | 0.144 |
| 4-OH androstenedione | Slope | Placental | 4 | -1.139 | 0.083 | -1.402 | -0.875 |
| 4-OH androstenedione | Slope | Recombinant | 4 | -1.050 | 0.045 | -1.194 | -0.907 |
| 4-OH androstenedione | Slope | Diff (1-2) | _ | -0.088 | 0.094 | -0.319 | 0.143 |
| 4-OH androstenedione | SE_Slope | Placental | 4 | 0.151 | 0.108 | -0.192 | 0.494 |
| 4-OH androstenedione | SE_Slope | Recombinant | 4 | 0.104 | 0.015 | 0.057 | 0.151 |
| 4-OH androstenedione | SE_Slope | Diff (1-2) | _ | 0.047 | 0.109 | -0.219 | 0.313 |
| Ketoconazole | LogIC50 | Placental | 4 | -4.928 | 0.023 | -5.001 | -4.855 |
| Ketoconazole | LogIC50 | Recombinant | 4 | -5.152 | 0.074 | -5.389 | -4.915 |
| Ketoconazole | LogIC50 | Diff (1-2) | _ | 0.224 | 0.078 | 0.033 | 0.415 |
| Ketoconazole | SE_LogIC50 | Placental | 4 | 0.025 | 0.004 | 0.011 | 0.039 |
| Ketoconazole | SE_LogIC50 | Recombinant | 4 | 0.054 | 0.020 | -0.008 | 0.117 |
| Ketoconazole | SE_LogIC50 | Diff (1-2) | _ | -0.029 | 0.020 | -0.079 | 0.020 |
| Ketoconazole | Slope | Placental | 4 | -0.995 | 0.031 | -1.092 | -0.897 |
| Ketoconazole | Slope | Recombinant | 4 | -1.027 | 0.048 | -1.179 | -0.874 |
| Ketoconazole | Slope | Diff (1-2) | _ | 0.032 | 0.057 | -0.108 | 0.171 |
| Ketoconazole | SE_Slope | Placental | 4 | 0.055 | 0.005 | 0.039 | 0.070 |
| Ketoconazole | SE_Slope | Recombinant | 4 | 0.085 | 0.021 | 0.019 | 0.152 |
| Ketoconazole | SE_Slope | Diff (1-2) | | -0.030 | 0.021 | -0.083 | 0.022 |

### 7.5 Supplementary Studies

In the prevalidation studies, the recombinant assay showed higher variability than the placental assay. This was unexpected and EPA decided that this matter should be pursued in additional studies. EPA was also concerned that at the concentrations tested, econazole and ketoconazole only generated partial inhibition curves, and EPA wanted these substances tested at concentrations that would generate complete inhibition curves. Thus, the primary objectives of this series of studies were to investigate the high variability found for 4-OH ASDN in the recombinant assay and generate a complete inhibition curve for econazole and ketoconazole; however, EPA also used this opportunity to demonstrate the equivalence of the tritiated water and estrone methods of analysis.

#### 7.5.1 Day-to-Day and Technician Variability Study

Day-to-day and technician variabilities were examined in both microsomal aromatase assays (human placental and human recombinant) using the optimized conditions determined in the pre-validation optimization study. A study (Table 7.5-1) 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 7.5-1. 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 |

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 x $10^{-7}$, $10^{-7.5}$ and 2.5 x $10^{-8}$, $10^{-8}$, and $10^{-9}$ M. In addition, full enzyme activity and background control samples were included for each study. Full enzyme activity controls contained substrate, NADPH, propylene glycol, buffer, vehicle (20 µL EtOH) and microsomes. Background activity controls contained all full activity control assay components except NADPH. Four full activity and four background controls were included with each study. The controls sets were split so that two tubes (of each full activity and background controls) were run at the beginning and two at the end of each study set.

Within a technician and day, full enzyme activity control values from samples run at the end of the assay set were always slightly (~ 10 percent) 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, full enzyme activity control values varied (average coefficient of variance was 15 percent) from technician to technician. This may have been due to slight differences in microsome dilution preparation. Most differences in aromatase activities in the in full activity controls were minor and even more so when normalized to percent control activities for the $IC_{50}$ determination.

The analysis of variance (ANOVA) results for the control samples are presented in Table 7.5-2. The analysis of the control samples shows no significant variance in the background control activities by technician, day, or portion (beginning vs. end) and no significant variance in the interaction between day and portion. Mean background control activities were near zero for all studies. In a few cases, higher background control activities were measured, probably due to inadvertent cross contamination of the negative control tubes with small amounts of NADPH. Full enzyme activity control values show significant variance by technician, day and portion but no significant variance in the interaction between day and portion (Reference 3).

**Table 7.5-2. ANOVA results for the control activities**

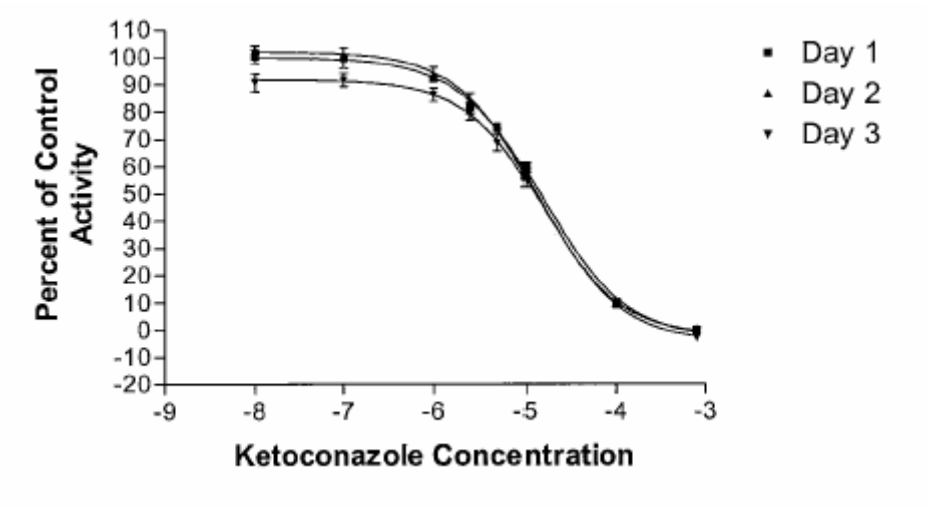
| Microsome Type | Control Type | Source | DF | Type I SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|---|---|
| Placental | Negative | Technician | 2 | 3.257E-33 | 1.628E-33 | 0.00 | 1.0000 |
| | | Day(Technician) | 4 | 8.844E-33 | 2.211E-33 | 0.00 | 1.0000 |
| | | Portion | 1 | 1.816E-05 | 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 |

Differences among technicians and day-to-day variability within technicians for enzymatic activity at various inhibitor concentrations were estimated and are presented in Tables B.7-11 and B.7-12 in Appendix B. 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 runs 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. No significant technician-to-technician variation was detected for log $IC_{50}$, slope, or their associated standard errors for either microsome type. For additional data, see Reference 3.

### 7.5.2 Inhibition Curves for Econazole and Ketoconazole

EPA decided to re-run the assay for econaole and ketoconazole because the testing of these substances, as described above in Section 7.4.2, failed to produce complete inhibition curves. Aromatase activity was assayed in both microsome types in the presence of varying concentrations of ketoconazole and econazole, the concentrations used for econazole being two orders of magnitude lower than those used in the studies described in Section 7.4 (Reference 2). Each inhibitor was assayed in triplicate on three separate days. Graphs of aromatase activity as percent of control activity versus ketoconazole and econazole concentrations are presented in Figures 7.5-1 and 7.5-2, respectively. These data are also presented in Tables 7.5-3 and 7.5-4.

**Placental**




**Recombinant**

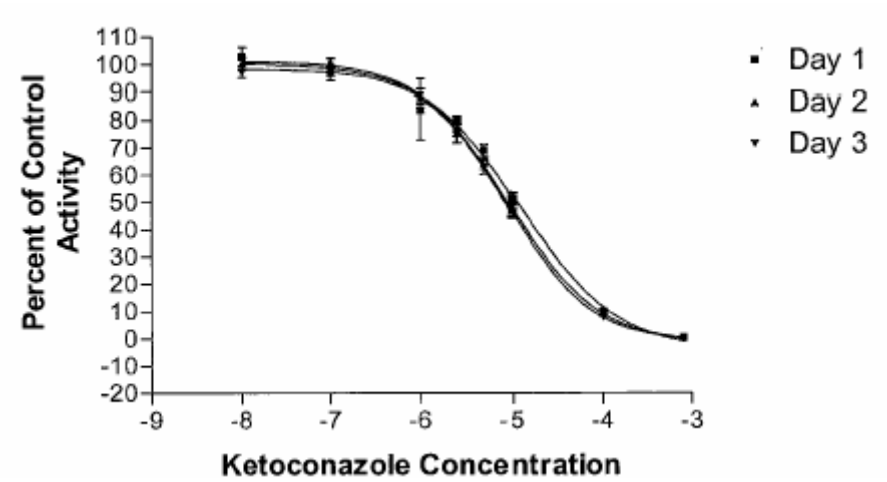
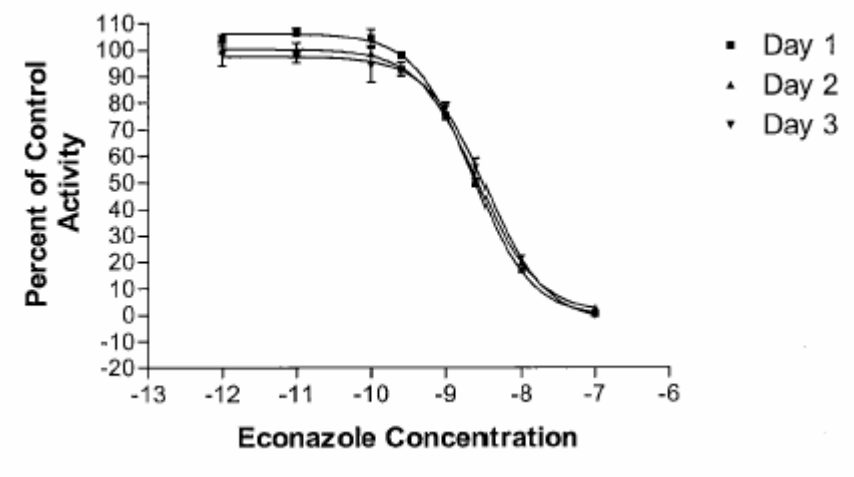



**Figure 7.5-1. Ketoconazole inhibition curves**
**Concentration units are log (M)**

**Table 7.5-3. Aromatase activity in the presence of ketoconazole (percent of full control activity)**

| Concentration Code | Ketoconazole Concentration (Log(M)) | Day 1 Replicate 1 | Day 1 Replicate 2 | Day 1 Replicate 3 | Day 2 Replicate 1 | Day 2 Replicate 2 | Day 2 Replicate 3 | Day 3 Replicate 1 | Day 3 Replicate 2 | Day 3 Replicate 3 |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Placental | | | | | | | | |
| 1 | -3.1 | -0.2 | -0.1 | -0.1 | 0.2 | 0.2 | 0.1 | -2.9 | -2.8 | -2.8 |
| 2 | -4.0 | 10.7 | 10.4 | 9.4 | 10.1 | 8.8 | 8.8 | 10.2 | 10.6 | 8.3 |
| 3 | -5.0 | 60.5 | 60.6 | 57.6 | 58.3 | 58.5 | 54.8 | 57.8 | 53.0 | 54.1 |
| 4 | -5.3 | 75.0 | 73.2 | 75.6 | 74.2 | 74.5 | 72.4 | 71.8 | 68.0 | 65.9 |
| 5 | -5.6 | 78.9 | 82.1 | 82.6 | 85.4 | 85.5 | 80.7 | 83.4 | 77.4 | 79.2 |
| 6 | -6.0 | 91.2 | 91.8 | 93.3 | 95.8 | 94.5 | 90.6 | 88.9 | 84.5 | 85.2 |
| 7 | -7.0 | 103.7 | 98.8 | 96.5 | 100.9 | 101.9 | 99.5 | 93.4 | 92.6 | 88.7 |
| 8 | -8.0 | 102.4 | 99.8 | 98.0 | 102.9 | 103.9 | 100.9 | 92.6 | 92.7 | 86.8 |
| | | Recombinant | | | | | | | | |
| 1 | -3.1 | 0.4 | 0.2 | 0.3 | 0.2 | 0.2 | 0.3 | 0.1 | 0.0 | 0.1 |
| 2 | -4.0 | 10.6 | 10.5 | 8.7 | 9.0 | 9.7 | 8.5 | 8.9 | 7.8 | 7.2 |
| 3 | -5.0 | 54.0 | 52.8 | 47.6 | 49.9 | 48.2 | 42.6 | 49.7 | 45.4 | 42.9 |
| 4 | -5.3 | 72.7 | 68.9 | 65.7 | 66.7 | 65.7 | 60.0 | 67.1 | 63.1 | 58.0 |
| 5 | -5.6 | 81.3 | 81.6 | 77.2 | 81.1 | 76.0 | 73.0 | 80.4 | 75.4 | 69.1 |
| 6 | -6.0 | 99.6 | 88.8 | 63.0 | 93.1 | 88.5 | 82.1 | 91.5 | 90.5 | 83.4 |
| 7 | -7.0 | 100.8 | 97.9 | 94.0 | 102.8 | 101.7 | 97.3 | 100.8 | 103.2 | 90.2 |
| 8 | -8.0 | 107.8 | 104.0 | 96.1 | 102.5 | 102.4 | 95.7 | 97.7 | 100.2 | 93.3 |

**Placental**




**Recombinant**

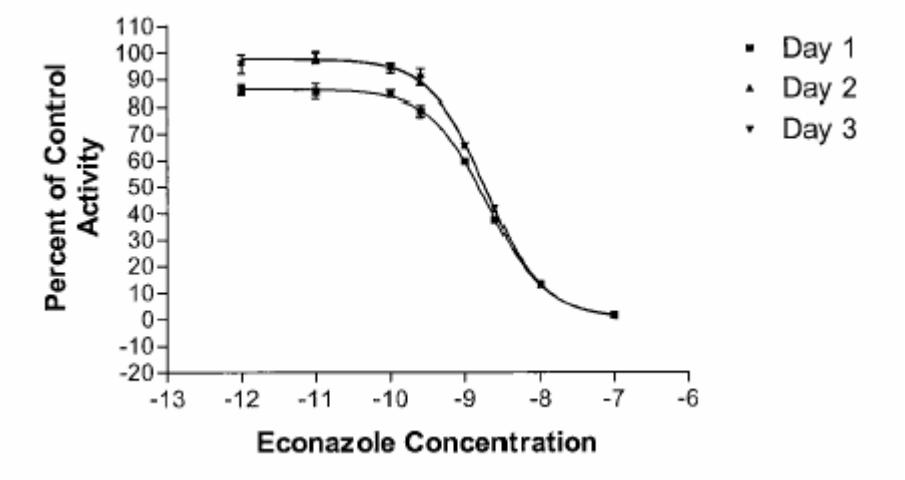



**Figure 7.5-2. Econazole inhibition curves**
**Concentration units are log(M)**

**Table 7.5-4. Aromatase activity in the presence of econazole (percent of full control activity)**

| Concentration Code | Econazole Concentration (Log(M)) | Day 1 Replicate 1 | Day 1 Replicate 2 | Day 1 Replicate 3 | Day 2 Replicate 1 | Day 2 Replicate 2 | Day 2 Replicate 3 | Day 3 Replicate 1 | Day 3 Replicate 2 | Day 3 Replicate 3 |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Placental | | | | | | | | |
| 1 | -7.0 | 0.7 | 0.4 | 0.7 | 2.3 | 2.4 | 2.4 | 0.1 | 0.0 | -0.2 |
| 2 | -8.0 | 17.9 | 16.8 | 16.2 | 19.8 | 20.1 | 19.9 | 21.5 | 21.2 | 21.4 |
| 3 | -8.6 | 49.4 | 50.1 | 49.2 | 51.6 | 51.5 | 51.7 | 51.1 | 57.4 | 57.9 |
| 4 | -9.0 | 74.1 | 75.8 | 76.0 | 74.0 | 75.5 | 75.2 | 80.4 | 77.3 | 76.5 |
| 5 | -9.6 | 97.7 | 99.2 | 97.1 | 94.0 | 94.5 | 89.9 | 93.9 | 91.1 | 90.7 |
| 6 | -10.0 | 101.3 | 104.8 | 107.6 | 98.6 | 99.1 | 98.0 | 97.4 | 98.5 | 86.7 |
| 7 | -11.0 | 108.0 | 106.9 | 105.4 | 101.9 | 100.9 | 97.2 | 99.3 | 97.0 | 95.3 |
| 8 | -12.0 | 105.6 | 102.4 | 103.8 | 101.3 | 99.0 | 99.4 | 103.2 | 97.8 | 94.5 |
| | | Recombinant | | | | | | | | |
| 1 | -7.0 | 1.4 | 1.5 | 1.6 | 1.5 | 1.6 | 1.6 | 1.6 | 1.5 | 1.8 |
| 2 | -8.0 | 13.1 | 13.1 | 13.2 | 13.6 | 12.8 | 12.7 | 13.7 | 13.7 | 13.0 |
| 3 | -8.6 | 38.1 | 38.3 | 36.3 | 42.4 | 41.7 | 40.6 | 42.2 | 41.6 | 41.7 |
| 4 | -9.0 | 61.4 | 59.5 | 58.0 | 67.5 | 65.8 | 63.5 | 64.3 | 67.2 | 64.7 |
| 5 | -9.6 | 83.4 | 75.7 | 76.6 | 91.4 | 89.7 | 86.4 | 96.4 | 89.8 | 85.7 |
| 6 | -10.0 | 87.1 | 84.4 | 82.6 | 96.4 | 92.0 | 95.7 | 93.3 | 96.3 | 91.5 |
| 7 | -11.0 | 90.7 | 84.6 | 81.1 | 101.3 | 97.5 | 94.7 | 101.0 | 97.4 | 99.7 |
| 8 | -12.0 | 89.9 | 83.7 | 84.5 | 99.0 | 95.9 | 95.0 | 102.2 | 94.9 | 89.9 |

The data demonstrate that there is little variance between replicate tubes (at each inhibitor concentration) within a study and across all inhibitor concentrations across three independently conducted assays (days). There was no discernable pattern toward differing variances between microsome types or inhibitors.

Complete inhibition curves for the interaction of the inhibitors ketoconazole and econazole with human placental and recombinant aromatase (CYP19) activity were established (Reference 4). Ketoconazole was tested over the range 10E-4 to 10E-8 M; econazole was assayed over the range 10E-7 to 10E-12 M. The measured $IC_{50}$ for ketoconazole in the recombinant assay was 10.08 ± 1.85 μM, while that for the placental assay was 15.00 ± 1.67 μM. These values are about one-sixth to one-fourth the literature value of >65 μM as discussed in Reference 2. However, White et al. (1999) reported an $IC_{50}$ for ketoconazole of 6 μM and noted that literature values vary widely (7-60 μM). Reference (5) reported an $IC_{50}$ for ketoconazole in human placental microsomes of 15 μM while Ayub and Levell (1988) reported an $IC_{50}$ of 7.3 μM for this inhibitor. The measured $IC_{50}$ for econazole in the recombinant assay was 1.93 ± 0.06 nM, while that for the placental assay was 2.79 ± 0.60 nM. These values are an order of magnitude less than the literature values of 30 to 50 nM reported in Reference 2. There was little variance in the $IC_{50}$ measured for a particular inhibitor using either microsome type. Table 7.5-5 shows the $IC_{50}$s by microsome type.

**Table 7.5-5. $IC_{50}$s by microsome type**

| Inhibitor | Microsome Type | Study | $IC_{50}$ | $IC_{50}$ Mean ± SD (CV) (by microsome type) |
|---|---|---|---|---|
| Econazole | Recombinant | 1 | 2.00 nM | |
| | | 2 | 1.89 nM | |
| | | 3 | 1.90 nM | 1.93 ± 0.06 nM (3.1%) |
| | Placental | 4 | 2.27 nM | |
| | | 5 | 2.65 nM | |
| | | 6 | 3.44 nM | 2.79 ± 0.60 nM (21.5%) |
| Ketoconazole | Recombinant | 7 | 12.12 µM | |
| | | 8 | 9.04 µM | |
| | | 9 | 8.98 µM | 10.05 ± 1.80 µM (17.0%) |
| | Placental | 10 | 15.38 µM | |
| | | 11 | 13.16 µM | |
| | | 12 | 16.44 µM | 14.99 ± 1.67 µM (11.1%) |

### 7.5.3 Comparison of Estrone and Tritiated Water Methods of Analysis

The estrone content of samples from the recombinant aromatase assay was measured by RIA and compared with the nmol of $^3H_20$ formed in the tritiated water assay (Reference 5). One mole of $^3H_20$ is formed for each mole of estrone formed in the aromatization of androstenedione. Generally, the amount of tritiated water measured in the assays was about 3-fold higher than the amount of estrone measured. At some high concentrations of inhibitor, the ratio was reduced, perhaps due to inhibition of another enzyme that may be involved in the further metabolism of estrone. It is possible that estrone may be further metabolized to another component that may not be detectable using RIA. Further investigations would be necessary to determine the source of the apparent discrepancy between the amount of estrone and $^3H_2O$ formed in microsomes.

Aromatase activity in the recombinant microsomes in the presence of ketoconazole or econazole was calculated based on estrone content measured by RIA. $IC_{50}$s calculated based on these activities were similar to those obtained based on the $^3H_2O$ release assay. Therefore, it appears that application of either method (estrone RIA or $^3H_2O$) gives similar results. Both methods require the use of radiolabeled materials, but the RIA requires the use of the higher energy (but lower half-life) 121 isotope. While the entire $^3H_2O$ assay can be completed within one day, an additional day may be required to analyze incubation aliquots by RIA. For these reasons, EPA selected the $^3H_2O$ assay for screening aromatase activity for the Endocrine Disruptor Screening Program.

# 8.0 Preliminary Interlaboratory Validation Studies

With confidence that both protocols were performing satisfactorily and obtaining good agreement, EPA decided to commence interlaboratory testing of the two protocols. EPA planned to have 4 to 5 laboratories conduct blinded studies with both the placental and recombinant microsomes on the 10 reference chemicals used previously in the prevalidation studies. Positive control studies (baseline hormone production and detection of inhibition with the positive control, 4-hydroxyandrostenedione) would be run to demonstrate the ability of the laboratories to conduct the protocol, after which studies with four reference chemicals would be conducted with placental microsomes prepared by the lead laboratory as described below in Section 8.3. Following this task, two laboratories would be selected to prepare placental microsomes. These would be distributed to all laboratories for the conduct of the main validation study using placental microsomes on all 10 chemicals. The main validation study would be repeated with the recombinant microsomes on the same 10 chemicals.

## 8.1 Selection and Training of Participating Laboratories

Battelle, the prime contractor to EPA for the validation of the aromatase assay, selected three contract laboratories (Battelle, In Vitro Technologies, and WIL Research Laboratories) to participate in the interlaboratory validation study. RTI, which conducted the prevalidation studies, served as the lead laboratory for the aromatase assay validation studies. Study directors and key technical staff of the participating laboratories were trained in the RTI laboratory. Training was conducted for the placental aromatase assay since it requires the additional step of microsome preparation. Table 8.1-1 lists the code designations and abbreviations for the participating laboratories.

**Table 8.1-1. Identification of Laboratories in the Preliminary Interlaboratory Studies**

| Lab ID | Laboratory Name | Abbreviation |
|---|---|---|
| RTI | RTI International | RTI |
| Lab A | In Vitro Technologies | In Vitro |
| Lab B | WIL Research Laboratories | WIL |
| Lab C | Battelle Memorial Institute | Battelle |

## 8.2 Positive Control Studies with Placental Microsomes in Participating Laboratories

Subsequent to training, positive control studies were conducted by staff from the three participating laboratories in both assays; however, only the placental aromatase assay studies are discussed in this section. The recombinant aromatase assays will be discussed in Section 8.5.

Each of the participating laboratories conducted at least three independent runs 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 replicates at each

of six concentrations of the 4-OH ASDN were tested for each of the three independent runs. Reagents and assay solutions were made fresh for each run so that the runs 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 the positive control studies (Table 8.2-1) were to evaluate the responsiveness of the human placental microsomal aromatase assay and the recombinant assay when performed by three participating laboratories that had the capability but only limited experience with this assay and 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.

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 replicates (within a single run) and between runs.

**Table 8.2-1. Positive control study design**

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

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

The $IC_{50}$ was calculated using Prism (version 4.0 or higher, GraphPad, San Diego, CA) as described in Chapter 5. Details of the intralaboratory data and statistical analysis are described in the individual participating laboratory reports included in the Positive Control Study Report (Reference 7)

The intralaboratory statistical analysis was done by the Data Coordination Center at Battelle for two of the three participating laboratories (Battelle and In Vitro) and the reports for these laboratories are included in their respective reports that can be found in the appendices to Reference 7. 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 to Reference 7.

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 run, times 100. The average percent of control among the four full enzyme activity control tubes is necessarily 100 percent within each run. The average percent of control among the four background activity control tubes is necessarily 0 percent.

### 8.2.1 Determination of Microsomal Protein

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 runs whereas the third laboratory reported analysis results for four runs (Table 8.2-2). 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 8.2-2. Human placental microsomal protein concentration determinations by run and laboratory[a]**

| Run | 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 |

### 8.2.2 Human Placental Aromatase Full Activity Controls

Full enzyme activity controls were conducted in duplicate replicates at the beginning and end of each run of the assay (a total of four tubes/run). Two of the laboratories reported analysis results for three runs whereas the third laboratory reported analysis results for four runs. The average full aromatase activity control values for all four replicates of a given run are shown in

Table 8.2-3 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 8.2.-3. Human placental aromatase activity control determinations by run and laboratory[a]**

| Run | Aromatase Activity (nmol/mg protein/min) | | |
|---|---|---|---|
| | 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 |

For each laboratory, the number of runs actually used in the analysis of the task was included

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 B.8-1 in Appendix B). The estimated variance among the laboratories for the background activity controls was near 0. 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.

### 8.2.3 Background Activity Controls

Background enzyme activity controls were conducted in duplicate replicates at the beginning and end of each run of the assay (a total of four-tubes/run). 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 B8-1 and B.8-2 in Appendix B.

### 8.2.4 Positive Control Values

Nominally for an inhibitor the percent of control activity values vary between approximately 0 percent near the high inhibition concentrations and approximately 100 percent 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 run 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 run.

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

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

Results for Laboratories A and B were reported based on three runs, while Laboratory C provided results based on runs 1 to 4, as well as results based on runs 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 runs for Laboratories A and C incorporated the among-run component of variation, while that for Laboratory B did not.

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 run 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 log10IC50 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 run 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 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 overall percent of control results by laboratory and the overall percent of control results for the task are summarized in Table 8.2-4.

**Table 8.2-4. 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 overall inhibition response curves by laboratory are shown in Figure 8.2-1. The curves in this figure 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 AS68DN 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.

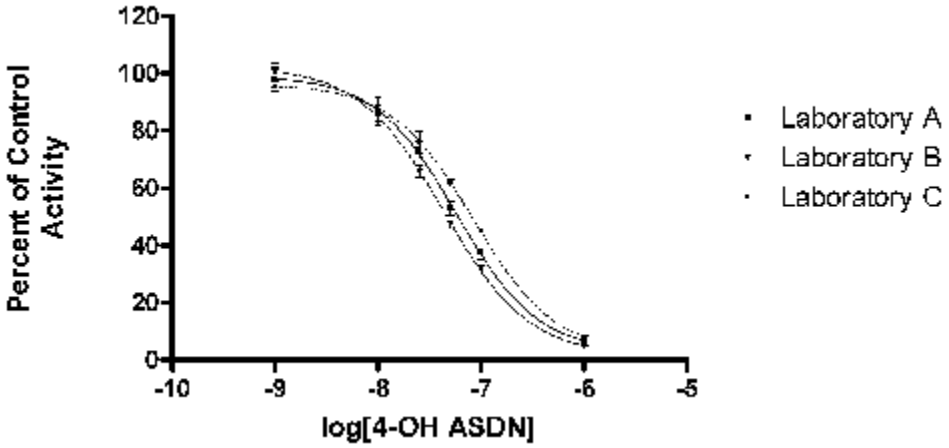



**Figure 8.2-1. Overall 4-OH ASDN inhibition response curve by laboratory**

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 run and laboratory are summarized in Table 8.2-5. 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 8.2-5. $IC_{50}$ values by run and laboratory[a]**

| Run | $IC_{50}$ Values | | |
|---|---|---|---|
| | 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 runs actually used in the analysis of the task was included.

The slope determinations by run and laboratory are summarized in Table 8.2-6 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 8.2-6. Slope values by run and laboratory[a]**

| Run | Slope Values | | |
|---|---|---|---|
| | 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 runs actually used in the analysis of the task was included.

Table B.8-1, shown in Appendix B, displays the estimated parameter values ($\log_{10}IC_{50}$ and for the slope) and associated within laboratory 95 percent confidence intervals about these values. It also displays the overall mean values across laboratories and their associated 95 percent confidence intervals, incorporating among laboratory variation based on the random effects analysis of variance. The overall mean was calculated with and without run 1 for Laboratory C and with and without the full enzyme activity control outlying value for laboratory A.

Table B.8-2 (Appendix B) 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 B.8-2 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 percent confidence intervals.

The results for the slope estimates were consistent among the three laboratories. The estimated variance among the laboratories was zero or near zero. The coefficients of variation among laboratories were 3.7 percent when run 1 in Laboratory C was included and 3.2 percent when run 1 in Laboratory C was excluded.

## 8.3 Studies with Placental Microsomes Prepared in the Lead Laboratory

In this task, the placental aromatase assay was conducted by staff from a lead laboratory (RTI International) and two participating laboratories (Battelle and WIL Research Laboratories). A third participating laboratory was planned, but scheduling constraints precluded its participation in this task. Each laboratory conducted at least three independent runs of the placental aromatase assay with aminoglutethimide, chrysin, econazole, and ketoconazole (reference chemicals), 4-hydroxyandrostenedione (positive control) and lindane (negative control). The human placental microsomes were prepared by RTI. The study design involved, for each reference chemical, conducting three replicates at each of eight concentrations, and for the positive and negative controls, conducting three replicates at a single concentration, for each of at least three independent runs. Reagents and assay solutions were made fresh for each run so that the runs

were truly independent. The reference chemicals and positive and negative controls were prepared and analyzed at a central laboratory (Chemical Repository at Battelle) before they were distributed to the laboratories.

An analysis of the microsomal protein analysis, full enzyme activity controls, background controls and positive controls were conducted prior to testing as described above in Section 8.2. The results of these analyses are not presented here but may be found in Reference 8.

### 8.3.1 Aminoglutethimide

The effect of increasing the concentrations of aminoglutethimide on aromatase activity was determined and the results were expressed as a percent of the control aromatase activity. The individual run percent of control results for each laboratory can be found in the appendices to Reference 8. The overall percent of control results by laboratory and the overall percent of control results for the task are summarized in Table 8.3-1.

The overall inhibition response curves by laboratory are shown in Figure 8.3-1. The curves in this figure 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 aminoglutethimide decreased the activity of the placental microsomal aromatase activity and the decrease was concentration-dependent. The shape of the enzyme activity vs aminoglutethimide cure was sigmoidal. At an aminoglutethimide concentration of $10^{-4}$ M, aromatase inhibition was almost complete; the laboratory percent of control values ranged from 4 to 5 percent. In contrast, at an aminoglutethimide concentration of $10^{-8}$ M, there was little to no aromatase inhibition; the laboratory percent of control values ranged from 98 to 100 percent. Overall task mean ± SEM percent of control values at $10^{-4}$ and $10^{-8}$ M were 4.50 ± 0.24 and 98.82 (n=2) percent, respectively. Table 8.3-2 shows the $IC_{50}$ and slope values for aminoglutethimide.

**Table 8.3-1. Effect of aminoglutethimide on aromatase activity (percent of control) by laboratory**

| Laboratory | Log Aminoglutethimide Conc (M) | Overall Percent of Control by Laboratory | | | |
|---|---|---|---|---|---|
| | | Mean | sd | SEM | %CV |
| RTI | -3.00 | 0.35 | 0.01 | 0.01 | 2.51 |
| | -4.00 | 4.38 | 0.14 | 0.08 | 3.14 |
| | -5.00 | 30.92 | 0.93 | 0.54 | 3.00 |
| | -5.30 | 47.73[b] | -- | -- | -- |
| | -5.60 | 63.18[b] | -- | -- | -- |
| | -6.00 | 78.09 | 2.53 | 1.46 | 3.24 |
| | -7.00 | 95.17 | 0.83 | 0.48 | 0.87 |
| | -8.00 | 97.93 | 1.38 | 0.80 | 1.41 |
| | -9.00 | NC[a] | NC | NC | NC |
| | -10.0 | NC | NC | NC | NC |
| Lab B | -3.00 | 0.91 | 1.73 | 1.00 | 190.45 |
| | -4.00 | 4.16 | 0.89 | 0.51 | 21.38 |
| | -5.00 | 30.61 | 2.15 | 1.24 | 7.04 |
| | -5.12 | 38.01[b] | -- | -- | -- |
| | -5.60 | 63.70[b] | -- | -- | -- |
| | -6.00 | 79.25 | 2.87 | 1.66 | 3.62 |
| | -7.00 | 95.40 | 2.76 | 1.59 | 2.89 |
| | -8.00 | NC | NC | NC | NC |
| | -9.00 | 95.84 | 3.36 | 1.94 | 3.50 |
| | -10.0 | NC | NC | NC | NC |
| Lab C | -3.00 | 0.39 | 0.13 | 0.06 | 34.35 |
| | -4.00 | 4.95 | 2.47 | 1.10 | 49.78 |
| | -5.00 | 30.46 | 10.11 | 4.52 | 33.20 |
| | -5.30 | 46.66 | 12.23 | 6.12 | 26.22 |
| | -5.60 | 63.03 | 12.91 | 6.45 | 20.48 |
| | -6.00 | 79.15 | 7.77 | 3.48 | 9.82 |
| | -7.00 | 92.20 | 8.79 | 3.93 | 9.53 |
| | -8.00 | 99.71 | 4.15 | 1.85 | 4.16 |
| | -9.00 | NC | NC | NC | NC |
| | -10.0 | NC | NC | NC | NC |
| Overall Task | Log Aminoglutethimide Conc (M) | Overall Mean | Overall sd[b] | Overall SEM[b] | Overall %CV[b] |
| | -3.00 | 0.55 | 0.31 | 0.18 | 56.8 |
| | -4.00 | 4.50 | 0.41 | 0.24 | 9.1 |
| | -5.00 | 30.66 | 0.23 | 0.14 | 0.8 |
| | -5.12 | (38.01)[c] | -- | -- | -- |
| | -5.30 | 47.20 | -- | -- | -- |
| | -5.60 | 63.30 | 0.35 | 0.20 | 0.6 |
| | -6.00 | 78.83 | 0.64 | 0.37 | 0.8 |
| | -7.00 | 94.26 | 1.78 | 1.03 | 1.9 |
| | -8.00 | 98.82 | -- | -- | -- |
| | -9.00 | (95.84)[c] | -- | -- | -- |
| | -10.00 | -- | -- | -- | -- |

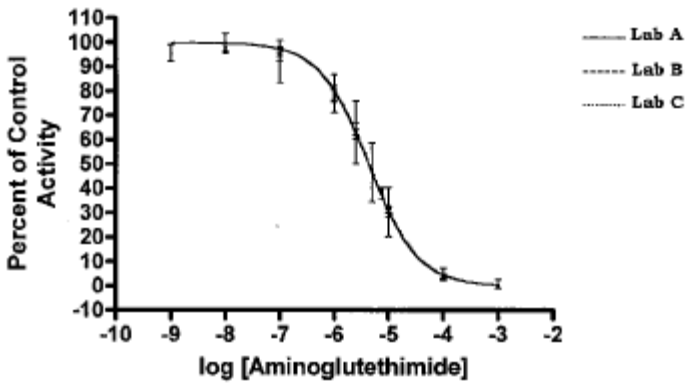



**Figure 8.3-1. Inhibition curve for aminogluthethimide**

**Table 8.3-2. Aminoglutethimide $IC_{50}$ and slope values**

| Run | $IC_{50}$ Values | | | Slope | | |
|---|---|---|---|---|---|---|
| | RTI | Lab B | Lab C | RTI | Lab B | Lab C |
| 1 | 4.09 | 3.98 | 3.39 | -0.9702 | -1.011 | -1.018 |
| 2 | 4.23 | 3.92 | 2.14 | -0.9578 | 0.9202 | -0.9532 |
| 3 | 4.46 | 4.93 | 3.66 | -0.9904 | -0.9984 | -0.9816 |
| 4 | -- | -- | 6.91 | -- | -- | -0.9950 |
| 5 | -- | -- | 6.60 | -- | -- | -0.9592 |
| Mean | 4.26 | 4.28 | 4.54 | -0.9728 | -0.9765 | -0.9814 |
| SD | 0.19 | 0.57 | 2.10 | 0.0165 | 0.0492 | 0.0265 |
| SEM | 0.11 | 0.33 | 0.94 | 0.0095 | 0.0284 | 0.0119 |
| %CV | 4.4 | 13.2 | 46.4 | 1.7 | 5.0 | 2.7 |

### 8.3.2 Chrysin

The effect of increasing the concentrations of chrysin on aromatase activity was determined and the results were expressed as a percent of the control aromatase activity. The individual run percent of control results for each laboratory can be found in the appendices to Reference 8. The overall percent of control results by laboratory and the overall percent of control results for the task are summarized in Table 8.3-3.

The individual chrysin inhibition response curves by run for each laboratory are reported in the appendices. The overall inhibition response curves by laboratory are shown in Figure 8.3-2. The curves in this figure 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 chrysin decreased the activity of the placental microsomal aromatase activity and the decrease was concentration-dependent. The shape of the enzyme activity vs. chrysin curve was sigmoidal. At a chrysin concentration of $10^{-4}$ M, aromatase inhibition was not complete; the laboratory percent of control values ranged from 9 to 29 percent. In contrast, at a chrysin concentration of $10^{-7}$ M, there was little to no aromatase inhibition; the laboratory percent of control values ranged from 93 to 97 percent. Overall task mean ± SEM percent of control values at $10^{-4}$ and $10^{-7}$ M were 20.53 ± 6.16 and 95.15 ± 0.94 percent, respectively. Table 8.3-4 shows the $IC_{50}$ and slope values for chrysin.

**Table 8.3-3. Effect of chrysin on aromatase activity (percent of control) by laboratory**

| Laboratory | Log Chrysin Conc (M) | Overall Percent of Control by Laboratory | | | |
|---|---|---|---|---|---|
| | | Mean | sd | SEM | %CV |
| RTI | -4.00 | 23.65 | 2.25 | 1.30 | 9.53 |
| | -5.00 | 28.20 | 2.50 | 1.44 | 8.86 |
| | -5.30 | 45.20 | 1.74 | 1.23 | 3.85 |
| | -5.60 | 62.29[b] | -- | -- | -- |
| | -6.00 | 78.20[b] | -- | -- | -- |
| | -6.30 | 85.90 | 2.48 | 1.43 | 2.88 |
| | -7.00 | 95.35 | 2.03 | 1.17 | 2.13 |
| | -8.00 | 96.66 | 0.58 | 0.34 | 0.60 |
| | -9.00 | NC[a] | NC | NC | NC |
| | -10.0 | NC | NC | NC | NC |
| Laboratory B | -4.00 | 8.65 | 6.11 | 3.53 | 70.59 |
| | -5.00 | 25.44 | 3.03 | 1.75 | 11.92 |
| | -5.30 | 38.30[b] | -- | -- | -- |
| | -5.60 | 56.08[b] | -- | -- | -- |
| | -6.00 | 77.95 | 6.04 | 3.49 | 7.75 |
| | -6.30 | 99.22[b] | -- | -- | -- |
| | -7.00 | 96.67 | 8.99 | 5.19 | 9.30 |
| | -7.30 | NC | NC | NC | NC |
| | -8.00 | 99.95 | 10.98 | 6.34 | 10.98 |
| | -9.00 | NC | NC | NC | NC |
| | -10.0 | NC | NC | NC | NC |
| Laboratory C | -4.00 | 29.29 | 6.28 | 3.14 | 21.46 |
| | -5.00 | 29.50 | 11.73 | 5.86 | 39.76 |
| | -5.60 | 64.90 | 8.13 | 4.69 | 12.53 |
| | -6.00 | 76.06 | 11.62 | 5.81 | 15.28 |
| | -6.30 | 88.23 | 5.05 | 2.92 | 5.73 |
| | -6.60 | 89.67 | 3.85 | 1.92 | 4.29 |
| | -7.00 | 93.42 | 3.63 | 1.82 | 3.89 |
| | -8.00 | 99.04 | 3.09 | 1.55 | 3.12 |
| | -9.00 | NC | NC | NC | NC |
| | -10.0 | NC | NC | NC | NC |
| Overall Task | Log Chrysin Conc (M) | Overall Mean | Overall sd[b] | Overall SEM[b] | Overall %CV[b] |
| | -4.00 | 20.53 | 10.67 | 6.16 | 52.0 |
| | -5.00 | 27.71 | 2.07 | 1.20 | 7.5 |
| | -5.30 | 41.75 | -- | -- | -- |
| | -5.60 | 61.09 | 4.53 | 2.62 | 7.4 |
| | -6.00 | 77.40 | 1.17 | 0.68 | 1.5 |
| | -6.30 | 91.12 | 7.11 | 4.11 | 7.8 |
| | -6.60 | (89.67)[c] | -- | -- | -- |
| | -7.00 | 95.15 | 1.63 | 0.94 | 1.7 |
| | -7.30 | NC | -- | -- | -- |
| | -8.00 | 98.55 | 1.70 | 0.98 | 1.7 |
| | -9.00 | NC | -- | -- | -- |
| | -10.00 | NC | -- | -- | -- |

a. NC – Not Calculated. Concentration only tested in one replicate so the mean, sd, SEM, and %CV were not calculated.
b. In those instances when n = 2 only the mean was reported.
c. Value is not an average of multiple laboratories as only one laboratory tested this concentration.

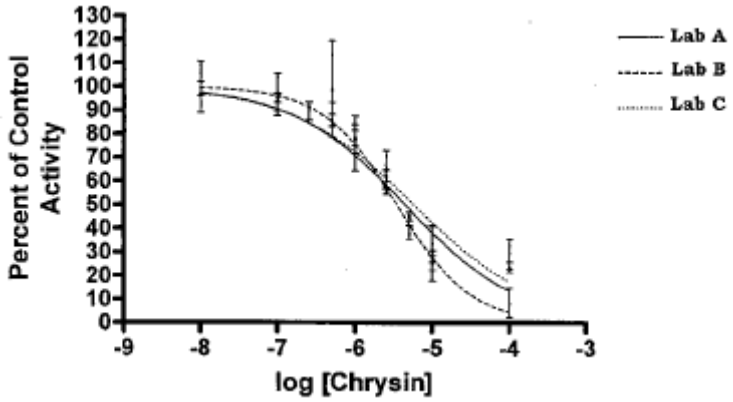



**Figure 8.3-2. Inhibition of chrysin by laboratory**

**Table 8.3-4. Chrysin $IC_{50}$ and slope values**

| Run | $IC_{50}$ Values | | | Slope | | |
|---|---|---|---|---|---|---|
| | RTI | Lab B | Lab C | RTI | Lab B | Lab C |
| 1 | 3.75 | 4.28 | 1.56 | -0.5892 | -0.9651 | -0.6870 |
| 2 | 5.50 | 3.32 | 4.04 | -0.5774 | -0.9219 | -0.5970 |
| 3 | 4.09 | 3.01 | 6.92 | -0.5976 | -0.9308 | -0.5723 |
| Mean | 4.45 | 3.54 | 4.17 | -0.5881 | -0.9393 | -0.6188 |
| SD | 0.93 | 0.66 | 2.68 | 0.0101 | 0.0228 | 0.0604 |
| SEM | 0.54 | 0.33 | 1.55 | 0.0059 | 0.0132 | 0.0302 |
| %CV | 20.9 | 18.7 | 64.3 | 1.7 | 2.4 | 9.8 |

### 8.3.3 Econazole

The effect of increasing the concentrations of econazole on aromatase activity was determined and the results were expressed as a percent of the control aromatase activity. The individual run percent of control results for each laboratory can be found in the individual lab reports. The overall percent of control results by laboratory and the overall percent of control results for the task are summarized in Table 8.3-5.

For Laboratory B, only run 1 was used to characterize the percent of control curve due to the large variability in the full enzyme control activity. The overall inhibition response curves by laboratory are shown in Figure 8.3-3. The curves in this figure are not fitted by the model but are representative of the curves as denoted by the symbols (mean data). For all three laboratories, increasing concentrations of econazole decreased the activity of the placental microsomal aromatase activity and the decrease was concentration-dependent. The shape of the enzyme activity vs econazole curve was sigmoidal. At an econazole concentration of $10^{-7}$ M, aromatase inhibition was almost complete; the laboratory percent of control was approximately 2 percent. In contrast, at an econazole concentration of $10^{-10}$ M, there was little to no aromatase inhibition; the laboratory percent of control values ranged from 93 to 102 percent. Overall task mean ± SEM percent of control values at $10^{-7}$ and $10^{-10}$ M were 2.05 ± 0.15 and 96.12 ± 2.8 percent, respectively. Table 8.3-6 shows the $IC_{50}$ and slope values for econazole.

**Table 8.3-5. Effect of econazole on aromatase activity (percent of control) by laboratory**

| Laboratory | Log Econazole Conc (M) | Overall Percent of Control by Laboratory | | | |
|---|---|---|---|---|---|
| | | Mean | sd | SEM | %CV |
| Laboratory B[d] | -3.00 | 0.58 | -- | -- | -- |
| | -4.00 | 0.54 | -- | -- | -- |
| | -5.00 | -0.03 | -- | -- | -- |
| | -6.00 | 0.17 | -- | -- | -- |
| | -7.00 | 2.34 | -- | -- | -- |
| | -8.00 | 16.56 | -- | -- | -- |
| | -9.00 | 66.46 | -- | -- | -- |
| | -10.0 | 92.99 | -- | -- | -- |
| Laboratory C | -3.00 | NC | NC | NC | NC |
| | -4.00 | NC | NC | NC | NC |
| | -5.00 | NC | NC | NC | NC |
| | -6.00 | 0.18 | 0.12 | 0.07 | 69.86 |
| | -7.00 | 1.85 | 0.18 | 0.10 | 9.69 |
| | -7.30 | NC | NC | NC | NC |
| | -7.60 | NC | NC | NC | NC |
| | -8.00 | 16.04 | 1.77 | 1.02 | 11.03 |
| | -8.30 | NC | NC | NC | NC |
| | -8.60 | 44.49 | NC | NC | NC |
| | -9.00 | 69.76 | 1.74 | 1.01 | 2.50 |
| | -9.60 | NC | NC | NC | NC |
| | -10.0 | 93.73 | NC | NC | NC |
| Overall Task | Log Econazole Conc (M) | Overall Mean | Overall sd[b] | Overall SEM[b] | Overall %CV[b] |
| | -3.00 | (0.58)[c] | -- | -- | -- |
| | -4.00 | (0.54)[c] | -- | -- | -- |
| | -5.00 | (-0.03)[c] | -- | -- | -- |
| | -6.00 | 0.17 | -- | -- | -- |
| | -7.00 | 2.05 | 0.26 | 0.15 | 12.4 |
| | -8.00 | 16.95 | 1.15 | 0.66 | 6.8 |
| | -8.30 | (28.54)[c] | -- | -- | -- |
| | -8.60 | 45.64 | -- | -- | -- |
| | -9.00 | 69.27 | 2.60 | 1.50 | 3.8 |
| | -9.30 | (89.00)[c] | -- | -- | -- |
| | -9.60 | (91.01)[c] | -- | -- | -- |
| | -10.0 | 96.12 | 4.79 | 2.77 | 5.0 |

a. NC – Not Calculated. Concentration only tested in one replicate so the mean, sd, SEM, and %CV were not calculated.
b. In those instances when n = 2 only the mean was reported.
c. Value is not an average of multiple laboratories as only one laboratory tested this concentration.
d. Variability in full enzyme activity control values precluded averaging replicates. Tabled values are the results of the first replicate only.

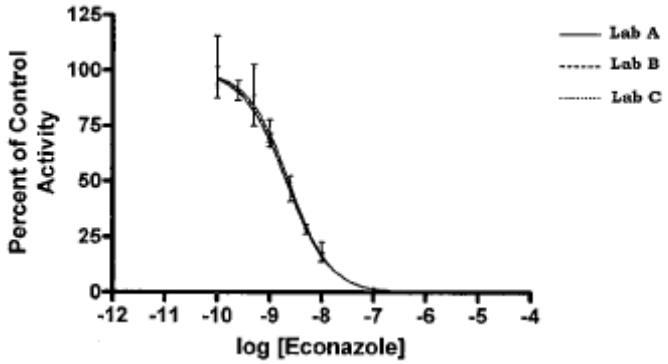



**Figure 8.3-3. Econazole inhibition by laboratory**

**Table 8.3-6. Econazole $IC_{50}$ and slope values**

| Run | $IC_{50}$ Values | | | Slope | | |
|---|---|---|---|---|---|---|
| | RTI | Lab B | Lab C | RTI | Lab B | Lab C |
| **1** | 2.11 | 2.00 | 2.36 | -1.037 | -0.9756 | -1.043 |
| **2** | 1.85 | a | 2.10 | -1.023 | a | -1.083 |
| **3** | 2.61 | a | 1.91 | -1.081 | a | -1.035 |
| **Mean** | 2.19 | a | 2.12 | -1.047 | a | -1.054 |
| **SD** | 0.39 | a | 0.23 | 0.030 | a | 0.026 |
| **SEM** | 0.22 | a | 0.13 | 0.017 | a | 0.015 |
| **%CV** | 17.6 | a | 10.6 | 2.9 | a | 2.4 |

a Variability of the full enzyme activity control value precluded determining $IC_{50}$ values.

### 8.3.4 Ketoconazole

The effect of increasing the concentrations of ketoconazole on aromatase activity was determined and the results were expressed as a percent of the control aromatase activity. The individual run percent of control results for each laboratory can be found in the original laboratory reports. The overall percent of control results by laboratory and the overall percent of control results for the task are summarized in Table 8.3-7.

The individual ketoconazole inhibition response curves by run for each laboratory are reported in the original laboratory reports. The overall inhibition response curves by laboratory are shown in Figure 8.3-4. The curves in this figure are not fitted by the model but are representative of the curves as denoted by the symbols (mean data). For all three laboratories, increasing concentrations of ketoconazole decreased the activity of the placental microsomal aromatase activity and the decrease was concentration-dependent. The shape of the enzyme activity vs. ketoconazole curve was sigmoidal. At a ketoconazole concentration of $10^{-4}$ M, aromatase inhibition was almost complete; the laboratory percent of control values ranged from 6 to 8 percent. In contrast, at a ketoconazole concentration of $10^{-7}$ M, there was little to no aromatase inhibition; the laboratory percent of control values ranged from 93 to 98 percent. Overall task mean ± SEM percent of control values at $10^{-4}$ and $10^{-7}$ M were 6.83 ± 0.78 and 95.77 (n=2) percent, respectively. Table 8.3-8 shows the $IC_{50}$ and slope values for ketoconazole.

**Table 8.3-7. Effect of ketoconazole on aromatase activity (percent of control) by laboratory**

| Laboratory | Log Ketoconazole Conc (M) | Overall Percent of Control by Laboratory | | | |
|---|---|---|---|---|---|
| | | Mean | sd | SEM | %CV |
| RTI | -4.00 | 6.05 | 0.08 | 0.05 | 1.40 |
| | -4.30 | 12.90[b] | -- | -- | -- |
| | -4.60 | 22.79[b] | -- | -- | -- |
| | -5.00 | 42.15 | 0.64 | 0.37 | 1.53 |
| | -5.30 | 57.92[b] | -- | -- | -- |
| | -6.00 | 86.57 | 1.37 | 0.79 | 1.59 |
| | -7.00 | 98.32 | 1.84 | 1.06 | 1.88 |
| | -8.00 | 98.80 | 1.62 | 0.94 | 1.64 |
| | -9.00 | NC[a] | NC | NC | NC |
| | -10.0 | NC | NC | NC | NC |
| | -11.0 | NC | NC | NC | NC |
| Laboratory B | -4.00 | 8.39 | 0.51 | 0.29 | 6.06 |
| | -4.40 | 18.20[b] | -- | -- | -- |
| | -4.80 | 35.79[b] | -- | -- | -- |
| | -5.00 | NC | NC | NC | NC |
| | -5.19 | 56.63[b] | -- | -- | -- |
| | -5.60 | 75.91[b] | -- | -- | -- |
| | -6.00 | 86.76 | 2.73 | 1.57 | 3.14 |
| | -7.00 | NC | NC | NC | NC |
| | -7.30 | 99.74 | 4.97 | 2.87 | 4.99 |
| | -8.00 | NC | NC | NC | NC |
| | -9.00 | 97.22 | 2.35 | 1.36 | 2.42 |
| | -10.0 | NC | NC | NC | NC |
| Laboratory C | -4.00 | 6.04 | 0.35 | 0.20 | 5.79 |
| | -4.60 | 21.93 | NC | NC | NC |
| | -5.00 | 42.20 | 2.19 | 1.26 | 5.18 |
| | -5.30 | 51.43 | NC | NC | NC |
| | -5.60 | 70.07 | NC | NC | NC |
| | -6.00 | 79.95 | 6.15 | 3.55 | 7.69 |
| | -6.60 | 92.28 | 6.57 | 3.79 | 7.12 |
| | -7.00 | 93.22 | 4.34 | 2.51 | 4.66 |
| | -8.00 | NC | NC | NC | NC |
| | -9.00 | NC | NC | NC | NC |
| | -10.0 | NC | NC | NC | NC |

| Laboratory | Log Ketoconazole Conc (M) | Overall Percent of Control by Laboratory | | | |
|---|---|---|---|---|---|
| | | Mean | sd | SEM | %CV |
| Overall Task | -4.00 | 6.83 | 1.35 | 0.78 | 19.8 |
| | -4.30 | (12.90)[c] | -- | -- | -- |
| | -4.40 | (18.20)[c] | -- | -- | -- |
| | -4.60 | 22.36 | -- | -- | -- |
| | -4.80 | (35.79)[c] | -- | -- | -- |
| | -5.00 | 42.18 | -- | -- | -- |
| | -5.20 | (56.63)[c] | -- | -- | -- |
| | -5.30 | 54.68 | -- | -- | -- |
| | -5.60 | 72.99 | -- | -- | -- |
| | -6.00 | 84.43 | 3.88 | 2.24 | 4.6 |
| | -6.60 | (92.28)[c] | -- | -- | -- |
| | -7.00 | 95.77 | -- | -- | -- |
| | -7.30 | (99.74)[c] | -- | -- | -- |
| | -8.00 | (98.80)[c] | -- | -- | -- |
| | -9.00 | (97.22)[c] | -- | -- | -- |
| | -10.00 | -- | -- | -- | -- |

a. NC – Not Calculated. Concentration only tested in one replicate so the mean, sd, SEM, and %CV were not calculated.
b. In those instances when n = 2 only the mean was reported.
c. Value is not an average of multiple laboratories as only one laboratory tested this concentration.

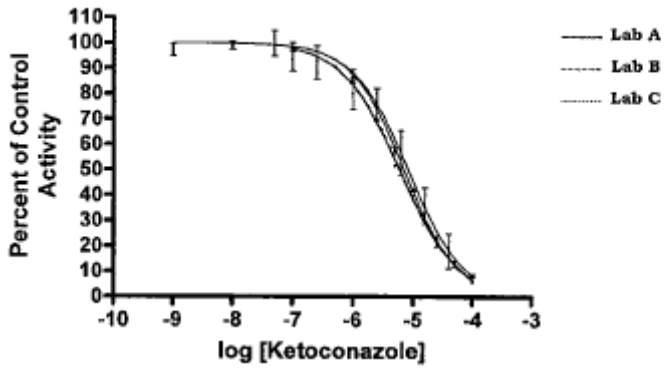



**Figure 8.3-4. Ketoconazole inhibition by laboratory**

**Table 8.3-8. Ketoconazole $IC_{50}$ and slope values**

| Run | $IC_{50}$ Values | | | Slope | | |
|---|---|---|---|---|---|---|
| | RTI | Lab B | Lab C | RTI | Lab B | Lab C |
| **1** | 7.44 | 8.81 | 7.83 | -1.047 | -1.001 | -1.043 |
| **2** | 6.85 | 10.77 | 6.08 | -0.9929 | -0.9947 | -0.9201 |
| **3** | 7.20 | 6.44 | 5.68 | -0.9865 | -1.009 | -0.08685 |
| **Mean** | 7.16 | 8.67 | 6.53 | -1.009 | -1.002 | -0.9439 |
| **SD** | 0.30 | 2.17 | 1.14 | 0.033 | 0.007 | 0.0896 |
| **SEM** | 0.17 | 1.08 | 0.66 | 0.019 | 0.004 | 0.0518 |
| **%CV** | 4.1 | 25.0 | 17.5 | 3.3 | 0.7 | 9.5 |

### 8.3.5 Discussion and Conclusions from Studies with Single Source Microsomes

All three laboratories characterized the concentration response curve as "Complete" for aminoglutethimide and ketoconazole since the percent of control data essentially spanned the 0 to 100 percent range. RTI and Laboratory B also characterized the econazole curve as complete for the same reason, although Laboratory C had one run that was not fully characterized due to a technical error. RTI and Laboratory C characterized chrysin as "Incomplete-Interpolated" since the percent of control data ranged from approximately 15 to 22 percent at the low concentrations tested and 100 percent at the high concentrations tested. Laboratory B characterized the concentration response curves for econazole and chrysin as complete. All classifications would support the correct conclusion that the substances were inhibitors of aromatase.

The full enzyme activity control value for this assay should be at least 0.03 nmol/mg protein/min according to the acceptance criterion in the study plan. For this task, the overall average full enzyme activity control values were 0.105 ± 0.009, 0.042 ± 0.013, and 0.049 ± 0.003 nmol/mg protein/min for RTI and Laboratories B and C, respectively. The lead laboratory did not have any runs below the acceptance criterion, whereas one of the participating laboratories had one run for aminoglutethimide with full enzyme activity value below the acceptance criterion and the other participating laboratory had two of three runs for ketoconazole and four of five runs for econazole below the acceptance criterion. There was no clear association between a run with full enzyme activity that was lower than the acceptance criterion and having higher variability (percent CV). For Laboratory B, the enzyme activity began to decrease over time and, for the ketoconazole and econazole analyses, the enzyme activity was very low and replicates within given runs were very erratic. Attempts to determine possible causes for the change in enzyme activity did not identify any specific factors that could be used to explain the problem. There was no evidence that the microsomes were damaged during storage or that the assay method was different than before.

There was one other consistent anomaly that should be mentioned. Laboratories reported significant differences between the beginning and the end portions for all control types. This implies a reduction in aromatase activity between the beginning and the end of a run. This finding suggests that the time required to analyze the samples decreased the enzyme activity and may have implications on the number of samples that can be analyzed at a given time.

The positive control, 4-0H ASDN, is a known aromatase inhibitor and inhibited aromatase activity in a consistent manner for all laboratories. 4-0H ASDN, at a final concentration of 5 x $10^{-8}$ M, resulted in laboratory overall group mean ± SEM inhibition values (as a percent of control) of 47.2 ± 2.3, 54.5 ± 7.1, and 55.9 ± 1.3 percent for RTI and Laboratories B and C, respectively. These results indicated that the target concentration, which was selected to inhibit aromatase approximately 50 percent based on results from previous studies, produced the desired degree of inhibition.

The $IC_{50}$ values measured for the four reference chemicals in this study compared well with those found in previous studies conducted in the validation program (References 2 and 3), Aromatase Detailed Review Paper (Reference 1), and the scientific literature (Chen, et al., 1997;

Le Bail, et al., 2001) for aminoglutethimide, chrysin, and ketoconazole but were about 10-fold lower than reported literature values for econazole.

For aminoglutethimide, the mean ± SEM $IC_{50}$ values in the present task for RTI and Laboratories B and C were 4.26 ± 0.11, 4.28 ± 0.33, and 4.5 ± 4 0.94 μM, respectively, with an overall task group mean SEM $IC_{50}$ value of 4.36 ± 0.09 μM. These values are in good agreement with a value ± from a previous work assignment (5.6 μM) and literature values (1.0 to 5.5 μM).

For chrysin, the mean ± SEM $IC_{50}$ values for RTI and Laboratories B and C were 4.45 ± 0.54, 3.54 ± 0.33, and 4.17 ± 1.55 μM, respectively with an overall task group mean ± SEM $IC_{50}$ value of 4.05 ± 0.27 μM. These values are in good agreement with a value from a previous work assignment (3.2 μM) and literature values (0.7 to 11 μM).

For econazole, the mean ± SEM $IC_{50}$ values for RTI and Laboratory C were 2.19 ± 0.22 and 2.12 ± 0.13 nM, respectively. There was no mean value calculated for Laboratory B. The overall task group mean ± SEM $IC_{50}$ value of 2.10 ± 0.06 nM. These values are in good agreement with a value from a previous work assignment (2.79 nM) but not from reported literature values (30 to 50 nM).

For ketoconazole, the mean ± SEM $IC_{50}$ values for RTI and Laboratories B and C were 7.16 ± 0.17, 8.67 ± 1.08, and 6.53 ± 0.66 μM, with an overall task group mean ± SEM $IC_{50}$ value of 7.46 ± 0.64 μM. These values are in good agreement with a value from a previous study (15.0 μM) and from literature values (6 to 60 μM).

There are two recommendations flowing from this work that were incorporated in later studies: (1) The assay should use a standard curve for protein determinations that encompasses the protein concentration of the microsomal dilution rather than extrapolate. This may reduce the variability that was seen with some of the laboratory results. (2) A four-parameter model (top, bottom, slope, and $IC_{50}$ are all variables) rather than a two-parameter model (top and bottom fixed; only slope and $IC_{50}$ are allowed to vary to fit the data) should be used for future analyzes. This conclusion was based on the results seen for chrysin. For chrysin, the two-parameter model did not span the entire 0 to 100 percent of control range.

### 8.4 Preliminary Studies with Placental Microsomes Prepared in Participating Laboratories

After completion of the studies with a single batch of microsomes prepared by the lead laboratory as discussed above in Section 8.3, two laboratories, Battelle and In Vitro, were selected to obtain human placenta and prepare microsomes. The two batches of microsomes were then sent to all other participating laboratories including the lead laboratory. This was done to demonstrate the ability of other laboratories to follow the protocol for preparing placental microsomes and to compare the results obtained with the use of different microsomal preparations as this would be the real world condition for using this assay. This characterization of the microsomal preparations was conducted in two stages. In the first stage, the preparing laboratory determined protein concentrations and aromatase activity of the preparations. In the second stage, the preparations were sent to the other laboratories for the determination of protein

concentration, aromatase activity, and study of the inhibition of aromatase with the positive control. Battelle's microsomal preparation was more concentrated than In Vitro's preparation, but both preparations were found to have acceptable concentrations of protein and enzyme activity to conduct the assay.

### 8.4.1 Protein Concentration

For the Battelle-supplied microsomes, the original protein concentration provided by Battelle at the time of distribution was 21 mg/mL. The laboratory group mean (± Standard Error of the Mean, SEM) protein concentration values were 26.8 ± 0.4, 25.0 ± 0.4, 18.6 ± 0.7, and 23.9 ± 0.8 mg/mL for RTI, Battelle, In Vitro, and WIL, respectively (Table 8.4-1). The measured protein concentration within a laboratory showed low variability, i.e., %CV values ranged from 5.3 to 11.5 percent. A comparison of the original reported protein concentration to the values determined by the laboratories resulted in a percent relative error (%RE) of 27.7, 19.1, -11.5, and 14.0 percent for RTI, Battelle, In Vitro, and WIL, respectively. In general, the laboratories obtained a similar protein concentration to that reported by the supplier. If a comparison is made to the results obtained by the lead laboratory (RTI), then the %RE was -6.7, -30.7, and -10.7 percent for Battelle, In Vitro, and WIL, respectively. The overall task mean ± SEM protein concentration was 23.6 ± 1.8 mg/mL with a percent CV of 15.0 percent.
For the In Vitro-supplied microsomes, the original protein concentration provided by In Vitro at the time of distribution was 8 mg/mL. The laboratory group mean ( ± Standard Error of the Mean, SEM) protein concentration values were 10.9 ± 0.4, 9.2 ± 0.1, 6.3 ± 0.6, and 8.5 ± 0.4 mg/mL for RTI, Battelle, In Vitro, and WIL, respectively (Table 8.4-1). The measured protein concentration within a laboratory showed low variability for three of four labs, i.e., %CV values ranged from 5.7 to 13.8 percent, whereas In Vitro had a larger measure of variability compared to its original reported value, i.e., 28.5 percent. A comparison of the original reported protein concentration to the values determined by the laboratories resulted in a percent relative error (%RE) of 36.1, 15.3, -21.4, and 6.5 percent for RTI, Battelle, In Vitro, and WIL, respectively. In general, the laboratories obtained a similar protein concentration to that reported by the supplier. If a comparison is made to the results obtained by the lead laboratory (RTI), then the %RE was -15.3, -42.2, and -21.7 percent for Battelle, In Vitro, and WIL, respectively. The overall task mean ± SEM protein concentration was 8.7 ± 1.0 mg/mL with a percent CV of 21.8 percent.

**Table 8.4-1. Human placental microsomal protein concentration**

| Parameter | RTI | Battelle | In Vitro | WIL |
|---|---|---|---|---|
| **Battelle-Supplied Microsomes** | | | | |
| Average (mg/mL) | 26.8 | 25.0 | 18.6 | 23.9 |
| sd | 1.4 | 1.6 | 2.1 | 2.5 |
| SEM | 0.4 | 0.4 | 0.7 | 0.8 |
| Minimum (mg/mL) | 25.3 | 20.9 | 14.8 | 19.4 |
| Maximum (mg/mL) | 29.8 | 27.3 | 22.4 | 27.9 |
| % CV | 5.3 | 6.5 | 11.5 | 10.6 |
| **In Vitro-Supplied Microsomes** | | | | |
| Average (mg/mL) | 10.9 | 9.2 | 6.3 | 8.5 |
| sd | 1.2 | 0.5 | 1.8 | 1.2 |
| SEM | 0.4 | 0.1 | 0.6 | 0.4 |
| Minimum (mg/mL) | 9.7 | 8.2 | 4.0 | 6.6 |
| Maximum (mg/mL) | 13.2 | 10.1 | 8.8 | 10.5 |
| % CV | 10.7 | 5.7 | 28.5 | 13.8 |

### 8.4.2 Full Enzyme Activity

Full enzyme activity controls were conducted in duplicate replicates at the beginning and end of each run of the assay (a total of four tubes/run). There were at least three runs conducted for each often reference chemicals by each of the four laboratories. In those instances where a laboratory assayed two reference chemicals on the same day and at the same time, there was only one set (four tubes) assayed, which resulted in one average enzyme activity for both reference chemicals. Thus, even though such controls were used for two reference chemicals, the control value was only used once in the calculation of any overall control value.

A comparison of the enzyme activity values obtained for a given laboratory when it used the Battelle- or In Vitro-supplied microsomes indicated that the activity was similar (taking into consideration the variability). The overall task mean ± SEM full enzyme activity control value for both sources of microsomes was 0.054 ± 0.005 nmol/mg protein/min with a percent CV of 17.9 percent. For this reason, it was believed reasonable to determine an overall enzyme activity value using the data generated by all laboratories and the microsomes from both suppliers (Table 8.4-2). These results indicated that microsomes prepared by different laboratories and analyzed by different laboratories can result in microsomes with similar aromatase activity.

**Table 8.4-2. Human placental full enzyme activity control determinations**

| Parameter | Aromatase Activity (nmol/mg protein/min) | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Overall Average | 0.043 | 0.051 | 0.066 | 0.055 |
| Overall sd | 0.008 | 0.010 | 0.023 | 0.008 |
| Overall SEM | 0.003 | 0.003 | 0.010 | 0.003 |
| % CV | 17.7 | 19.0 | 35.4 | 15.3 |

### 8.4.3 Background Activity

Background enzyme activity controls were conducted in duplicate replicates at the beginning and end of each run of the assay (a total of four tubes/run). There were at least three runs conducted for each of ten reference chemicals by each of the four laboratories. For the most part, the aromatase activity in these samples for all laboratories and reference chemicals was negligible, indicating that there was no background activity that interfered with the interpretation of the results.

Lindane was added as a negative control for all of these studies at a single concentration of $10^{-6}$ M, but this procedure provided no additional benefit over the background activity control and was dropped as a feature of the final protocol.

### 8.4.4 Positive Control Activity

4-0H ASDN, at a final concentration of 5 x $10^{-8}$ M, was used as the positive control with each run of the assay because it is a known aromatase inhibitor and this concentration has been shown to produce an approximately 50 percent inhibition of the enzyme. Since the full enzyme activity values were similar for both sources of microsomes, the results using microsomes from the two sources were combined to report the results by laboratory and task. Table 8.4-3 shows the placental enzyme activity per unit of protein.

**Table 8.4-3. Human placental enzyme activity of the positive control (5 x $10^{-8}$ M 4-OH ASDN)**

| Parameter | Percent of Control | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Overall Average | 48.0 | 48.7 | 40.5 | 42.7 |
| Overall sd | 1.1 | 3.3 | 18.0 | 7.5 |
| Overall SEM | 0.4 | 1.0 | 7.4 | 3.1 |
| % CV | 2.3 | 6.8 | 44.5 | 17.5 |

After laboratories had demonstrated their ability to perform these analyses with acceptable agreement between laboratories, the laboratories were authorized to begin the main interlaboratory study.

## 8.5 Positive Control Studies with Recombinant Microsomes

Virtually identical interlaboratory studies were also carried out with recombinant microsomes with the 10 reference chemicals to validate both the human placental and recombinant assay methods and enable a comparison of their performance. The effect of increasing concentrations of the reference chemicals on aromatase activity was determined and the results were expressed as a percent of the control aromatase activity. A summary of these results by chemical, laboratory, and overall task are provided in Chapter 9. The individual run percent of control results for each laboratory can be found in the appendices to Reference 12.

Recombinant microsomes (Human CYPL9 + P450 Reductase SUPERSOMES) were provided to each of the laboratories by RTI (Lot # 5, 4.9 mg protein/mL, purchased from Gentest, Woburn, MA). The microsomes were stored at approximately -70°C until the time of the assay.

### 8.5.1 Protein Concentration

The microsomal protein concentration was determined on the day that the microsomes were used in the assay. The number of protein concentrations differed somewhat because some laboratories performed two independent runs of the assay but used the same protein analysis results for both runs, thereby reducing the total number of protein determinations for a given laboratory.

For In Vitro, there were four instances when the determined protein concentration of the final dilution was very near or below the lowest standard. As a result, the determined values were

very low and, in at least one instance, the value was a negative number, thereby suggesting that the determined protein concentrations were inaccurate. For this reason, the laboratory was directed to use an intermediate dilution sample to determine the protein concentration. However, the four instances when the final dilutions were analyzed prior to this directive were used by the laboratory and are included in the results reported in Table 8.5-1, except for the one negative value. Inclusion of these values accounts for the much of the disparity reported for In Vitro.

The supplier documented protein concentration provided by RTI at the time of distribution was 4.9 mg/mL. The laboratory group mean (± Standard Error of the Mean, SEM) protein concentration values were 4.7 ± 0.2, 5.2 ± 0.0, 3.3 ± 0.4, and 6.6 ± 0.4 mg/mL for RTI, Battelle, In Vitro, and WIL, respectively (Table 8.5-1). The precision (%CV) ranged from 4.2 to 27.l percent, except for In Vitro which had a %CV value of 51.9 percent. A comparison of the original reported protein concentration to the values determined by the laboratories resulted in a percent relative error (%RE) of -4.l, 5.5, -33.6, and 35.5 percent for RTI, Battelle, In Vitro, and WIL, respectively. If a comparison is made to the results obtained by the lead laboratory (RTI), then the %RE was 10.0, -30.8, and 41.2 percent for Battelle, In Vitro, and WIL, respectively. The overall task mean ± SEM protein concentration was 4.9:1 0.7 mg/mL with a percent CV of 28.3 percent.

**Table 8.5-1. Recombinant microsomal protein concentration[a]**

| Parameter | RTI | Battelle | In Vitro[b] | WIL |
|---|---|---|---|---|
| Average (mg/mL) | 4.7 | 5.2 | 3.3 | 6.6 |
| sd | 1.1 | 0.2 | 1.7 | 1.8 |
| SEM | 0.2 | 0.0 | 0.4 | 0.4 |
| Minimum (mg/mL) | 2.9 | 4.6 | 0.1 | 3.8 |
| Maximum (mg/mL) | 6.3 | 5.6 | 5.4 | 9.5 |
| % CV | 22.6 | 4.2 | 51.9 | 27.1 |

a. Table values were based on all protein concentrations reported by labs for all runs (regardless of whether the runs were used or not for reporting enzyme activity and $IC_{50}$ value).

b. Includes aberrant values (see text for explanation) attributed to low sample concentration. Aberrant values were 0.261, 0.570, and 0.142 mg/mL, as well as one negative value that was not included in the summary calculations.

Protein QCs were analyzed with the unknowns during the study. The target QC concentrations were 10 and 100 g/mL (RTI's reported concentrations were 12 and 110 µg/mL). Accuracy (%RE) ranged from -2.3 to l6.3 percent for the low QC, except for In Vitro which had a value of -39.7 percent. Accuracy ranged from -0.7 to 14.7 percent for the high QC, thereby indicating that an acceptable level of accuracy could be achieved for both QC standards (based on acceptance criteria of 15 percent). Precision (%CV) ranged from 10.9 to 27.2 percent for the low QC, except for In Vitro that reported a value of 67.9 percent, whereas the precision ranged from 2.4 to 7.6 for the high QC, thereby indicating that precision was poor for the low QC but within an acceptable level for the high QC (based on acceptance criterion of 15 percent). In Vitro's poorer accuracy and precision results were attributed to their use of a six-point standard curve (included the 250 µg/mL standard) rather than the 5-point standard cure (excluded the 250 µg/mL standard), which was used by the other laboratories when they found increased variability and poorer fit when the 250 µg/mL standard was included

### 8.5.2 Full Enzyme Activity Controls

Full enzyme activity controls were conducted in duplicate replicates at the beginning and end of each run of the assay (a total of four tubes/run). There were at least three runs conducted for each of ten reference chemicals by each of the four laboratories. In those instances where a laboratory assayed two reference chemicals on the same day and at the same time, there was only one set (four tubes) assayed, which resulted in one average enzyme activity for both reference chemicals. Thus, even though such controls were used for two reference chemicals, the control value was only used once in the calculation of any overall control value.

A comparison of the before and ending activity tubes indicated that activity changed. For all four laboratories, the activity for the before tubes was higher than it was for the ending tubes. The statistical analysis provides a measure of this finding. The laboratory overall average ± SEM (%CV) full enzyme activity control values were 0.378 ± 0.029 (18.6%), 0.312 ± 0.016 (16.1%), 1.258 ± 0.400 (100.4%) and 0.340 ± 0.052 (37.3%) nmol/mg protein/min for RTI, Battelle, In Vitro, and WIL, respectively (Table 8.5-2). The higher activity and lack of precision for In Vitro was attributed to the inaccurate protein concentration determinations (see protein concentration section). The slightly lower precision for WIL was attributed to low activity for run 2 for prochloraz and fenarimol. The overall task mean ± SEM full enzyme activity control value (excluding In Vitro) was 0.343 ± 0.019 nmol/mg protein/min with a percent CV of 9.6 percent. (If In Vitro is included, the overall task mean ± SEM and %CV were 0.572 ± 0.229 nmol/mg protein/min and 80.1 percent.) Comparison of the lead laboratory's results relative to the individual laboratory results produced %RE values of -17.4, 232.8, and –10.l percent for Battelle, In Vitro, and WIL, respectively.

**Table 8.5-2. Recombinant full enzyme activity control determinations**

| Parameter | Aromatase Activity (nmol/mg protein/min) | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro[b] | WIL |
| Overall Average | 0.378 | 0.312 | 1.258 | 0.340 |
| Overall sd | 0.070 | 0.050 | 1.264 | 0.127 |
| Overall SEM | 0.029 | 0.016 | 0.400 | 0.052 |
| % CV | 18.6 | 16.1 | 100.4 | 37.3 |

### 8.5.3 Background Enzyme Activity Controls

Background enzyme activity controls were conducted in duplicate replicates at the beginning and end of each run of the assay (a total of four tubes/run). There were at least three runs conducted for each of ten reference chemicals by each of the four laboratories. For the most part, the aromatase activity in these samples for all laboratories and reference chemicals was negligible, indicating that there was no background activity that interfered with the interpretation of the results.

### 8.5.4 Positive Activity Controls

4-0H ASDN, at a final concentration of 5 x $10^{-8}$ M, was used as the positive control with each run of the assay because it is a known aromatase inhibitor and this concentration has been shown to produce an approximately 50 percent inhibition of the enzyme. The laboratory overall average ± SEM (%CV) enzyme activity values in the presence of 4-0H ASDN were 0.216 ± 0.017 (19.8 %), 0.169 ± 0.008 (14.9%), 0.642 ± 0.226 (111.0%), and 0.166 ± 0.019 (27.5%) nmol/mg protein/min for RTI, Battelle, In Vitro, and WIL, respectively (Table 8.5-3). Comparison of the lead laboratory to the individual laboratories resulted in %RE values of -21.7, 198.0, and -23.2 percent for Battelle, In Vitro, and WIL, respectively. The higher activity and lack of precision for In Vitro was attributed to the inaccurate protein concentration determinations (see protein concentration section). The overall task mean ± SEM full enzyme activity control value (excluding In Vitro) was 0.183 0.016 nmol/mg protein/min with a percent CV of 15.3 percent. (If In Vitro is included, the overall task mean ± SEM and %CV were 0.298 ±0.1115 nmol/mg protein/min and 77.4 percent.)

**Table 8.5-3. Recombinant enzyme activity in the presence of 4-OH ASDN (positive control)[a]**

| Parameter | Aromatase Activity (nmol/mg protein/min) | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro[b] | WIL |
| Overall Average | 0.216 | 0.169 | 0.642 | 0.166 |
| Overall sd | 0.043 | 0.025 | 0.713 | 0.046 |
| Overall SEM | 0.017 | 0.008 | 0.226 | 0.019 |
| % CV | 19.8 | 14.9 | 111.0 | 27.5 |

a. The overall average value for the laboratory was calculated using the mean values for the ten reference chemicals, (mean values from paired reference chemicals were only used once).
b. Includes aberrant values (see text for explanation) attributed to low protein concentration determinations. Aberrant values were 1.517, 1.192, and 2.148 nmol/mg protein/min.

# 9.0 Interlaboratory Validation Studies with Placental and Recombinant Microsomes

This section provides a summary description of the interlaboratory studies conducted by four laboratories (RTI, Battelle, In Vitro and WIL) for the validation of the placental and recombinant aromatase assays. Individual laboratory reports provide all of the details including the statistical analysis of within run and run to run data. The subsections below will focus on a comparison of results between laboratories. All laboratories followed GLPs and the same model protocol; however, laboratories were free to use judgment to vary some concentrations when confronted with issues such as insolubility at higher concentrations. These deviations from the model protocol reflect real world conditions and are noted.

Overviews of the placental and recombinant assays are presented, followed by chemical-specific results. Within each chemical, the placental and recombinant results are given, including respective results on IC50 and slope determinations. Based on the curve-fit of the percent of control aromatase activity values across the various number of concentrations for each reference chemical, the calculated IC50 values by run and laboratory are summarized.

**Overview of Placental Assay.** Human placental microsomes were provided to each of the laboratories by Battelle (Lot # 6-041305, 21 mg protein/mL, ~200 µL/vial) and In Vitro (Lot # BAA, 8 mg protein/mL, ~ 150 µL/vial). Reference chemicals were matched with microsome sources in the testing laboratories according to the scheme shown in Table 9.0-1.

**Table 9.0-1. Source of microsomes by reference chemical and laboratory**

| Reference Chemical (RC Blind Code) | RTI | Battelle | In Vitro | WIL |
|---|---|---|---|---|
| Aminoglutethimide (1) | BATTELLE | BATTELLE | *IN VITRO* | *IN VITRO* |
| Ketoconazole (2) | BATTELLE | BATTELLE | *IN VITRO* | *IN VITRO* |
| Prochloraz (3) | *IN VITRO* | *IN VITRO* | BATTELLE | BATTELLE |
| 4-Nonylphenol (4) | BATTELLE | *IN VITRO* | *IN VITRO* | BATTELLE |
| Dibenz[a,h]anthracene (5) | *IN VITRO* | BATTELLE | BATTELLE | *IN VITRO* |
| Fenarimol (6) | *IN VITRO* | *IN VITRO* | BATTELLE | BATTELLE |
| Econazole (7) | BATTELLE | *IN VITRO* | BATTELLE | *IN VITRO* |
| Chrysin (8) | BATTELLE | *IN VITRO* | BATTELLE | *IN VITRO* |
| Dicofol (9) | *IN VITRO* | BATTELLE | *IN VITRO* | BATTELLE |
| Atrazine (10) | *IN VITRO* | BATTELLE | *IN VITRO* | BATTELLE |

a. BATTELLE – denotes Battelle was the source of the microsomes. *IN VITRO* - denotes In Vitro was the source of the microsomes.

**Overview of Recombinant Assay.** Virtually identical interlaboratory studies were also carried out with recombinant microsomes with the 10 reference chemicals to validate both the human placental and recombinant assay methods and enable a comparison of their performance. The effect of increasing concentrations of the reference chemicals on aromatase activity was determined and the results were expressed as a percent of the control aromatase activity. A summary of these results by chemical, laboratory, and overall task are provided in Sections 9.1

through 9.10. The individual run percent of control results for each laboratory can be found in the appendices to Reference 12.

Recombinant microsomes (Human CYPL9 + P450 Reductase SUPERSOMES) were provided to each of the laboratories by RTI (Lot # 5, 4.9 mg protein/mL, purchased fromGentest, Woburn, MA). The microsomes were stored at approximately -70°C until the time of the assay.

**Protein Concentration:** The microsomal protein concentration was determined on the day that the microsomes were used in the assay. The number of protein concentrations differed somewhat because some laboratories performed two independent runs of the assay but used the same protein analysis results for both runs, thereby reducing the total number of protein determinations for a given laboratory.

For In Vitro, there were four instances when the determined protein concentration of the final dilution was very near or below the lowest standard. As a result, the determined values were very low and, in at least one instance, the value was a negative number, thereby suggesting that the determined protein concentrations were inaccurate. For this reason, the laboratory was directed to use an intermediate dilution sample to determine the protein concentration. However, the four instances when the final dilutions were analyzed prior to this directive were used by the laboratory and are included in the results reported in Table 9.0-2, except for the one negative value. Inclusion of these values accounts for the much of the disparity reported for In Vitro.

The supplier documented protein concentration provided by RTI at the time of distribution was 4.9 mg/mL. The laboratory group mean (± Standard Error of the Mean, SEM) protein concentration values were 4.7 ± 0.2, 5.2 ± 0.0, 3.3 ± 0.4, and 6.6 ± 0.4 mg/mL for RTI, Battelle, In Vitro, and WIL, respectively (Table 9.0-2). The precision (%CV) ranged from 4.2 to 27.l percent, except for In Vitro which had a %CV value of 51.9 percent. A comparison of the original reported protein concentration to the values determined by the laboratories resulted in a percent relative error (%RE) of -4.l, 5.5, -33.6, and 35.5 percent for RTI, Battelle, In Vitro, and WIL, respectively. If a comparison is made to the results obtained by the lead laboratory (RTI), then the %RE was 10.0, -30.8, and 41.2 percent for Battelle, In Vitro, and WIL, respectively. The overall task mean ± SEM protein concentration was 4.9:1 0.7 mg/mL with a percent CV of 28.3 percent.

**Table 9.0-2. Recombinant microsomal protein concentration[a]**

| Parameter | RTI | Battelle | In Vitro[b] | WIL |
|---|---|---|---|---|
| Average (mg/mL) | 4.7 | 5.2 | 3.3 | 6.6 |
| Sd | 1.1 | 0.2 | 1.7 | 1.8 |
| SEM | 0.2 | 0.0 | 0.4 | 0.4 |
| Minimum (mg/mL) | 2.9 | 4.6 | 0.1 | 3.8 |
| Maximum (mg/mL) | 6.3 | 5.6 | 5.4 | 9.5 |
| %CV | 22.6 | 4.2 | 51.9 | 27.1 |

a. Table values were based on all protein concentrations reported by labs for all runs (regardless of whether the runs were used or not for reporting enzyme activity and $IC_{50}$ value).

b. Includes aberrant values (see text for explanation) attributed to low sample concentration. Aberrant values were 0.261, 0.570, and 0.142 mg/mL, as well as one negative value that was not included in the summary calculations.

Protein QCs were analyzed with the unknowns during the study. The target QC concentrations were 10 and 100 g/mL (RTI's reported concentrations were l2 and 110 µg/mL). Accuracy (%RE) ranged from -2.3 to l6.3 percent for the low QC, except for In Vitro which had a value of -39.7 percent. Accuracy ranged from -0.7 to l4.7 percent for the high QC, thereby indicating that an acceptable level of accuracy could be achieved for both QC standards (based on acceptance criteria of l5 percent). Precision (%CV) ranged from 10.9 to 27.2 percent for the low QC, except for In Vitro that reported a value of 67.9 percent, whereas the precision ranged from 2.4 to 7.6 for the high QC, thereby indicating that precision was poor for the low QC but within an acceptable level for the high QC (based on acceptance criterion of l5 percent). In Vitro's poorer accuracy and precision results were attributed to their use of a six-point standard curve (included the 250 µg/mL standard) rather than the 5-point standard cure (excluded the 250 µg/mL standard), which was used by the other laboratories when they found increased variability and poorer fit when the 250 µg/mL standard was included

**Full Enzyme Activity Controls:** Full enzyme activity controls were conducted in duplicate replicates at the beginning and end of each run of the assay (a total of four tubes/run). There were at least three runs conducted for each of ten reference chemicals by each of the four laboratories. In those instances where a laboratory assayed two reference chemicals on the same day and at the same time, there was only one set (four tubes) assayed, which resulted in one average enzyme activity for both reference chemicals. Thus, even though such controls were used for two reference chemicals, the control value was only used once in the calculation of any overall control value.

A comparison of the before and ending activity tubes indicated that activity changed. For all four laboratories, the activity for the before tubes was higher than it was for the ending tubes. The statistical analysis provides a measure of this finding. The laboratory overall average ± SEM (%CV) full enzyme activity control values were 0.378 ± 0.029 (18.6%), 0.312 ± 0.016 (16.1%), 1.258 ± 0.400 (100.4%) and 0.340 ± 0.052 (37.3%) nmol/mg protein/min for RTI, Battelle, In Vitro, and WIL, respectively (Table 9.0-3). The higher activity and lack of precision for In Vitro was attributed to the inaccurate protein concentration determinations (see protein concentration section). The slightly lower precision for WIL was attributed to low activity for run 2 for prochloraz and fenarimol. The overall task mean ± SEM full enzyme activity control value (excluding In Vitro) was 0.343 ± 0.019 nmol/mg protein/min with a percent CV of 9.6 percent. (If In Vitro is included, the overall task mean ± SEM and %CV were 0.572 ± 0.229 nmol/mg protein/min and 80.1 percent.) Comparison of the lead laboratory's results relative to the individual laboratory results produced %RE values of -17.4, 232.8, and –10.1 percent for Battelle, In Vitro, and WIL, respectively.

**Table 9.0-3. Recombinant full enzyme activity control determinations[a]**

| Parameter | Aromatase Activity (nmol/mg protein/min) | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro[b] | WIL |
| Overall Average | 0.378 | 0.312 | 1.258 | 0.340 |
| Overall sd | 0.070 | 0.050 | 1.264 | 0.127 |
| Overall SEM | 0.029 | 0.016 | 0.400 | 0.052 |
| %CV | 18.6 | 16.1 | 100.4 | 37.3 |

a. The overall average value for the laboratory was calculated using the mean values for the ten reference chemicals, (mean values from paired reference chemicals were only used once).

b. Includes aberrant values (see text for explanation) attributed to low protein concentration determinations. Aberrant values were 2.905, 2.850, and 3.446 nmol/mg protein/min.

**Background enzyme activity controls:** Background enzyme activity controls were conducted in duplicate replicates at the beginning and end of each run of the assay (a total of four tubes/run). There were at least three runs conducted for each of ten reference chemicals by each of the four laboratories. For the most part, the aromatase activity in these samples for all laboratories and reference chemicals was negligible, indicating that there was no background activity that interfered with the interpretation of the results.

**Positive Activity Controls:** 4-0H ASDN, at a final concentration of $5 \times 10^{-8}$ M, was used as the positive control with each run of the assay because it is a known aromatase inhibitor and this concentration has been shown to produce an approximately 50 percent inhibition of the enzyme. The laboratory overall average ± SEM (%CV) enzyme activity values in the presence of 4-0H ASDN were 0.216 ± 0.017 (19.8%), 0.169 ± 0.008 (14.9%), 0.642 ± 0.226 (111.0%), and 0.166 ± 0.019 (27.5%) nmol/mg protein/min for RTI, Battelle, In Vitro, and WIL, respectively (Table 9.0-4). Comparison of the individual laboratories to the lead laboratory resulted in % relative error values of -21.7, 198.0, and -23.2 percent for Battelle, In Vitro, and WIL, respectively. The higher activity and lack of precision for In Vitro was attributed to the inaccurate protein concentration determinations (see protein concentration section). The overall task mean ± SEM full enzyme activity control value (excluding In Vitro) was 0.183 ± 0.016 nmol/mg protein/min with a percent CV of 15.3 percent. (If In Vitro is included, the overall task mean ± SEM and %CV were 0.298 ± 0.115 nmol/mg protein/min and 77.4 percent.)

**Table 9.0-4. Recombinant enzyme activity in the presence of 4-OH ASDN (positive control)[a]**

| Parameter | Aromatase Activity (nmol/mg protein/min) | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro[b] | WIL |
| Overall Average | 0.216 | 0.169 | 0.642 | 0.166 |
| Overall sd | 0.043 | 0.025 | 0.713 | 0.046 |
| Overall SEM | 0.017 | 0.008 | 0.226 | 0.019 |
| % CV | 19.8 | 14.9 | 111.0 | 27.5 |

a. The overall average value for the laboratory was calculated using the mean values for the ten reference chemicals, (mean values from paired reference chemicals were only used once).
b. Includes aberrant values (see text for explanation) attributed to low protein concentration determinations. Aberrant values were 1.517, 1.192, and 2.148 nmol/mg protein/min.

The following subsections present the placental and recombinant assay results, respectively, for each of the 10 compounds under study. The by-laboratory results for each compound are presented in Appendix B, in the supplemental materials for Section 9.

## 9.1 Aminoglutethimide

**Aminoglutethimide Assay Overview.** The effect of increasing concentrations of aminoglutethimide on aromatase activity is reported by laboratory in Tables B.9-1 and B.9-2 (Appendix B) and by overall task in Tables 9.1-1 and 9.1-2. In addition, the results are graphically presented by laboratory (individual runs and average) in Figures 9.1-1 and 9.1-2.

After the first run was completed, only the Battelle study director modified the aminoglutethimide concentrations selected for testing in runs 2 and 3 by adding a single intermediate concentration of $5 \times 10^{-6}$ M, which was used in place of the $10^{-9}$ M (Tables B.9-1 and B.9-2). For the other three labs, since the highest concentration that could be tested was $10^{-3}$ M, one additional concentration had to be selected, which was $5 \times 10^{-6}$ M for RTI, $10^{-10}$ M for In Vitro, and $5 \times 10^{-5}$ M for WIL. The laboratories tested a dilution higher than the lowest planned concentration, i.e., $10^{-9}$ M rather than $10^{-10}$ M, due to a miscommunication by the CR regarding the correct RC blind number and its corresponding concentration**.**

**Table 9.1-1. Placental assay: effect of aminoglutethimide on aromatase activity by overall task**

| Reference Chemical Log Conc (M) | Number of Labs[a] | Overall Mean | Overall sd | Overall SEM | Overall %CV |
|---|---|---|---|---|---|
| -3.00 | 4 | 0.17 | 0.20 | 0.10 | 120.3 |
| -4.00 | 4 | 4.15 | 0.41 | 0.21 | 9.9 |
| -4.30 | 1 | 8.26 | NC[b] | NC | NC |
| -5.00 | 4 | 29.43 | 2.07 | 1.04 | 7.0 |
| -5.30 | 2 | 46.10 | NC | NC | NC |
| -5.60 | 1 | 64.88 | NC | NC | NC |
| -6.00 | 4 | 79.05 | 2.84 | 1.42 | 3.6 |
| -7.00 | 4 | 95.16 | 1.56 | 0.78 | 1.6 |
| -8.00 | 4 | 98.59 | 1.65 | 0.82 | 1.7 |
| -9.00 | 4 | 98.78 | 2.17 | 1.08 | 2.2 |
| -10.00 | 1 | 98.75 | NC | NC | NC |

a. Number of labs that conducted the assay at the cited reference chemical concentration. The run number varied because each laboratory selected some concentrations for testing that differed, as well as Battelle revised the concentrations used for runs 2 and 3 after reviewing their run 1 results.

b. NC – Not Calculated. If the overall mean was calculated using an n ≤ 2, then the sd, SEM, and %CV were not calculated.

**Table 9.1-2. Recombinant assay: effect of aminoglutethimide on aromatase activity (percent of control) by overall task**

| Reference Chemical Log Conc (M) | Number of Labs[a] | Overall Mean | Overall sd | Overall SEM | Overall %CV |
|---|---|---|---|---|---|
| -3.00 | 4 | 0.38 | 0.09 | 0.05 | 23.8 |
| -4.00 | 4 | 4.62 | 0.54 | 0.27 | 11.6 |
| -4.30 | 1 | 7.99 | NC[b] | NC | NC |
| -5.00 | 4 | 32.08 | 2.33 | 1.17 | 7.3 |
| -5.30 | 2 | 49.69 | NC | NC | NC |
| -5.60 | 1 | 67.62 | NC | NC | NC |
| -6.00 | 4 | 81.02 | 3.68 | 1.84 | 4.5 |
| -7.00 | 4 | 97.17 | 6.37 | 3.19 | 6.6 |
| -8.00 | 4 | 100.08 | 7.24 | 3.62 | 7.2 |
| -9.00 | 4 | 101.37 | 7.69 | 3.84 | 7.6 |
| -10.00 | 1 | 109.98 | NC | NC | NC |

a. Number of labs that conducted the assay at the cited reference chemical concentration. The run number varied because each laboratory selected some concentrations for testing that differed, as well as Battelle revised the concentrations used for runs 2 and 3 after reviewing their run 1 results.

b. NC – Not Calculated. If the overall mean was calculated using an n ≤ 2, then the sd, SEM, and %CV were not calculated.

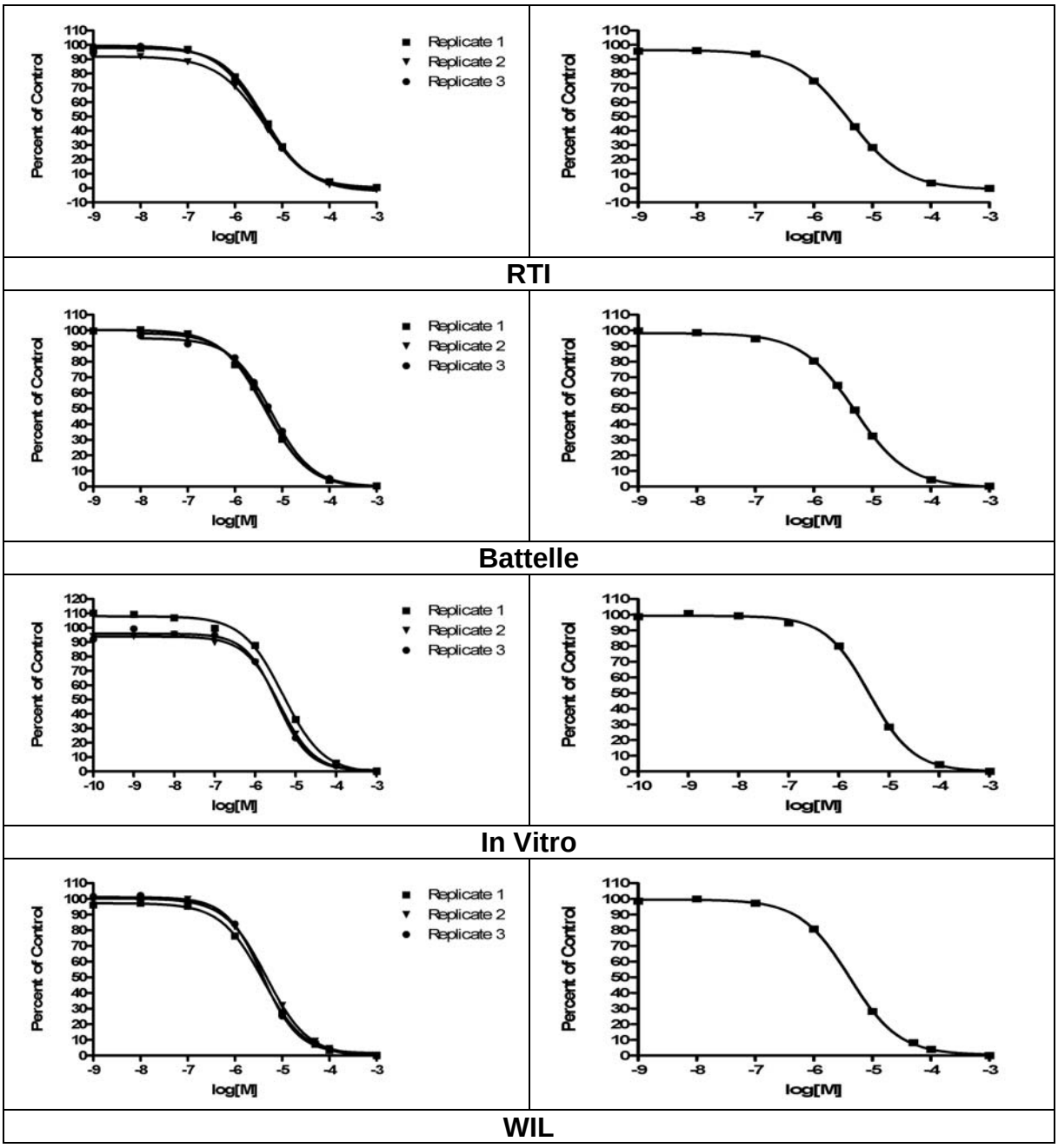



**Figure 9.1-1. Placental assay: individual run and average aminoglutethimide inhibition response curves (percent of control) by laboratory**

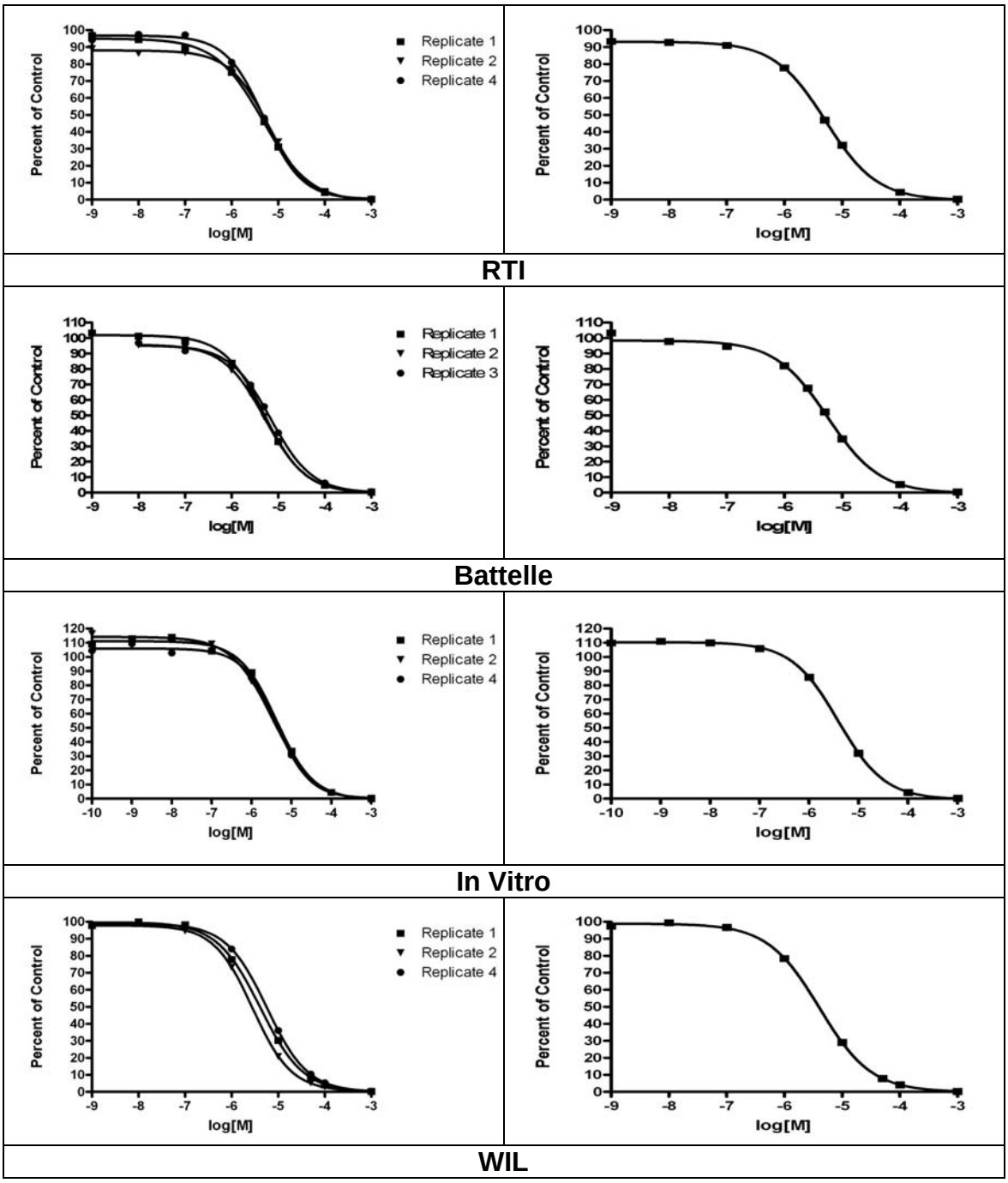



**Figure 9.1-2. Recombinant assay: individual run and average aminoglutethimide inhibition response curves (percent of control) by laboratory**

For all four laboratories, increasing concentrations of aminoglutethimide decreased the activity of the placental and recombinant microsomal aromatase and the decrease was concentration-dependent (Tables 9.1-1 and 9.1-2). The shapes of the enzyme activity vs. aminoglutethimide curves were sigmoidal (Figures 9.1-1 and 9.1-2). At an aminoglutethimide concentration of $10^{-3}$ M, the aromatase inhibition was complete; the laboratory percent of control values were less than 1 percent. In contrast, at an aminoglutethimide concentration of approximately $10^{-7}$ M, there was little to no aromatase inhibition; the laboratory percent of control values were greater than or equal to 94 percent in the placental assay and greater than or equal to 91 percent in the recombinant assay for all labs.

For the placental assay, the overall task mean ± SEM percent of control values at $10^{-3}$ and $10^{-7}$ M were 0.17 ± 0.10 and 95.16 ± 0.78 percent, respectively (Table 9.1-1). For the recombinant assay, the overall task mean ± SEM percent of control values at $10^{-3}$ and $10^{-7}$ M were 0.38 ± 0.05 and 97.17± 3.19 percent, respectively (Table 9.1-2).

For the recombinant assay, all labs reported assay precision values (%CV) that were less than or equal to 15 percent for those concentrations used in at least three runs, except at a concentration of $10^{-3}$ M (and to $10^{-5}$ M for WIL) where the precision varied widely, i.e., 24 to 68 percent (Table B.9-2). The overall task assay precision ranged from 5 to 12 percent for those concentrations that were tested by 3 or more labs, except at a concentration of $10^{-3}$ M where the %CV value was 24 percent (Table 9.1-2).

**Aminoglutethimide Placental IC50:** For aminoglutethimide, the average ± SEM IC50 values for RTI, Battelle, In Vitro, and WIL were 4.02 ± 0.15, 4.98 ± 0.51, 4.09 ± 0.40, and 4.11 ± 0.27 µM, respectively (Table 9.1-3). The percent CV values were 6.5, 17.6, 17.1, and 11.5 percent, respectively. The IC50 values were reasonably precise for all runs and laboratories, which suggested that the IC50 could be precisely estimated using the run 1 starting concentrations. The overall task group mean ± SEM IC50 value was 4.30± 0.23 µM and the percent CV was 10.6 percent.

**Table 9.1-3. Placental $IC_{50}$ values by laboratory for aminoglutethimide**

| Run | $IC_{50}$ Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Aminoglutethimide (µM) | | | | |
| 1 | 4.16 | 4.26 | 4.85 | 3.89 |
| 2 | 4.19 | 4.73 | 3.95 | 4.65 |
| 3 | 3.72 | 5.96 | 3.47 | 3.78 |
| Average | 4.02 | 4.98 | 4.09 | 4.11 |
| sd | 0.26 | 0.88 | 0.70 | 0.47 |
| SEM | 0.15 | 0.51 | 0.40 | 0.27 |
| %CV | 6.5 | 17.6 | 17.1 | 11.5 |

Source: Reference 10.

**Aminoglutethimide Placental Slope:** The average ± SEM slope values for RTI, Battelle, In Vitro, and WIL were -0.9609 ± 0.0121, -0.9806 ± 0.0506, -2.3068 ± 1.4254, and -0.9800 ± 0.0638, respectively (Table 9.1-4). The percent CV value were 2.2, 8.9, 107.0, and 11.3 percent, respectively. The higher %CV values for In Vitro was attributed to a slope obtained from run 1.

The results from RTI, Battelle, and WIL indicate that the slope could be precisely estimated using the run 1 starting concentrations. However, three of four labs showed that modifications to the run 1 concentrations tested did improve the characterization of the steep portion of the inhibition curve and slope estimate. The run 1 slope value reported by In Vitro was not included in the calculation used to determine the overall task values. The overall task group mean ± SEM slope value was -0.9510 ± 0.0232 and the percent CV was 4.9 percent.

**Table 9.1-4. Placental slope values by laboratory for aminoglutethimide**

| Run | Slope Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Aminoglutethimide | | | | |
| 1 | -0.9437 | -0.8987 | -5.155 | -0.8613 |
| 2 | -0.9842 | -1.073 | -0.9889 | -1.0799 |
| 3 | -0.9547 | -0.9702 | -0.7764 | -0.9987 |
| Average | -0.9609 | -0.9806 | -2.3068 | -0.9800 |
| sd | 0.0209 | 0.0876 | 2.4689 | 0.1105 |
| SEM | 0.0121 | 0.0506 | 1.4254 | 0.0638 |
| %CV | 2.2 | 8.9 | 107.0 | 11.3 |

Source: Reference 10

**Aminoglutethimide Recombinant IC50:** The average ± SEM IC50 values for RTI, Battelle, In Vitro, and WIL were 5.23 ± 0.45, 5.71 ± 0.63, 3.89 ± 0.28, and 4.22 ± 0.85 µM, respectively (Table 9.1-5). The percent CV values were 15.0, 19.2, 12.3, and 34.7 percent, respectively. Only one of the laboratories modified the concentrations tested after the first run, thereby suggesting that the initial range of concentrations selected were adequate to characterize the curve. The overall task group mean ± SEM IC50 value was 4.76 ± 0.43 µM and the percent CV was 17.9 percent.

**Table 9.1-5. Recombinant $IC_{50}$ values by laboratory for aminoglutethimide**

| Run | $IC_{50}$ Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Aminoglutethimide (µM) | | | | |
| 1 | 4.656 | 4.969 | 4.22 | 4.105 |
| 2 | 6.124 | 5.196 | 3.34 | 2.821 |
| 3 | 4.920 | 6.972 | 4.10 | 5.745 |
| Average | 5.23 | 5.71 | 3.89 | 4.22 |
| Sd | 0.78 | 1.10 | 0.48 | 1.47 |
| SEM | 0.45 | 0.63 | 0.28 | 0.85 |
| %CV | 15.0 | 19.2 | 12.3 | 34.7 |

Source: Reference 12

**Aminoglutethimide Recombinant Slope:** The average ± SEM slope values for RTI, Battelle, In Vitro, and WIL were -0.9774± 0.0615, -0.9861 ± 0.0060, -0.9278 ± 0.0489, and -0.9894 ± 0.03l1, respectively (Table 9.1-6). The percent CV values were 10.9, 1.0, 9.1, and 5.4 percent, respectively. Only one of the laboratories modified the concentrations tested after the first run, thereby suggesting that the initial range of concentrations selected were adequate to characterize the curve. The slopes of the curves were determined with a high degree of precision for all

laboratories. The overall task group mean ± SEM slope value was -0.9702 ± 0.0144 and the percent CV was 3.0 percent.

**Table 9.1-6. Recombinant slope values by laboratory for aminoglutethimide**

| Run | Slope Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Aminoglutethimide | | | | |
| 1 | -0.8562 | -0.9977 | -0.9324 | -0.9349 |
| 2 | -1.020 | -0.9779 | -0.8409 | -1.0425 |
| 3 | -1.056 | -0.9826 | -1.010 | -0.9907 |
| Average | -0.9774 | -0.9861 | -0.9278 | -0.9894 |
| sd | 0.1065 | 0.0103 | 0.0846 | 0.0538 |
| SEM | 0.0615 | 0.0060 | 0.0489 | 0.0311 |
| %CV | 10.9 | 1.0 | 9.1 | 5.4 |

Source: Reference 12

## 9.2 Atrazine

**Atrazine Assay Overview.** The effect of increasing concentrations of atrazine on aromatase activity is reported by laboratory in Tables B.9-3 and B.9-4 (Appendix B) and by overall task in Tables 9.2-1 and 9.2-2. In addition, the results are graphically presented by laboratory (individual runs and average) in Figures 9.2-1 and 9.2-2.

After the first run was completed, the study directors from two of the four laboratories modified the atrazine concentrations selected for testing in runs 2 and 3 (Tables B.9-3 and B.9-4). In Vitro and WIL used the same eight concentrations for their three runs. For the placental assay, in general, the modifications that were made in an attempt to characterize the shape of the curve included adding mid-level concentrations between $10^{-3}$ and $10^{-5}$ M. Even with the modifications, there was no further characterization of the curve beyond what was originally determined by the first run.

In the recombinant assay, at an atrazine concentration of $10^{-3}$ M (or $10^{-4}$ M for In Vitro), aromatase activity approximated 86 to 92 percent of control. Similarly, at an atrazine concentration of approximately $10^{-10}$ M, there was little to no aromatase inhibition; the laboratory percent of control values were greater than 90 percent for all labs. The overall task mean ± SEM percent of control values at $10^{-3}$ and $10^{-10}$ M were 88.06 ± 2.02 and 96.46 ± 3.51 percent, respectively (Table 9.2-2).

For all four laboratories, increasing concentrations of atrazine did not decrease the activity of the placental or recombinant microsomal aromatase (Tables 9.2-1 and 9.2-2). In the recombinant assay, however, there was a trend toward inhibition at the highest concentration (Table 9.2-2).

The shapes of the enzyme activity vs atrazine curves approached a horizontal line (Figures 9.2-1 and 9.2-2). In the placental assay, at an atrazine concentration of $10^{-3}$ M, aromatase activity approximated 80 to 90 percent of control. The overall task mean ± SEM percent of control values at $10^{-3}$ and $10^{-10}$ M were 81.31 ± 1.46 and 93.62 ± 1.57 percent, respectively (Table 9.2-1). All labs have assay precision values (%CV) that were less than 8 percent for those concentrations used in at least three runs (Table 9.2-1). The overall task assay precision ranged from 2 to 5 percent for those concentrations that were tested by 3 or more labs (Table 9.2-2).

**Table 9.2-1. Placental assay: effect of atrazine on aromatase activity (percent of control) by overall task**

| Reference Chemical Log Conc (M) | Number of Labs[a] | Overall Mean | Overall sd | Overall SEM | Overall %CV |
|---|---|---|---|---|---|
| -3.00 | 3 | 81.31 | 2.53 | 1.46 | 3.1 |
| -3.05 | 1 | 83.63 | NC[b] | NC | NC |
| -3.10 | 1 | 81.99 | NC | NC | NC |
| -3.12 | 1 | 84.21 | NC | NC | NC |
| -3.30 | 2 | 81.48 | NC | NC | NC |
| -3.60 | 1 | 85.75 | NC | NC | NC |
| -4.00 | 4 | 92.02 | 2.33 | 1.16 | 2.5 |
| -4.30 | 1 | 94.17 | NC | NC | NC |
| -4.48 | 1 | 89.83 | NC | NC | NC |
| -5.00 | 4 | 96.71 | 1.82 | 0.91 | 1.9 |
| -6.00 | 4 | 96.01 | 2.47 | 1.23 | 2.6 |
| -7.00 | 4 | 95.38 | 4.16 | 2.08 | 4.4 |
| -8.00 | 4 | 95.24 | 4.62 | 2.31 | 4.9 |
| -9.00 | 4 | 94.69 | 2.99 | 1.50 | 3.2 |
| -10.00 | 4 | 93.62 | 3.15 | 1.57 | 3.4 |

a. Number of labs that conducted the assay at the cited reference chemical concentration. The run number varied because each laboratory used the results from their first run to select concentrations to be used in runs 2 and 3 that were believed would further (In Vitro and WIL) or better characterize the percent inhibition curve.

b. NC – Not Calculated. If the overall mean was calculated using an n ≤ 2, then the sd, SEM, and %CV were not calculated.

**Table 9.2-2. Recombinant assay: effect of atrazine on aromatase activity (percent of control) by overall task**

| Reference Chemical Log Conc (M) | Number of Labs[a] | Overall Mean | Overall sd | Overall SEM | Overall %CV |
|---|---|---|---|---|---|
| -3.00 | 3 | 88.06 | 3.51 | 2.02 | 4.0 |
| -3.05 | 1 | 90.66 | NC[b] | NC | NC |
| -3.10 | 1 | 88.29 | NC | NC | NC |
| -3.12 | 1 | 89.41 | NC | NC | NC |
| -3.30 | 2 | 88.87 | NC | NC | NC |
| -3.60 | 1 | 90.43 | NC | NC | NC |
| -4.00 | 4 | 95.60 | 5.56 | 2.78 | 5.8 |
| -4.30 | 1 | 95.94 | NC | NC | NC |
| -4.48 | 1 | 92.14 | NC | NC | NC |
| -5.00 | 4 | 99.03 | 5.51 | 2.75 | 5.6 |
| -6.00 | 4 | 97.73 | 6.81 | 3.40 | 7.0 |
| -7.00 | 4 | 100.58 | 7.31 | 3.65 | 7.3 |
| -8.00 | 4 | 98.56 | 7.36 | 3.68 | 7.5 |
| -9.00 | 4 | 98.51 | 6.12 | 3.06 | 6.2 |
| -10.00 | 4 | 96.46 | 7.02 | 3.51 | 7.3 |

a. Number of labs that conducted the assay at the cited reference chemical concentration. The run number varied because each laboratory used the results from their first run to select concentrations to be used in runs 2 and 3 that were believed would further (In Vitro and WIL) or better characterize the percent inhibition curve.

b. NC – Not Calculated. If the overall mean was calculated using an $n \leq 2$, then the sd, SEM, and %CV were not calculated.

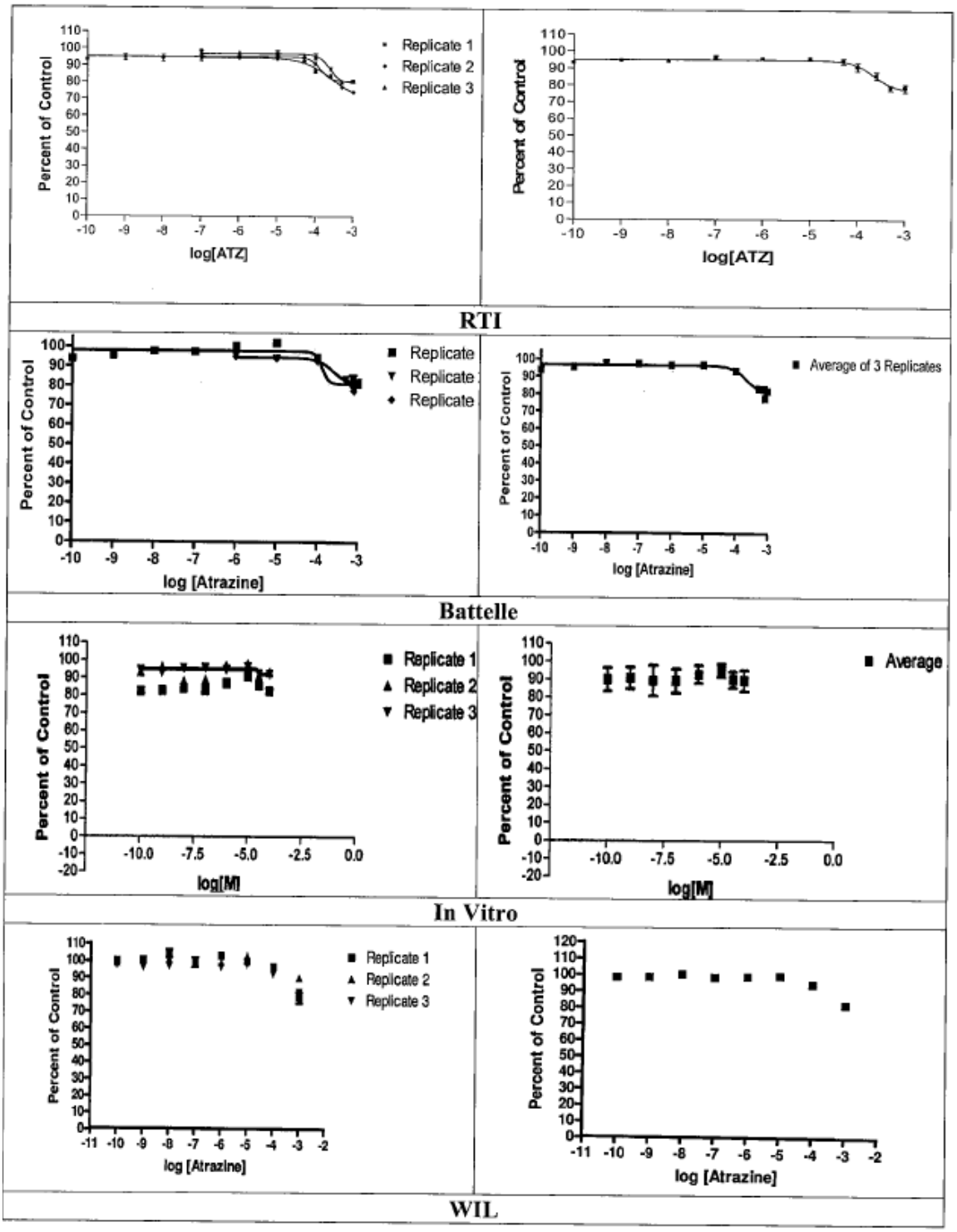



**Figure 9.2-1. Placental assay: individual run and average atrazine inhibition response curves by laboratory**

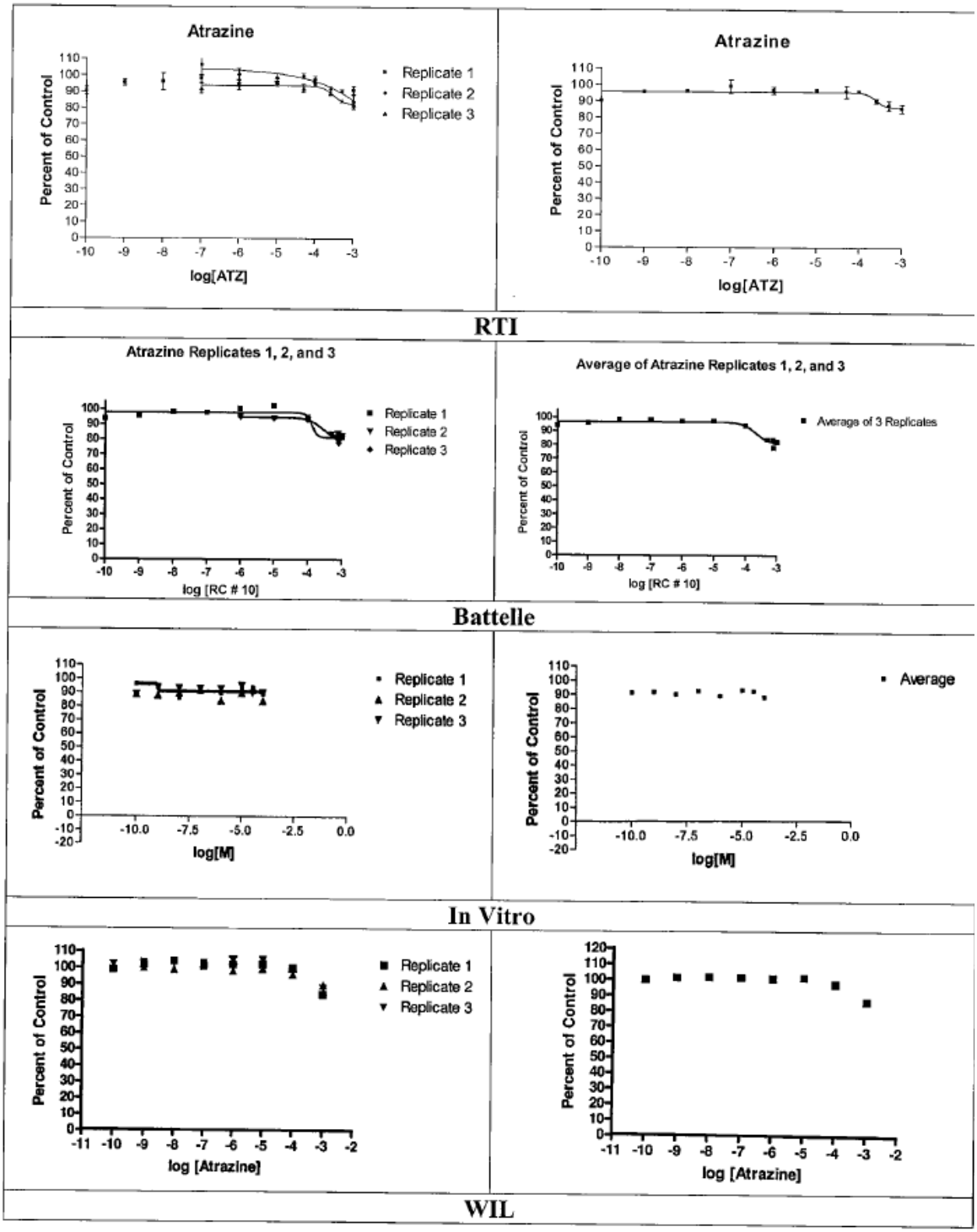



**Figure 9.2-2. Recombinant assay: individual run and average atrazine inhibition response curves (percent of control) by laboratory**

**Atrazine Placental IC50.** This compound did not inhibit aromatase (Table 9.2-3). There were no valid IC50 values reported by the laboratories. An examination of the percent of control curves indicated that there was no inhibition.

**Table 9.2-3. Placental $IC_{50}$ values by laboratory for atrazine**

| Run | $IC_{50}$ Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| | Atrazine (µM) | | | |
| 1 | no inhibition[a] | no inhibition[a] | no inhibition | no inhibition |
| 2 | no inhibition[a] | no inhibition[a] | no inhibition | no inhibition |
| 3 | no inhibition[a] | no inhibition[a] | no inhibition[a] | no inhibition |

a. Although a value was reported, the percent of control curve indicated that there was no inhibition.

Source: Reference 10

**Atrazine Placental Slope:** This compound did not inhibit aromatase (Table 9.2-4). There were no valid $IC_{50}$ values reported by the laboratories. An examination of the percent of control curves indicated that there was no inhibition.

**Table 9.2-4. Placental slope values by laboratory for atrazine**

| Run | Slope Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| | Atrazine | | | |
| 1 | no inhibition[a] | no inhibition[a] | no inhibition | no inhibition |
| 2 | no inhibition[a] | no inhibition[a] | no inhibition | no inhibition |
| 3 | no inhibition[a] | no inhibition[a] | no inhibition[a] | no inhibition |

a. Although a value was reported, the percent of control curve indicated that there was no inhibition.

Source: Reference 10

**Atrazine Recombinant IC50:** The % of control curves for each run and all laboratories indicated that no graded inhibition occurred, thereby precluding any determination of a valid IC50 value (Table 9.2-5). Although there were IC50 values determined for some of the laboratories, the Prism analysis for these runs indicated that the error associated with all of the parameter estimates was so large that the results were considered unreliable. Thus, atrazine did not inhibit aromatase.

**Table 9.2-5. Recombinant $IC_{50}$ values by laboratory for atrazine**

| Run | $IC_{50}$ Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| | Atrazine (µM) | | | |
| 1 | no inhibition | no inhibition[a] | no inhibition[a] | no inhibition |
| 2 | no inhibition[a] | no inhibition[a] | no inhibition | no inhibition |
| 3 | no inhibition | no inhibition[a] | no inhibition | no inhibition |

a. Although a value was reported, the percent of control curve indicated that there was no inhibition.

Source: Reference 12

**Atrazine Recombinant Slope:** The percent of control curves had no valid measurable slopes (Table 9.2-6). For the most part, the curves appeared as horizontal lines. There was no indication that any inhibition of aromatase by atrazine occurred.

**Table 9.2-6. Recombinant slope values by laboratory for atrazine**

| Run | Slope Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| **Atrazine** | | | | |
| 1 | no inhibition | no inhibition[a] | no inhibition[a] | no inhibition |
| 2 | no inhibition[a] | no inhibition[a] | no inhibition | no inhibition |
| 3 | no inhibition | no inhibition[a] | no inhibition | no inhibition |

a. Although a value was reported, the percent of control curve indicated that there was no inhibition.

Source: Reference 12

## 9.3 Chrysin

**Chrysin Assay Overview.** The effect of increasing concentrations of chrysin on aromatase activity is reported by laboratory in Tables B.9-5 and B.9-6 (Appendix B) and by overall task in Tables 9.3-1 and 9.3-2. In addition, the results are graphically presented by laboratory (individual runs and average) in Figures 9.3-1 and 9.3-2.

After the first placental run was completed, the study directors from all laboratories modified the chrysin concentrations selected for testing in runs 2 and 3 (Table B.9-5). In general, the modifications involved including additional mid-concentrations between $10^{-4}$ and $10^{-7}$ M and using them in place of concentrations at $10^{-9}$ to $10^{-10}$ M that were tested in run 1.

After the first recombinant run was completed, the study directors from three of four laboratories modified the chrysin concentrations selected for testing in runs 2 and 3 (Table B.9-6). RTI tested the same concentrations for all three runs (a fourth run was used when the first run did not show any dose dependence). The modifications involved including additional mid-concentrations between $10^{-5}$ and $10^{-6}$ M and using them in place of concentrations at the high end, i.e., $10^{-4}$ M (In Vitro), or the low end, i.e., $10^{-9}$ to $10^{-10}$ M (Battelle and WIL), of the concentrations that were tested in run 1.

For all four laboratories, increasing concentrations of chrysin decreased the activity of the microsomal aromatase and the decrease was concentration-dependent (Tables 9.3-1 and 9.3-2). The shapes of the enzyme activity vs chrysin curves were sigmoidal (Figures 9.3-1 and 9.3-2). At a chrysin concentration of $10^{-4}$ M (the highest achievable concentration), aromatase inhibition percent of control values ranged from 14 to 23 percent in the placental assay, and from approximately 15 to 25 percent for the recombinant assay.

In the placental assay, at chrysin concentrations $\leq 10^{-7}$ M there was little aromatase inhibition with most laboratories reporting percent of control values greater than 90 percent. The overall task mean ± SEM percent of control values at $10^{-4}$ and $10^{-7}$ M were 18.74 ± 1.88 and 89.51 ± 1.69 percent, respectively (Table 9.3-1).

The highest chrysin concentrations tested in the recombinant assay did not result in a percent response. By contrast, at chrysin concentrations $\leq 10^{-8}$ M in the recombinant assay, there was little aromatase inhibition with most laboratories reporting percent of control values greater than or equal to approximately 85 percent. The overall task mean ± SEM percent of control values at $10^{-4}$ and $10^{-8}$ M were 20.84 ± 2.20 and 91.23 ± 2.16 percent, respectively (Table 9.3-2).

**Table 9.3-1. Placental assay: effect of chrysin on aromatase activity by overall task**

| Reference Chemical Log Conc (M) | Number of Labs[a] | Overall Mean | Overall sd | Overall SEM | Overall %CV |
|---|---|---|---|---|---|
| -4.00 | 4 | 18.74 | 3.76 | 1.88 | 20.0 |
| -4.48 | 1 | 8.19 | NC[b] | NC | NC |
| -5.00 | 4 | 24.28 | 2.23 | 1.12 | 9.2 |
| -5.30 | 3 | 38.33 | 2.50 | 1.45 | 6.5 |
| -5.48 | 1 | 47.21 | NC | NC | NC |
| -5.60 | 3 | 53.64 | 3.11 | 1.80 | 5.8 |
| -6.00 | 4 | 72.93 | 2.29 | 1.15 | 3.1 |
| -6.30 | 1 | 80.70 | NC | NC | NC |
| -6.60 | 1 | 86.99 | NC | NC | NC |
| -7.00 | 4 | 89.51 | 3.39 | 1.69 | 3.8 |
| -8.00 | 4 | 92.31 | 1.32 | 0.66 | 1.4 |
| -9.00 | 4 | 94.82 | 5.68 | 2.84 | 6.0 |
| -10.00 | 4 | 90.05 | 5.12 | 2.56 | 5.7 |

a. Number of labs that conducted the assay at the cited reference chemical concentration. The run number varied because each laboratory used the results from their first run to select concentrations to be used in runs 2 and 3 that were believed would better characterize the percent inhibition curve.

b. NC – Not Calculated. If the overall mean was calculated using an n ≤ 2, then the sd, SEM, and %CV were not calculated.

**Table 9.3-2. Recombinant assay: effect of chrysin on aromatase activity (percent of control) by overall task**

| Reference Chemical Log Conc (M) | Number of Labs[a] | Overall Mean | Overall sd | Overall SEM | Overall %CV |
|---|---|---|---|---|---|
| -4.00 | 4 | 20.84 | 4.41 | 2.20 | 21.1 |
| -4.48 | 1 | 18.13 | NC[b] | NC | NC |
| -5.00 | 4 | 27.05 | 2.26 | 1.13 | 8.4 |
| -5.30 | 3 | 41.24 | 4.02 | 2.32 | 9.7 |
| -5.48 | 1 | 67.65 | NC | NC | NC |
| -5.60 | 3 | 57.19 | 4.46 | 2.57 | 7.8 |
| -6.00 | 4 | 70.22 | 7.89 | 3.95 | 11.2 |
| -6.30 | 1 | 82.17 | NC | NC | NC |
| -6.60 | 1 | 64.93 | NC | NC | NC |
| -7.00 | 4 | 89.73 | 6.36 | 3.18 | 7.1 |
| -8.00 | 4 | 91.23 | 4.33 | 2.16 | 4.7 |
| -9.00 | 3 | 91.54 | 7.65 | 4.41 | 8.4 |
| -10.00 | 3 | 85.01 | 8.72 | 5.04 | 10.3 |

a. Number of labs that conducted the assay at the cited reference chemical concentration. The run number varied because three of four laboratories used the results from their first run to select concentrations to be used in runs 2 and 3 that were believed would better characterize the percent inhibition curve. RTI used the same concentrations for all three runs.

b. NC – Not Calculated. If the overall mean was calculated using an n ≤ 2, then the sd, SEM, and %CV were not calculated.

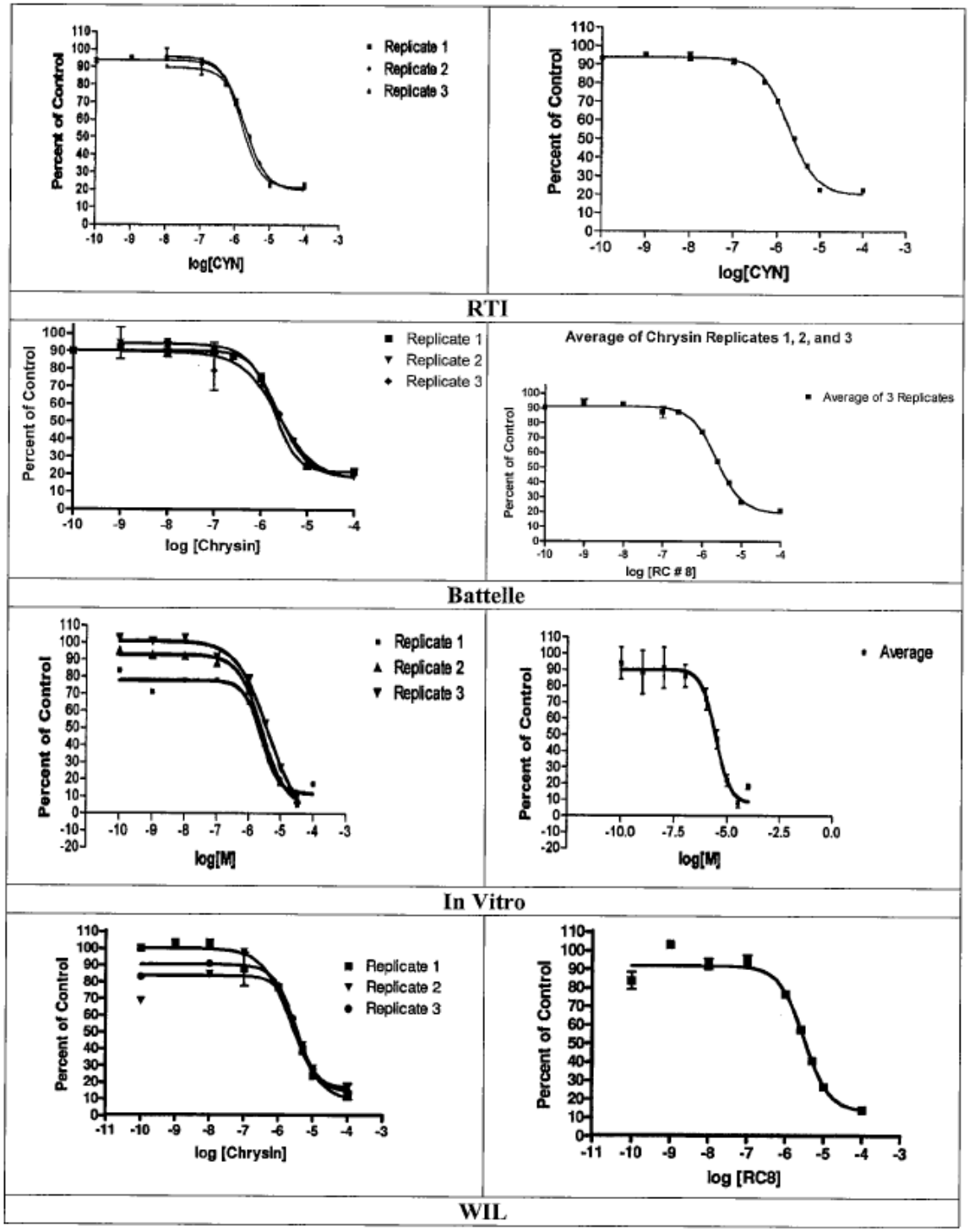



**Figure 9.3-1. Placental assay: individual run and average chrysin inhibition curves by laboratory**

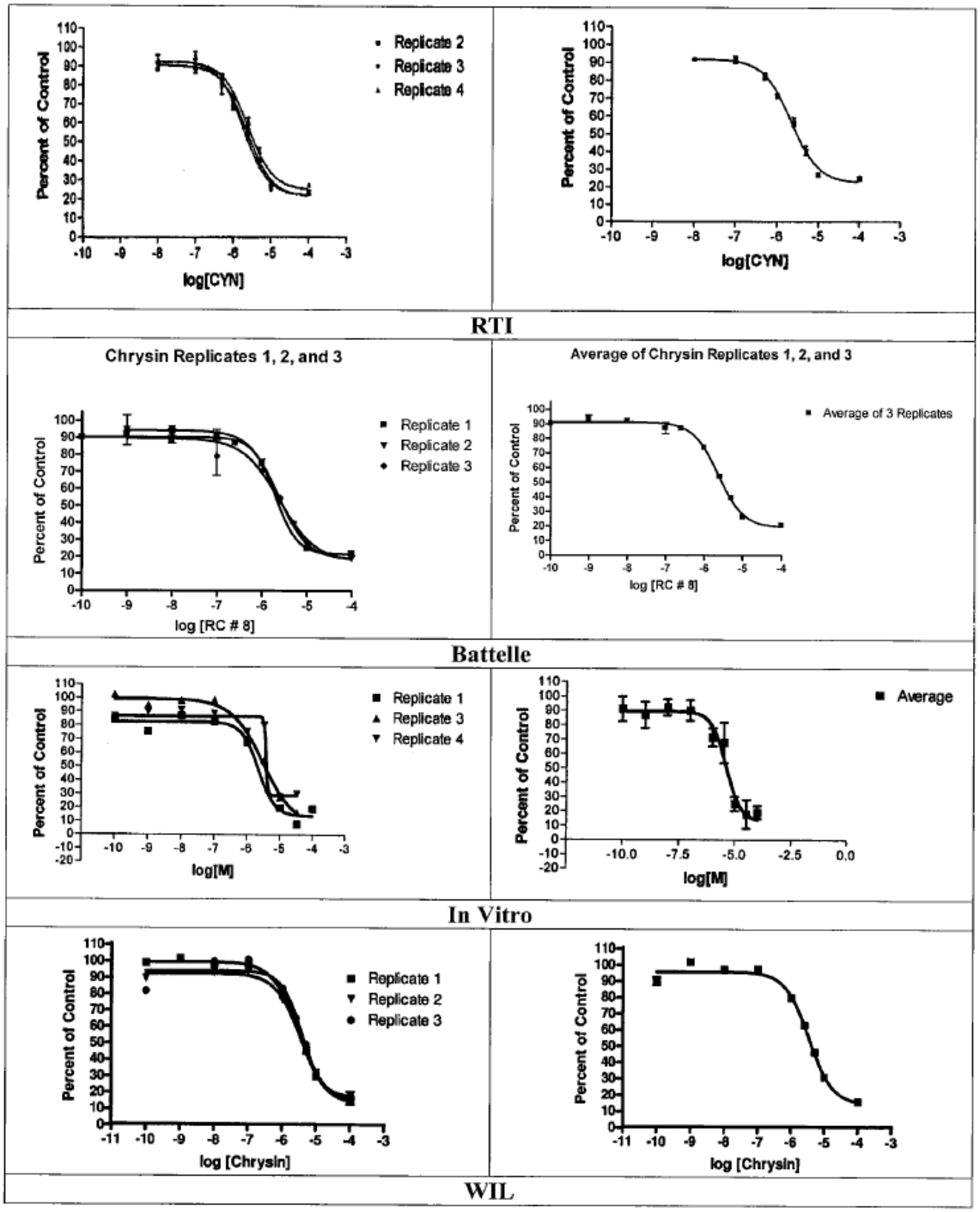



**Figure 9.3-2. Recombinant assay: individual run and average chrysin inhibition response curves (percent of control) by laboratory**

**Chrysin Placental IC50:** The average ± SEM $IC_{50}$ values for RTI, Battelle, In Vitro, and WIL were 1.72 ± 0.13, 2.21 ± 0.13, 3.02 ± 0.48, and 3.06 ± 0.33 µM, respectively (Table 9.3-3). The percent CV values were 13.4, 10.0, 27.4, and 18.4 percent, respectively. The higher %CV values for In Vitro and WIL were attributed to the IC50 value obtained from one of the three runs. For In Vitro, run 3 was relatively higher and, for WIL, run 1 was relatively lower. The overall task group mean ± SEM IC50 value was 2.50 ± 0.33 µM and the percent CV was 26.1 percent.

**Table 9.3-3. Placental $IC_{50}$ values by laboratory for chrysin**

| Run | $IC_{50}$ Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| | Chrysin (µM) | | | |
| 1 | 1.48 | 1.97 | 2.47 | 2.44 |
| 2 | 1.93 | 2.25 | 2.62 | 3.54 |
| 3 | 1.75 | 2.40 | 3.97 | 3.21 |
| Average | 1.72 | 2.21 | 3.02 | 3.06 |
| sd | 0.23 | 0.22 | 0.83 | 0.56 |
| SEM | 0.13 | 0.13 | 0.48 | 0.33 |
| %CV | 13.4 | 10.0 | 27.4 | 18.4 |

Source: Reference 10

**Chrysin Placental Slope:** The average ± SEM slope values for RTI, Battelle, In Vitro, and WIL were -1.421 ± 0.066, -1.395 ± 0.208, -1.213 ± 0.211, and -1.317 ± 0.158, respectively (Table 9.3-4). The percent CV values were 8.0, 25.8, 30.2, and 20.8 percent, respectively. The higher %CV values for Battelle, In Vitro, and WIL were attributed to a slope obtained from run 1. The results from RTI indicate that the slope could be precisely estimated using the run 1 starting concentrations. However, all four labs showed that modifications to the run 1 concentrations tested did improve the characterization of the steep portion of the inhibition curve and slope estimate.

Regardless, the slope estimates were in good agreement for the labs. The overall task group mean ± SEM slope value was -1.336 ± 0.047 and the percent CV was 7.0 percent.

**Table 9.3-4. Placental slope values by laboratory for chrysin**

| Run | Slope Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| | Chrysin | | | |
| 1 | -1.528 | -1.785 | -1.599 | -1.0139 |
| 2 | -1.432 | -1.325 | -1.167 | -1.5485 |
| 3 | -1.302 | -1.075 | -0.872 | -1.3875 |
| Average | -1.421 | -1.395 | -1.213 | -1.317 |
| sd | 0.114 | 0.360 | 0.366 | 0.274 |
| SEM | 0.066 | 0.208 | 0.211 | 0.158 |
| %CV | 8.0 | 25.8 | 30.2 | 20.8 |

Source: Reference 12

**Chrysin Recombinant $IC_{50}$:** The average ± SEM $IC_{50}$ values for RTI, Battelle, In Vitro, and WIL were 2.18 ± 0.17, 1.48 ± 0.36, 3.02 ± 0.46, and 3.41 ± 0.20 µM, respectively (Table 9.3-5). The percent CV values were 13.1, 42.0, 26.5, and 10.2 percent, respectively. The higher %CV values for Battelle and In Vitro were attributed to the $IC_{50}$ value obtained from one of the three runs. For Battelle, run 1 produced a relatively lower $IC_{50}$ value, which was attributed to the lower concentrations producing a higher level of inhibition that was not observed for runs 2 and 3. This is a good example of how the variability was increased by including the results from the first run in the average. For In Vitro, run 3 produced a relatively higher $IC_{50}$ value, which was attributed to an oddly shaped percent of control curve with a very steep slope, i.e., almost perpendicular with the x-axis. It appears that the $IC_{50}$ could be adequately estimated without modifying the concentrations tested, although the data from WIL is the only laboratory that can be used to draw such a conclusion because of the issues addressed above for Battelle and In Vitro, as well as the fact that RTI used the same concentrations for all three runs. It is also important to note that for all of the laboratories, the percent of control values at the highest chrysin concentrations tested did not achieve a zero percent response. Rather, the overall task average percent of control value achieved was approximately 2l percent. The overall task group mean ± SEM IC50 value was 2.52 ± 0.43 µM and the percent CV was 34.3 percent.

**Table 9.3-5. Recombinant $IC_{50}$ values by laboratory for chrysin**

| Run | $IC_{50}$ Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| | Chrysin (µM) | | | |
| 1 | 2.265 | 0.8299 | 2.19 | 3.030 |
| 2 | 1.862 | 1.536 | 3.09 | 3.472 |
| 3 | 2.415 | 2.066 | 3.79 | 3.713 |
| Average | 2.18 | 1.48 | 3.02 | 3.41 |
| Sd | 0.29 | 0.62 | 0.80 | 0.35 |
| SEM | 0.17 | 0.36 | 0.46 | 0.20 |
| %CV | 13.1 | 42.0 | 26.5 | 10.2 |

Source: Reference 12.

**Chrysin Recombinant Slope:** The average ± SEM slope values for RTI, Battelle, In Vitro, and WIL were -1.305 ± 0.008, -1.135 ± 0.l71, -1.289 ± 0.402, and -1.23l ± 0.101, respectively (Table 9.3-6). The percent CV values were 1.1, 26.1, 44.0, and 14.2 percent, respectively. The higher %CV value for Battelle was attributed to the shape of the run 1 curve, which did not produce a full response at the low and high concentrations. As for In Vitro, the values reported above were calculated only using runs 1 and 2 because the shape of the curve for the last run was very skewed, i.e., the slope was virtually perpendicular to the x-axis. If all three runs were used, then the average ± SEM slope and %CV values for In Vitro were -6.1 00 calculated only using runs 1 and 2 because the shape of the curve for the last run was very skewed, i.e., the slope was virtually perpendicular to the x-axis. If all three runs were used, then the average ± SEM slope and %CV values for In Vitro were -6.1 00 ± l 4.8l6 and 136.7 percent. The relatively high degree of precision achieved by RTI was attributed to their use of the same concentrations for all three runs. Regardless, it appears that the slope of the curve was precisely determined over the concentration range tested. The overall task group mean SEM slope value was -1.240 ± 0.038

and the percent CV was 6.2 percent. (Note: overall values do not include the aberrant value for In Vitro.)

**Table 9.3-6. Recombinant slope values by laboratory for chrysin**

| Run | Slope Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Chrysin | | | | |
| 1 | -1.295 | -1.426 | -1.691 | -1.1072 |
| 2 | -1.321 | -0.8334 | -0.8879 | -1.1553 |
| 3 | -1.298 | -1.145 | -15.72[a] | -1.4316 |
| Average | -1.305 | -1.135 | -6.100 | -1.231 |
| sd | 0.014 | 0.296 | 8.341 | 0.175 |
| SEM | 0.008 | 0.171 | 4.816 | 0.101 |
| %CV | 1.1 | 26.1 | 136.7 | 14.2 |

a. Considered an aberrant value. See source text for results without the value included.

Source: Reference 12

## 9.4 Dicofol

**Dicofol Assay Overview.** The effect of increasing concentrations of dicofol on aromatase activity is reported by laboratory in Tables B.9-7 and B.9-8 (Appendix B) and by overall task in Tables 9.4-1 and 9.4-2. In addition, the results are graphically presented by laboratory (individual runs and average) in Figures 9.4-1 and 9.4-2.

After the first run was completed, the study directors from three of four laboratories modified the dicofol concentrations selected for testing in runs 2 and 3 (Tables B.9-7 and B.9-8). In the placental assay, In Vitro used the same dicofol concentrations for all three runs. It is not clear why In Vitro did not test at a concentration of $10^{-3}$ M. In general, the modifications involved including additional mid-concentrations between $10^{-3}$ and $10^{-6}$ M, which replaced concentrations ranging from $10^{-7}$ to $10^{-10}$ M that were tested in run 1.

In the recombinant assay, In Vitro used the same dicofol concentrations for all three runs and may have done so based on information from the previous work assignment. It is not clear why In Vitro's highest concentration tested was $10^{-4}$ M, rather than $10^{-3}$ M. In general, the modifications involved including additional mid-concentrations between $10^{-3}$ and $10^{-6}$ M, which replaced concentrations at the low end, i.e.$10^{-7}$ to $10^{-10}$ M (RTI, Battelle, and WIL) of the concentration range that was tested in run 1.

For all four laboratories, increasing concentrations of dicofol decreased the activity of the microsomal aromatase in a concentration-dependent sigmoidal curve (Figures 9.4-1 and 9.4-2). In the placental assay, the curve for In Vitro was a partial sigmoidal curve, since they did not test at the highest possible concentration. At a dicofol concentration of $10^{-3}$ M, aromatase inhibition was almost complete for the three of four laboratories that tested at this concentration. The laboratory percent of control value was 3 percent at $10^{-3}$ M. For In Vitro, the percent of control value at its highest tested concentration ($10^{-4}$ M) was 45 percent. In contrast, at a dicofol

concentration of approximately $10^{-6}$ M, there was little to no aromatase inhibition; the laboratory percent of control values were approximately 90 percent or higher for all labs. The overall task mean ± SEM percent of control values at $10^{-3}$ and $10^{-6}$ M were 2.86 ± 0.15 and 91.01 ± 0.80 percent, respectively (Table 9.4-1).

In the recombinant assay, at a dicofol concentration of $10^{-3}$ M, aromatase inhibition was almost complete for the three of four laboratories that tested at this concentration. The laboratory percent of control value was 2 to 3 percent at $10^{-3}$ M. It is important to note that the curves for In Vitro did not result in achieving activity that was a percent of control at the highest concentration tested, i.e., the percent of control value at $10^{-4}$ M was 38 percent. In contrast, at a dicofol concentration of approximately $10^{-7}$ M, there was little to no aromatase inhibition; the laboratory percent of control values were approximately 90 percent or higher for all labs. The overall task mean ± SEM percent of control values at $10^{-3}$ and $10^{-7}$ M were 2.65 ± 0.32 and 93.32 ± l.66 percent, respectively (Table 9.4-2).

.

**Table 9.4-1. Placental assay: effect of dicofol on aromatase activity by overall task**

| Reference Chemical Log Conc (M) | Number of Labs[a] | Overall Mean | Overall sd | Overall SEM | Overall %CV |
|---|---|---|---|---|---|
| -3.00 | 3 | 2.86 | 0.25 | 0.15 | 8.9 |
| -3.30 | 1 | 5.59 | NC[b] | NC | NC |
| -3.52 | 1 | 13.57 | NC | NC | NC |
| -3.60 | 1 | 12.05 | NC | NC | NC |
| -4.00 | 4 | 31.78 | 9.47 | 4.73 | 29.8 |
| -4.30 | 1 | 33.03 | NC | NC | NC |
| -4.48 | 1 | 61.52 | NC | NC | NC |
| -4.52 | 1 | 60.61 | NC | NC | NC |
| -5.00 | 4 | 67.41 | 1.09 | 0.55 | 1.6 |
| -5.52 | 1 | 84.67 | NC | NC | NC |
| -6.00 | 4 | 91.01 | 1.60 | 0.80 | 1.8 |
| -7.00 | 4 | 97.36 | 1.53 | 0.76 | 1.6 |
| -8.00 | 4 | 97.36 | 4.05 | 2.02 | 4.2 |
| -9.00 | 4 | 98.21 | 3.36 | 1.68 | 3.4 |
| -10.00 | 4 | 98.35 | 1.59 | 0.79 | 1.6 |

a. Number of labs that conducted the assay at the cited reference chemical concentration. The run number varied because each laboratory used the results from their first run to select concentrations to be used in runs 2 and 3 that were believed would further (In Vitro) or better characterize the percent inhibition curve.
b. NC – Not Calculated. If the overall mean was calculated using an n ≤ 2, then the sd, SEM, and %CV were not calculated.

**Table 9.4-2. Recombinant assay: effect of dicofol on aromatase activity (percent of control) by overall task**

| Reference Chemical Log Conc (M) | Number of Labs[a] | Overall Mean | Overall sd | Overall SEM | Overall %CV |
|---|---|---|---|---|---|
| -3.00 | 3 | 2.65 | 0.55 | 0.32 | 20.8 |
| -3.30 | 1 | 6.25 | NC[b] | NC | NC |
| -3.52 | 1 | 12.10 | NC | NC | NC |
| -3.60 | 1 | 12.42 | NC | NC | NC |
| -4.00 | 4 | 27.06 | 8.85 | 4.43 | 32.7 |
| -4.30 | 1 | 22.95 | NC | NC | NC |
| -4.48 | 1 | 47.10 | NC | NC | NC |
| -4.52 | 1 | 59.51 | NC | NC | NC |
| -5.00 | 4 | 66.07 | 8.48 | 4.24 | 12.8 |
| -5.52 | 1 | 86.35 | NC | NC | NC |
| -6.00 | 4 | 93.02 | 3.77 | 1.88 | 4.1 |
| -7.00 | 4 | 93.32 | 3.32 | 1.66 | 3.6 |
| -8.00 | 4 | 95.77 | 7.66 | 3.83 | 8.0 |
| -9.00 | 4 | 96.65 | 5.68 | 2.84 | 5.9 |
| -10.00 | 4 | 95.12 | 4.75 | 2.38 | 5.0 |

a. Number of labs that conducted the assay at the cited reference chemical concentration. The run number varied because each laboratory used the results from their first run to select concentrations to be used in runs 2 and 3 that were believed would further (In Vitro) or better characterize the percent inhibition curve.

b. NC – Not Calculated. If the overall mean was calculated using an $n \leq 2$, then the sd, SEM, and %CV were not calculated.

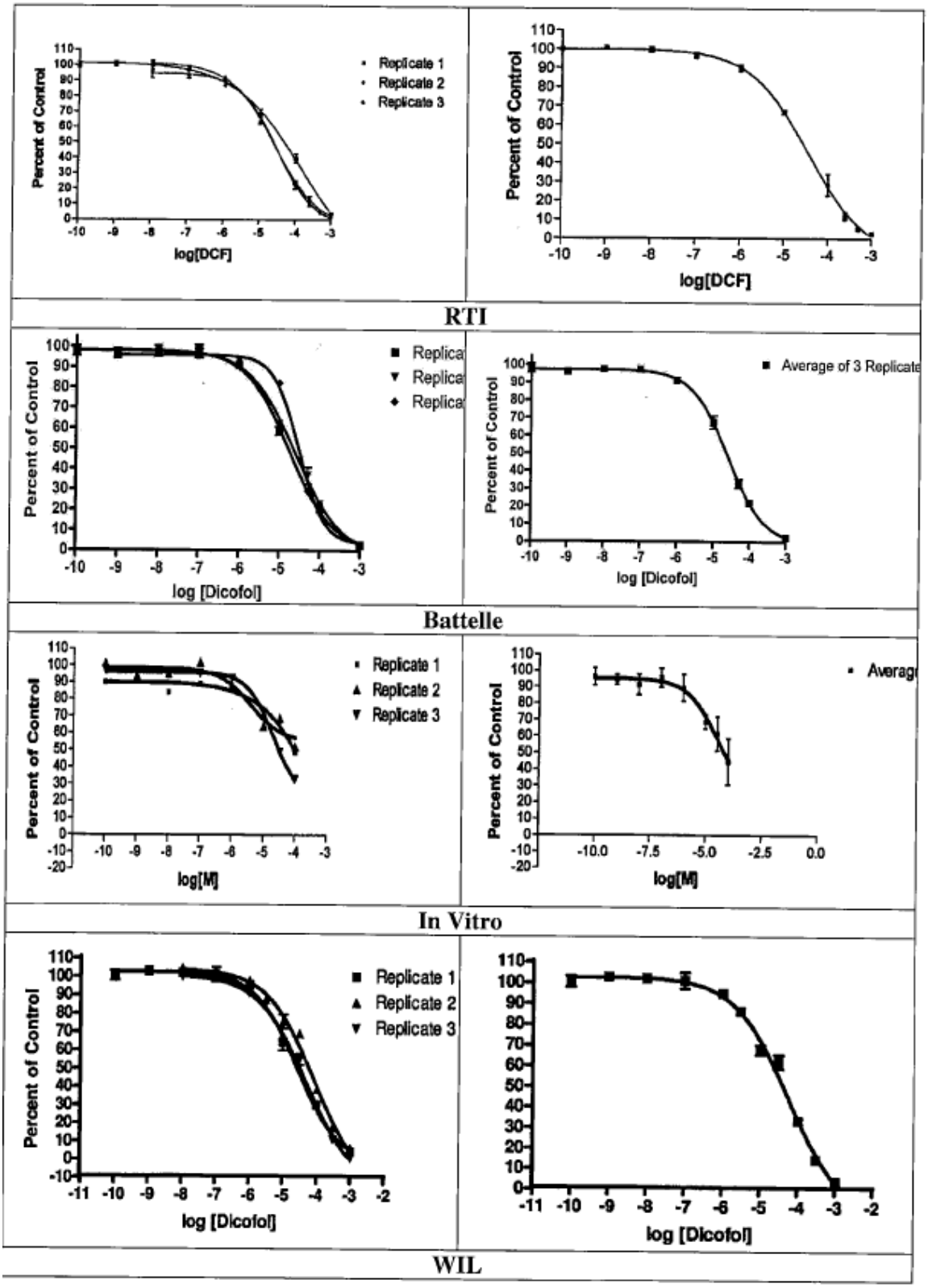



**Figure 9.4-1. Placental assay: individual run and average dicofol inhibition response curves by laboratory**

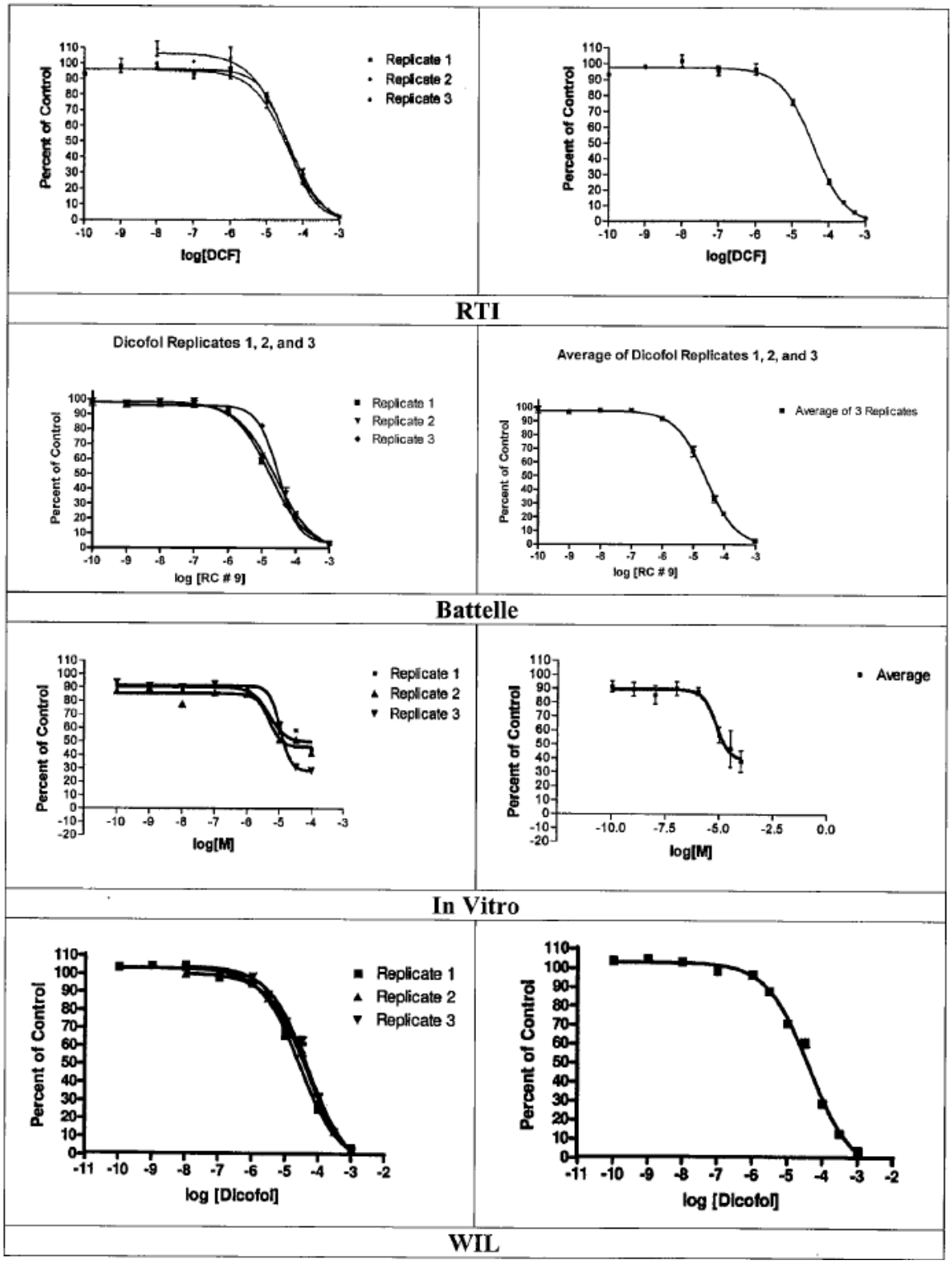



**Figure 9.4-2. Recombinant assay: individual run and average dicofol inhibition response curves (percent of control) by laboratory**

**Dicofol Placental $IC_{50}$:** The average ± SEM $IC_{50}$ values for RTI, Battelle, In Vitro, and WIL were 62.91 ± 35.86, 24.14 ± 3.72, 501 ± 489, and 53.13 ± 16.56 µM, respectively (Table 9.4-3). The percent CV values were 98.7, 26.7, 169.0, and 54.0 percent, respectively. The higher %CV values for RTI and In Vitro were attributed to the $IC_{50}$ value obtained from run 1. Modifications to the concentrations tested by RTI and In Vitro after run 1 resulted in $IC_{50}$ values that were in better agreement with subsequent estimates, as well as estimates by the other labs. The results from Battelle and WIL indicate that the $IC_{50}$ could be precisely estimated using the run 1 starting concentrations. However, three of four labs showed that modifications to the run 1 concentrations tested did improve the characterization of the steep portion of the inhibition curve and IC50 estimate. Due to the extremely high $IC_{50}$ values reported by RTI and In Vitro from their run 1 results, these values were not included in the overall task estimate. The overall task group mean ± SEM $IC_{50}$ value was 29.13 ± 8.62 µM and the percent CV was 59.2 percent.

**Table 9.4-3. Placental $IC_{50}$ values by laboratory for dicofol**

| Run | $IC_{50}$ Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| | Dicofol (µM) | | | |
| 1 | 134.59 | 17.41 | 1478 | 26.56 |
| 2 | 29.24 | 24.75 | 3.966 | 83.54 |
| 3 | 24.89 | 30.27 | 20.42 | 49.28 |
| Mean | 62.91 | 24.14 | 501 | 53.13 |
| sd | 62.12 | 6.45 | 846 | 28.68 |
| SEM | 35.86 | 3.72 | 489 | 16.56 |
| %CV | 98.7 | 26.7 | 169.0 | 54.0 |

Source: Reference 10

**Dicofol Placental Slope:** For dicofol, the average ± SEM slope values for RTI, Battelle, In Vitro, and WIL were -0.6671 ± 0.1075, -0.9984 ± 0.2055, -0.7978 ± 0.1659, and -0.6394 ± 0.0270, respectively (Table 9.4-4). The percent CV values were 27.9, 35.7, 36.0, and 7.3 percent, respectively. The higher %CV values for RTI and In Vitro were attributed to a slope obtained from run 1, whereas for Battelle it was associated with the slope determined from run 3. The results from WIL indicate that the slope could be precisely estimated using the run 1 starting concentrations. However, three of four labs showed that modifications to the run 1 concentrations tested did improve the characterization of the steep portion of the inhibition curve and slope estimate. The overall task group mean :: SEM slope value was -0.7757 ± 0.0819 and the percent CV was 21.1 percent.

**Table 9.4-4. Placental slope values by laboratory for dicofol**

| Run | Slope Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Dicofol | | | | |
| 1 | -0.4643 | -0.8235 | -0.4795 | -0.6920 |
| 2 | -0.8303 | -0.7637 | -0.8759 | -0.6235 |
| 3 | -0.7067 | -1.408 | -1.038 | -0.6028 |
| Mean | -0.6671 | -0.9984 | -0.7978 | -0.6394 |
| Sd | 0.1862 | 0.3560 | 0.2873 | 0.0467 |
| SEM | 0.1075 | 0.2055 | 0.1659 | 0.0270 |
| %CV | 27.9 | 35.7 | 36.0 | 7.3 |

Source: Reference 10

**Dicofol Recombinant $IC_{50}$:** The average ± SEM $IC_{50}$ values for RTI, Battelle, In Vitro, and WIL were 35.77 ± 0.79, 17.54 ± 2.13, 6.71 ± 1.95, and 39.96 ± 6.55 µM, respectively (Table 9.4-5). The percent CV values were 3.8, 21.0, 50.3, and 28.4 percent, respectively. The higher %CV value for In Vitro was attributed to the response curves for all three runs. Unlike the other laboratories, the percent of control values at the higher concentrations did not achieve a zero percent response for In Vitro, presumably because they didn't test at $10^{-3}$ M, like the other labs. Not only was there a larger measure of variability in the $IC_{50}$ value, but it was also several fold lower than what was estimated by the other laboratories. It is unclear why In Vitro's results differed at the higher concentrations tested. The results from the other laboratories indicate that the $IC_{50}$ can be precisely estimated using the run 1 starting concentrations. However, the results also indicated that modifications to the run 1 concentrations tested did improve the characterization of the steep portion of the inhibition curve and $IC_{50}$ estimate. The overall task group mean ± SEM $IC_{50}$ value was 25.00 ± 7.80 µM and the percent CV was 62.4 percent.

**Table 9.4-5. Recombinant $IC_{50}$ values by laboratory for dicofol**

| Run | $IC_{50}$ Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Dicofol (µM) | | | | |
| 1 | 37.33 | 15.25 | 4.74 | 27.22 |
| 2 | 35.24 | 15.57 | 4.78 | 43.67 |
| 3 | 34.75 | 21.79 | 10.6 | 49.00 |
| Mean | 35.77 | 17.54 | 6.71 | 39.96 |
| Sd | 1.37 | 3.69 | 3.37 | 11.35 |
| SEM | 0.79 | 2.13 | 1.95 | 6.55 |
| %CV | 3.8 | 21.0 | 50.3 | 28.4 |

Source: Reference 12

**Dicofol Recombinant Slope:** The average ± SEM slope values for RTI, Battelle, In Vitro, and WIL were -0.9773 ± 0.0951, -1.1443 ± 0.1409, -2.1567 ± 0.2645, and -0.725 ± 0.0056, respectively (Table 9.4-6). The percent CV values were 16.9, 21.3, 21.2, and 1.3 percent, respectively. The shapes of the curves were similar for RTI, Battelle, and WIL but dissimilar for In Vitro in that the higher concentrations did not result in a 0 percent response for any of the runs. This finding led to a steeper slope estimate for In Vitro than for the other laboratories.

Although three of the four laboratories modified the concentrations tested after run 1, there wasn't an obvious improvement in characterizing the curve or estimating the slope. Thus, the initial concentrations tested were adequate in providing this information. The overall task group mean ± SEM slope value was -1.2511 ± 0.3139 and the percent CV was 50.2 percent.

**Table 9.4-6. Recombinant slope values by laboratory for dicofol**

| Run | Slope Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Dicofol | | | | |
| 1 | -1.159 | -1.246 | -1.664 | -0.7327 |
| 2 | -0.8375 | -0.8659 | -2.236 | -0.7303 |
| 3 | -0.9354 | -1.321 | -2.570 | -0.7148 |
| Mean | -0.9773 | -1.1443 | -2.1567 | -0.7259 |
| sd | 0.1648 | 0.2440 | 0.4582 | 0.0097 |
| SEM | 0.0951 | 0.1409 | 0.2645 | 0.0056 |
| %CV | 16.9 | 21.3 | 21.2 | 1.3 |

Source: Reference 12

## 9.5 Dibenz[a,h]anthracene

**Dibenz[a,h]anthracene Assay Overview.** The effect of increasing concentrations of dibenz[a,h]anthracene on aromatase activity is reported by laboratory in Tables B.9-9 and B.9-10 (Appendix B) and by overall task in Tables 9.5-1 and 9.5-2. In addition, the results are graphically presented by laboratory (individual runs and average) in Figures 9.5-1 and 9.5-2.

For all four laboratories, increasing concentrations of dibenz(a,h) anthracene did not decrease the activity of the microsomal aromatase (Tables B.9-9 and B.9-10). The shapes of the enzyme activity vs dibenz(a,h)anthracene curves approached a horizontal line (Figures 9.5-1 and 9.5-2). At a dibenz(a,h)anthracene concentration of $10^{-4}$ M (or $3.3 \times 10^{-5}$ M for In Vitro in the placental assay) to $10^{-10}$M, there was no indication of any aromatase inhibition.

In the placental assay, the overall task mean ± SEM percent of control values at $10^{-4}$ and $10^{-10}$ M were 100.77 ± 2.23 and 97.56 ± 3.96 percent, respectively (Table 9.5-1).

In the recombinant assay, after the first run was completed, the study directors from three of four laboratories did not make any modifications of the dibenz[a,h]anthracene concentrations selected for testing in runs 2 and 3 (Table B.9-10). In Vitro modified the run 1 concentrations tested by deleting the $3.3 \times 10^{-6}$ M concentration and adding a high-dilution concentration, i.e., $10^{-4}$ M. Based on the run 1 results and the absence of any inhibition, the other laboratories apparently didn't believe it was necessary to modify the run 1 concentrations.

In the recombinant assay, the overall task mean ± SEM percent of control values at $10^{-4}$ and $10^{-10}$ M were 95.98 ± 3.83 and 96.49 ± 4.24 percent, respectively (Table 9.5-2).

**Table 9.5-1. Placental assay: effect of dibenz[a,h]anthracene on aromatase activity by overall task**

| Reference Chemical Log Conc (M) | Number of Labs[a] | Overall Mean | Overall sd | Overall SEM | Overall %CV |
|---|---|---|---|---|---|
| -4.00 | 3 | 100.77 | 3.86 | 2.23 | 3.8 |
| -4.48 | 1 | 83.46 | NC[b] | NC | NC |
| -5.00 | 4 | 100.74 | 5.90 | 2.95 | 5.9 |
| -5.30 | 1 | 105.04 | NC | NC | NC |
| -5.48 | 1 | 88.28 | NC | NC | NC |
| -6.00 | 4 | 98.14 | 11.07 | 5.53 | 11.3 |
| -6.30 | 1 | 101.70 | NC | NC | NC |
| -6.60 | 1 | 104.17 | NC | NC | NC |
| -7.00 | 4 | 101.08 | 5.65 | 2.83 | 5.6 |
| -8.00 | 4 | 99.37 | 6.82 | 3.41 | 6.9 |
| -9.00 | 4 | 99.29 | 10.59 | 5.30 | 10.7 |
| -10.00 | 4 | 97.56 | 7.92 | 3.96 | 8.1 |

a. Number of labs that conducted the assay at the cited reference chemical concentration. The run number varied because the concentrations selected by the laboratories for testing in the runs differed in some instances.
b. NC – Not Calculated. If the overall mean was calculated using an n ≤ 2, then the sd, SEM, and %CV were not calculated.

**Table 9.5-2. Recombinant assay: effect of dibenz(a,h)anthracene on aromatase activity (percent of control) by overall task**

| Reference Chemical Log Conc (M) | Number of Labs[a] | Overall Mean | Overall sd | Overall SEM | Overall %CV |
|---|---|---|---|---|---|
| -4.00 | 4 | 95.98 | 7.65 | 3.83 | 8.0 |
| -4.48 | 1 | 89.56 | NC[b] | NC | NC |
| -5.00 | 4 | 98.56 | 5.96 | 2.98 | 6.0 |
| -5.30 | 1 | 104.28 | NC | NC | NC |
| -5.48 | 1 | 94.50 | NC | NC | NC |
| -6.00 | 4 | 98.64 | 7.78 | 3.89 | 7.9 |
| -6.30 | 1 | 94.48 | NC | NC | NC |
| -6.60 | 1 | 102.86 | NC | NC | NC |
| -7.00 | 4 | 95.79 | 4.43 | 2.21 | 4.6 |
| -8.00 | 4 | 98.05 | 7.04 | 3.52 | 7.2 |
| -9.00 | 4 | 96.67 | 8.84 | 4.42 | 9.1 |
| -10.00 | 4 | 96.49 | 8.47 | 4.24 | 8.8 |

a. Number of labs that conducted the assay at the cited reference chemical concentration. The run number varied because the concentrations selected by the laboratories for testing in the runs differed in some instances.
b. NC – Not Calculated. If the overall mean was calculated using an n ≤ 2, then the sd, SEM, and %CV were not calculated.

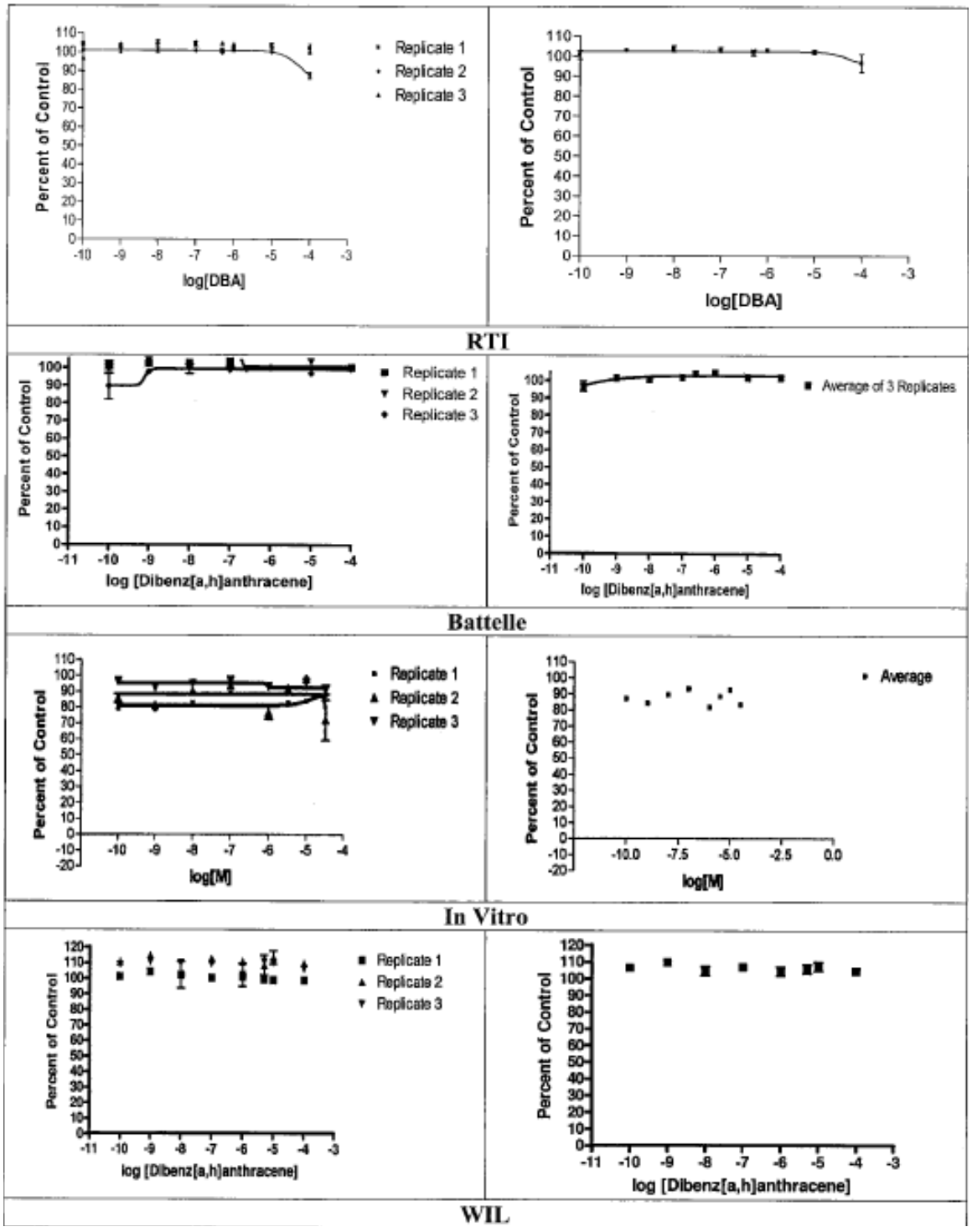



**Figure 9.5-1. Placental assay: individual run and average dibenz(a,h)anthracene inhibition response curves (percent of control) by laboratory**

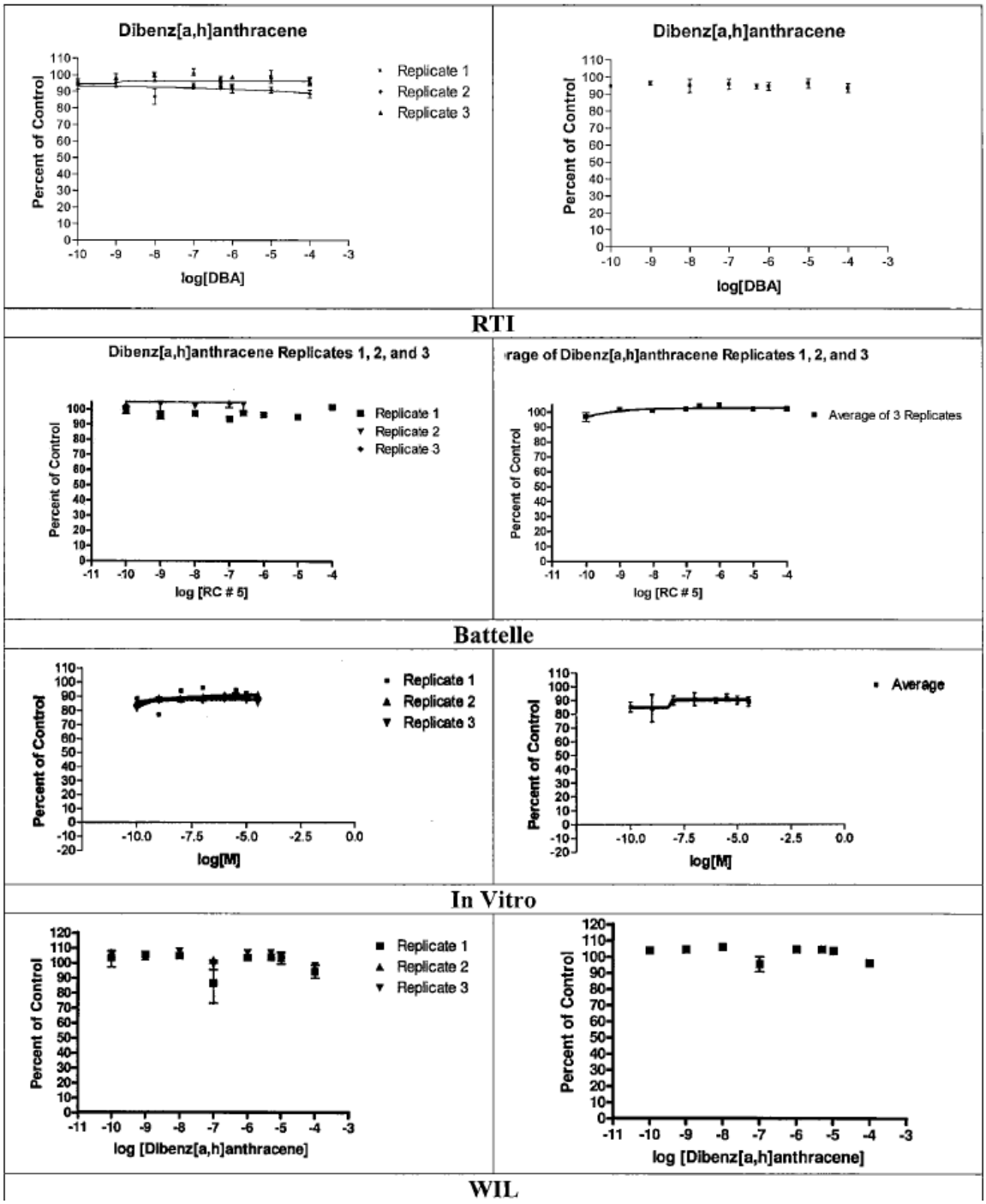



**Figure 9.5-2. Recombinant assay: individual run and average dibenz(a,h)anthracene inhibition response curves (percent of control) by laboratory**

**Dibenz(a,h)anthracene Placental $IC_{50}$.** This compound did not inhibit aromatase (Table 9.5-3). There were no valid $IC_{50}$ values reported by the laboratories. An examination of the percent of control curves indicated that there was no inhibition.

**Table 9.5-3. Placental $IC_{50}$ values by laboratory for dibenz(a,h)anthracene**

| Run | $IC_{50}$ Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Dibenz[a,h]anthracene (µM) | | | | |
| 1 | no inhibition | no inhibition | no inhibition[a] | no inhibition |
| 2 | no inhibition[a] | no inhibition[a] | no inhibition[a] | no inhibition |
| 3 | no inhibition | no inhibition[a] | no inhibition[a] | no inhibition |

a. Although a value was reported, the percent of control curve indicated that there was no inhibition.
Source: Reference 10

**Dibenz(a,h)anthracene Placental Slope:** This compound did not inhibit aromatase (Table 9.5-4). There were no valid $IC_{50}$ values reported by the laboratories. An examination of the percent of control curves indicated that there was no inhibition.

**Table 9.5-4. Placental slope values by laboratory for dibenz(a,h)anthracene**

| Run | Slope Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Dibenz[a,h]anthracene | | | | |
| 1 | no inhibition | no inhibition | no inhibition[a] | no inhibition |
| 2 | no inhibition[a] | no inhibition[a] | no inhibition[a] | no inhibition |
| 3 | no inhibition | no inhibition[a] | no inhibition[a] | no inhibition |

a. Although a value was reported, the percent of control curve indicated that there was no inhibition.

Source: Reference 10

**Dibenz(a,h)anthracene Recombinant $IC_{50}$:** The percent of control curves for each run and all laboratories indicated that no graded inhibition occurred, thereby precluding any determination of a valid $IC_{50}$ value (Table 9.5-5). Although there were $IC_{50}$ values determined for some of the laboratories, the Prism analysis for these runs indicated that the error associated with all of the parameter estimates was so large that the results were considered unreliable. Thus, dibenz[a,h]anthracene did not inhibit aromatase.

**Table 9.5-5. Recombinant $IC_{50}$ values by laboratory for dibenz(a,h)anthracene**

| Run | $IC_{50}$ Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Dibenz[a,h]anthracene (µM) | | | | |
| 1 | no inhibition | no inhibition | no inhibition | no inhibition |
| 2 | no inhibition[a] | no inhibition | no inhibition[a] | no inhibition |
| 3 | no inhibition | no inhibition[a] | no inhibition[a] | no inhibition |

a. Although a value was reported, the percent of control curve indicated that there was no inhibition.

Source: Reference 12

**Dibenz(a,h)anthracene Recombinant Slope:** The percent of control curves had no valid measurable slopes (Table 9.5-6). For the most part, the curves appeared as horizontal lines. There was no indication that any inhibition of aromatase by dibenz(a,h) anthracene occurred.

**Table 9.5-6. Recombinant slope values by laboratory for dibenz(a,h)anthracene**

| Run | Slope Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Dibenz[a,h]anthracene | | | | |
| 1 | no inhibition | no inhibition | No inhibition | no inhibition |
| 2 | no inhibition[a] | no inhibition | No inhibition[a] | no inhibition |
| 3 | no inhibition | no inhibition[a] | No inhibition[a] | no inhibition |

a. Although a value was reported, the percent of control curve indicated that there was no inhibition.

Source: Reference 12

## 9.6 Econazole

**Econazole Assay Overview.** The effect of increasing concentrations of econazole on aromatase activity is reported by laboratory in Tables B.9-11 and B.9-12 (Appendix B) and by overall task in Tables 9.6-1 and 9.6-2. In addition, the results are graphically presented by laboratory (individual runs and average) in Figures 9.6-1 and 9.6-2.

For all laboratories, increasing concentrations of econazole decreased the activity of the microsomal aromatase in a concentration-dependent sigmoidal curve (Figures 9.6-1 and 9.6-2). In the placental assay, after the first run was completed, the study directors from all four laboratories modified the econazole concentrations selected for testing in runs 2 and 3 (Table B.9-11). In general, the modifications involved including additional mid-concentrations between $10^{-8}$ and $10^{-10}$ M, which replaced the concentrations ranging from $10^{-3}$ and $10^{-4}$ M that were tested in run 1.

However, the top portions of the curves (lowest inhibitor concentrations) were not clearly defined to the extent of a well characterized plateau. Lower concentrations would have provided better characterization. Even so, the curve was sufficiently defined to evaluate the effect of econazole on aromatase activity.

In the placental assay, approximately 1 to 2 percent of the enzyme activity remained at econazole concentrations of $10^{-7}$ M. In contrast, at an econazole concentration of approximately $10^{-10}$ M, there was little to no aromatase inhibition; the laboratory percent of control values were approximately 90 percent or higher for three of four labs and approximately 80 percent for Battelle. The overall task mean ± SEM percent of control values at $10^{-7}$ and $10^{-10}$ M were 1.52 ± 0.07 and 89.94 ± 3.34 percent, respectively (Table 9.6-1).

In the recombinant assay, after the first run was completed, the study directors from three of four laboratories modified the econazole concentrations selected for testing in runs 2 and 3 (Table B.9-12). In general, the modifications involved including additional mid-concentrations between $10^{-8}$ and $10^{-10}$ M, which replaced the concentrations ranging from $10^{-3}$ and 10-5 M that were

tested in run 1. RTI used the same concentrations for all three runs and tested between the range of $10^{-6}$ M and $10^{-10}$ M, presumably because these were the concentrations found to be optimal. (RTI replaced the first run because it didn't show concentration dependence.)

In the recombinant assay, less than or equal to 2 percent of the enzyme activity remained at econazole concentrations of $10^{-7}$ M. In contrast, at an econazole concentration of approximately $10^{-10}$ M, there was little to no aromatase inhibition; the laboratory percent of control values were approximately 90 percent or higher for all four labs. The overall task mean ± SEM percent of control values at $10^{-7}$ and $10^{-10}$ M were 1.76 ± 0.17 and 94.21 ± 2.33 percent, respectively (Table 9.6-2).

**Table 9.6-1. Placental assay: effect of econazole on aromatase activity (percent of control) by overall task**

| Reference Chemical Log Conc (M) | Number of Labs[a] | Overall Mean | Overall sd | Overall SEM | Overall %CV |
|---|---|---|---|---|---|
| -3.00 | 3 | 0.01 | 0.31 | 0.18 | 4701.3 |
| -4.00 | 4 | -0.11 | 0.20 | 0.10 | 180.9 |
| -4.48 | 1 | -0.35 | NC[b] | NC | NC |
| -5.00 | 4 | -0.02 | 0.27 | 0.14 | 1566.6 |
| -6.00 | 4 | -0.03 | 0.21 | 0.10 | 751.3 |
| -7.00 | 4 | 1.52 | 0.14 | 0.07 | 9.0 |
| -8.00 | 4 | 12.86 | 1.78 | 0.89 | 13.9 |
| -8.30 | 1 | 22.32 | NC | NC | NC |
| -8.48 | 1 | 29.15 | NC | NC | NC |
| -8.52 | 1 | 35.32 | NC | NC | NC |
| -8.60 | 1 | 44.40 | NC | NC | NC |
| -9.00 | 4 | 58.22 | 5.69 | 2.84 | 9.8 |
| -9.30 | 1 | 76.05 | NC | NC | NC |
| -9.48 | 1 | 72.39 | NC | NC | NC |
| -9.52 | 1 | 89.83 | NC | NC | NC |
| -9.60 | 1 | 86.43 | NC | NC | NC |
| -10.00 | 4 | 89.94 | 6.67 | 3.34 | 7.4 |

a. Number of labs that conducted the assay at the cited reference chemical concentration. The run number varied because each laboratory used the results from their first run to select concentrations to be used in runs 2 and 3 that were believed would better characterize the percent inhibition curve.

b. NC – Not Calculated. If the overall mean was calculated using an n ≤ 2, then the sd, SEM, and %CV were not calculated.

**Table 9.6-2. Recombinant assay: effect of econazole on aromatase activity (percent of control) by overall task**

| Reference Chemical Log Conc (M) | Number of Labs[a] | Overall Mean | Overall sd | Overall SEM | Overall %CV |
|---|---|---|---|---|---|
| -3.00 | 2 | 0.08 | NC[b] | NC | NC |
| -4.00 | 3 | 0.02 | 0.04 | 0.03 | 217.9 |
| -4.48 | 1 | -0.06 | NC | NC | NC |
| -5.00 | 3 | 0.08 | 0.11 | 0.06 | 143.1 |
| -6.00 | 4 | 0.23 | 0.03 | 0.01 | 12.8 |
| -7.00 | 4 | 1.76 | 0.34 | 0.17 | 19.3 |
| -8.00 | 4 | 15.53 | 1.96 | 1.96 | 12.6 |
| -8.30 | 1 | 31.41 | NC | NC | NC |
| -8.48 | 1 | 32.94 | NC | NC | NC |
| -8.52 | 1 | 43.76 | NC | NC | NC |
| -8.60 | 1 | 49.15 | NC | NC | NC |
| -9.00 | 4 | 68.82 | 7.32 | 3.66 | 10.6 |
| -9.30 | 1 | 87.85 | NC | NC | NC |
| -9.48 | 1 | 84.05 | NC | NC | NC |
| -9.52 | 1 | 94.03 | NC | NC | NC |
| -9.60 | 1 | 93.78 | NC | NC | NC |
| -10.00 | 4 | 94.21 | 4.65 | 2.33 | 4.9 |

a. Number of labs that conducted the assay at the cited reference chemical concentration. The run number varied because three out of the four laboratories used the results from their first run to select concentrations to be used in runs 2 and 3 that were believed would better characterize the percent inhibition curve. RTI used the same concentrations for all three runs.

b. NC – Not Calculated. If the overall mean was calculated using an $n \leq 2$, then the sd, SEM, and %CV were not calculated.

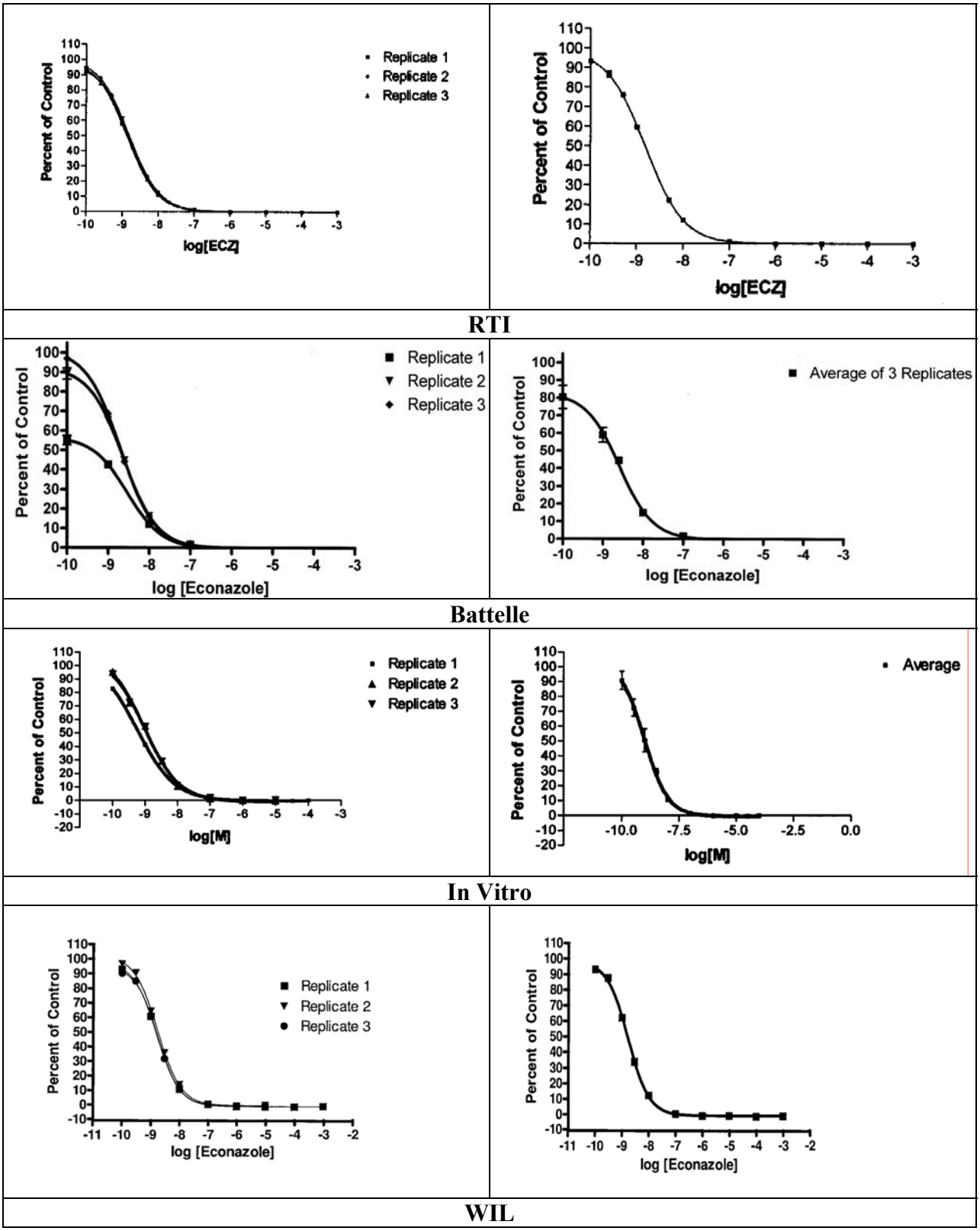



**Figure 9.6-1. Placental assay: individual run and average econazole inhibition response curves (percent of control) by laboratory**

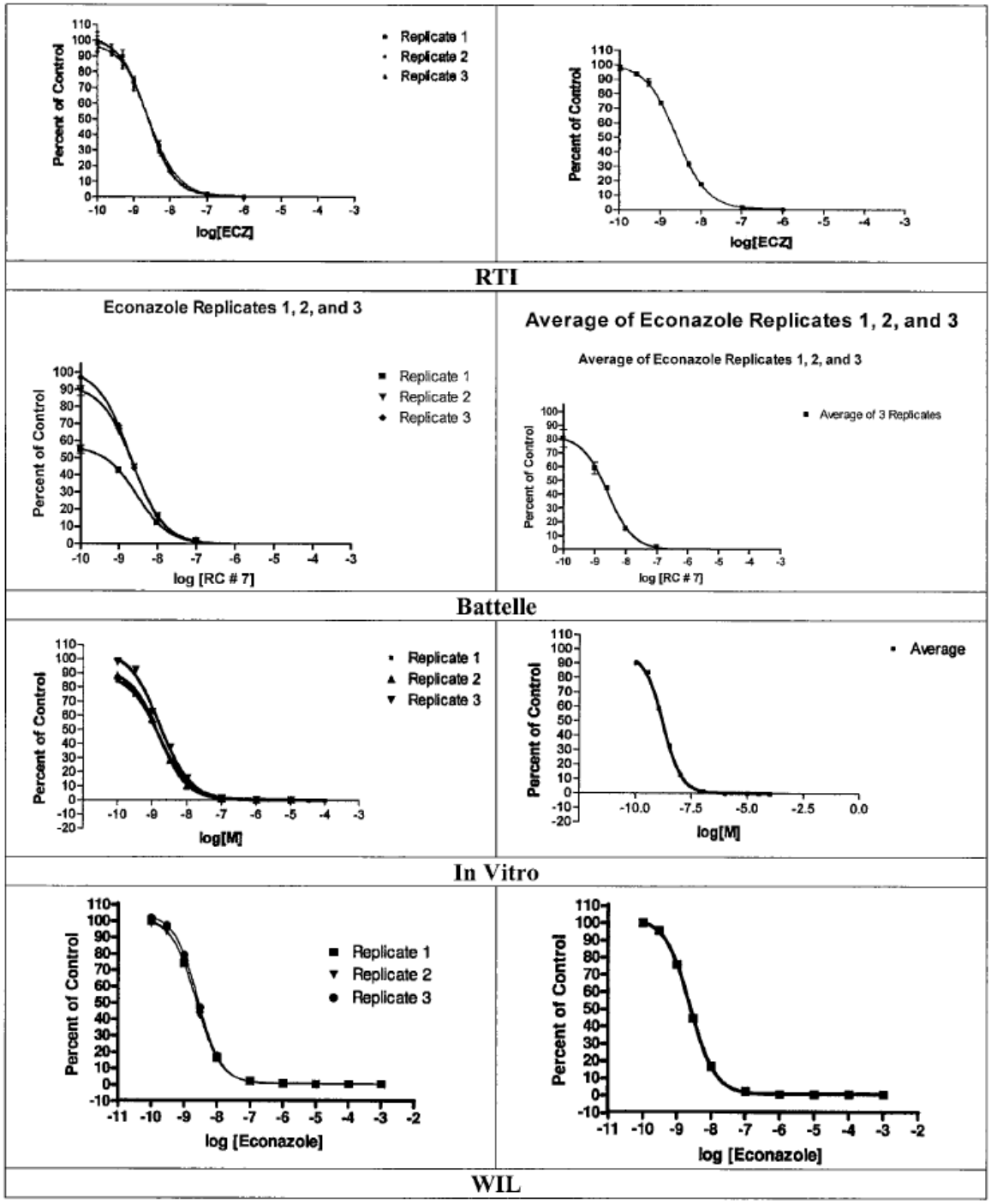



**Figure 9.6-2. Recombinant assay: individual run and average econazole inhibition response curves (percent of control) by laboratory**

**Econazole Placental $IC_{50}$:** The average ± SEM $IC_{50}$ values for RTI, Battelle, In Vitro, and WIL were 1.50 ± 0.06, 2.43 ± 0.27,0.818 ± 0.127, and 1.69 ± 0.04 nM, respectively (Table 9.6-3). The percent CV values were 6.8, 19.5, 27.0, and 4.2 percent, respectively. The higher %CV values for Battelle and In Vitro were attributed to the $IC_{50}$ value obtained from run 1. In general, modifications to the concentrations tested after run 1 seemed to result in $IC_{50}$ values that were in closer agreement for runs 2 and 3. Although, the $IC_{50}$ values determined were in reasonably good agreement regardless of the modifications. The overall task group mean ± SEM $IC_{50}$ value was 1.61 ± 0.33 nM and the percent CV was 41.3 percent.

**Table 9.6-3. Placental $IC_{50}$ values by laboratory for econazole**

| Run | $IC_{50}$ Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Econazole (µM) | | | | |
| 1 | 0.00138 | 0.00294 | 0.000566 | 0.00161 |
| 2 | 0.00154 | 0.00235 | 0.000914 | 0.00172 |
| 3 | 0.00157 | 0.00201 | 0.000975 | 0.00173 |
| Mean | 0.00150 | 0.00243 | 0.000818 | 0.00169 |
| sd | 0.00010 | 0.00047 | 0.000221 | 0.00007 |
| SEM | 0.00006 | 0.00027 | 0.000127 | 0.00004 |
| %CV | 6.8 | 19.5 | 27.0 | 4.2 |

Source: Reference 10

**Econazole Placental Slope:** The average ± SEM slope values for RTI, Battelle, In Vitro, and WIL were -1.0503 ± 0.0125, -1.0370 ± 0.0067, -0.7915 ± 0.0229, and -1.0996 ± 0.0185, respectively (Table 9.6-4). The percent CV values were 2.1, 1.1, 5.0, and 2.9 percent, respectively. The runs for a given lab and between labs were in close agreement. Modifications to concentrations or lack thereof did not seem to have any overt effect on slope values. The overall task group mean ± SEM slope value was -0.9946 ± 0.0690 and the percent CV was 13.9 percent.

**Table 9.6-4. Placental slope values by laboratory for econazole**

| Run | Slope Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Econazole | | | | |
| 1 | -1.048 | -1.025 | -0.7479 | -1.0946 |
| 2 | -1.030 | -1.048 | -0.8011 | -1.0703 |
| 3 | -1.073 | -1.038 | -0.8255 | -1.1339 |
| Mean | -1.0503 | -1.0370 | -0.7915 | -1.0996 |
| sd | 0.0216 | 0.0115 | 0.0397 | 0.0321 |
| SEM | 0.0125 | 0.0067 | 0.0229 | 0.0185 |
| %CV | 2.1 | 1.1 | 5.0 | 2.9 |

Source: Reference 10

**Econazole Recombinant $IC_{50}$:** The average ± SEM $IC_{50}$ values for RTI, Battelle, In Vitro, and WIL were 2.47 ± 0.05, 2.39 ± 0.34, 1.63 ± 0.03, and 2.38 ± 0.09 nM, respectively (Table 9.6-5). The percent CV values were 3.6, 24.7, 3.6, and 6.4 percent, respectively. Only Battelle's $IC_{50}$ estimate had a relative low measure of precision (%CV). However, a review of Battelle's percent of control curves indicated that there wasn't a run that was disparate from the others; rather, the three runs simply varied to the extent determined. The high precision measured by RTI may be attributed to using information from the previous work assignment in determining the concentrations tested (see percent of control results). However, In Vitro and WIL modified their concentrations after run 1 and still achieved a measure of precision that approximated 4 to 6 percent, thereby indicating that a high degree of precision was still attainable by following the study design. The overall task group mean ± SEM $IC_{50}$ value was 2.22 ± 0.20 nM and the percent CV was 17.9 percent.

**Table 9.6-5. Recombinant $IC_{50}$ values by laboratory for econazole**

| Run | $IC_{50}$ Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| | Econazole (µM) | | | |
| 1 | 0.002432 | 0.001901 | 0.00165 | 0.002268 |
| 2 | 0.002576 | 0.003049 | 0.00156 | 0.002314 |
| 3 | 0.002415 | 0.002226 | 0.00167 | 0.002551 |
| Mean | 0.00247 | 0.00239 | 0.00163 | 0.00238 |
| Sd | 0.00009 | 0.00059 | 0.00006 | 0.00015 |
| SEM | 0.00005 | 0.00034 | 0.00003 | 0.00009 |
| %CV | 3.6 | 24.7 | 3.6 | 6.4 |

Source: Reference 12

**Econazole Recombinant Slope:** The average ± SEM slope values for RTI, Battelle, In Vitro, and WIL were -1.1450 ± 0.0523, -1.1517 ± 0.0751, -1.0181 ± 0.0310, and -1.1846 ± 0.0198, respectively (Table 9.6-6). The percent CV values were 7.9, 11.3, 5.3, and 2.9 percent, respectively. The runs for a given lab and between labs were in close agreement. Modifications to concentrations or lack thereof did not seem to have any affect on being able to characterize the curve or estimate the slope values. The overall task group mean ± SEM slope value was -1.1248 ± 0.0366 and the percent CV was 6.5 percent.

**Table 9.6-6. Recombinant slope values by laboratory for econazole**

| Run | Slope Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| | Econazole | | | |
| 1 | -1.216 | -1.046 | -0.9904 | -1.1489 |
| 2 | -1.176 | -1.297 | -1.080 | -1.1875 |
| 3 | -1.043 | -1.112 | -0.9838 | -1.2174 |
| Mean | -1.1450 | -1.1517 | -1.0181 | -1.1846 |
| sd | 0.0906 | 0.1301 | 0.0537 | 0.0343 |
| SEM | 0.0523 | 0.0751 | 0.0310 | 0.0198 |
| %CV | 7.9 | 11.3 | 5.3 | 2.9 |

Source: Reference 12

## 9.7 Fenarimol

**Fenarimol Assay Overview.** The effect of increasing concentrations of fenarimol on aromatase activity is reported by laboratory in Tables B.9-13 and B.9-14 (Appendix B) and by overall task in Tables 9.7-1 and 9.7-2. In addition, the results are graphically presented by laboratory (individual runs and average) in Figures 9.7-1 and 9.7-2.

After the first run was completed, the study directors from three of four laboratories modified the fenarimol concentrations selected for testing in runs 2 and 3 (Tables B.9-13 and B.9-14). In general, the modifications for these three laboratories involved including additional mid-concentrations between $10^{-4}$ and $10^{-6}$ M, which replaced one to two of the concentrations from $10^{-8}$ to $10^{-10}$ M that were tested in run 1. In Vitro tested the same concentrations for their three runs, starting at a concentration of $3.3 \times 10^{-5}$ M. This starting concentration was selected by In Vitro because they reported seeing visible precipitate at concentrations of $10^{-3}$ and $10^{-4}$ M. In the recombinant assay, In Vitro used the same concentrations as was used for the placental assay, which explains why no adjustments were made after the first run.

For all four laboratories, increasing concentrations of fenarimol decreased the activity of the placental and recombinant microsomal aromatase and the decrease was concentration-dependent (Tables 9.7-1 and 9.7-2). The shapes of the enzyme activity vs. fenarimol curves were sigmoidal (Figures 9.7-1 and 9.7-2).

In the placental assay, at a fenarimol concentration of $10^{-3}$ M, aromatase inhibition was almost complete; the laboratory percent of control values ranged from 1 to -6 percent for three of the labs and, for In Vitro at its highest concentration of $3.3 \times 10^{-5}$ M, the percent of control value was 15 percent. In contrast, at a fenarimol concentration of approximately $10^{-7}$ M, there was little to no aromatase inhibition; the laboratory percent of control values were greater than 90 percent for all labs, except WIL with a value of 86 percent. The overall task mean ± SEM percent of control values at $10^{-3}$ and $10^{-7}$ M were -1.11 ± 2.40 and 91.88 ± 2.27 percent, respectively (Table 9.7-2).

In the recombinant assay, at a fenarimol concentration of $10^{-3}$ M, aromatase inhibition was almost complete; the laboratory percent of control values for RTI and Battelle were approximately 1 percent. For WIL, two of three runs had percent of control values of approximately 1percent, but the low enzyme activity measured for the second run resulted in a higher percent of control value at $10^{-3}$ M, i.e., 23 percent, which resulted in an average percent of control at this concentration of 8 percent. For In Vitro at its highest concentration tested ($3.3 \times 10^{-5}$ M), the percent of control value was 20 percent. In contrast, at a fenarimol concentration of approximately $10^{-7}$ M, there was little to no aromatase inhibition; the laboratory percent of control values were greater than 90 percent for all labs. The overall task mean ± SEM percent of control values at $10^{-3}$ and $10^{-7}$ M were 3.52 ± 2.32 and 95.67± 1.90 percent, respectively (Table 10.7-2).

**Table 9.7-1. Placental assay: effect of fenarimol on aromatase activity (percent of control) by overall task**

| Reference Chemical Log Conc (M) | Number of Labs[a] | Overall Mean | Overall sd | Overall SEM | Overall %CV |
|---|---|---|---|---|---|
| -3.00 | 3 | -1.11 | 4.16 | 2.40 | 374.5 |
| -4.00 | 3 | 3.66 | 4.13 | 2.39 | 112.8 |
| -4.48 | 1 | 14.85 | NC[b] | NC | NC |
| -4.52 | 1 | 9.44 | NC | NC | NC |
| -4.60 | 1 | 20.26 | NC | NC | NC |
| -5.00 | 4 | 34.31 | 2.85 | 1.43 | 8.3 |
| -5.30 | 1 | 52.63 | NC | NC | NC |
| -5.48 | 1 | 55.23 | NC | NC | NC |
| -5.52 | 1 | 57.22 | NC | NC | NC |
| -5.60 | 1 | 69.43 | NC | NC | NC |
| -6.00 | 4 | 80.73 | 2.55 | 1.27 | 3.2 |
| -7.00 | 4 | 91.88 | 4.53 | 2.27 | 4.9 |
| -8.00 | 4 | 98.03 | 5.34 | 2.67 | 5.4 |
| -9.00 | 4 | 92.88 | 6.78 | 3.39 | 7.3 |
| -10.00 | 4 | 93.40 | 8.06 | 4.03 | 8.6 |

a. Number of labs that conducted the assay at the cited reference chemical concentration. The run number varied because each laboratory used the results from their first run to select concentrations to be used in runs 2 and 3 that were believed would further (In Vitro) or better characterize the percent inhibition curve.

b. NC – Not Calculated. If the overall mean was calculated using an n ≤ 2, then the sd, SEM, and %CV were not calculated.

**Table 9.7-2. Recombinant assay: effect of fenarimol on aromatase activity (percent of control) by overall task**

| Reference Chemical Log Conc (M) | Number of Labs[a] | Overall Mean | Overall sd | Overall SEM | Overall %CV |
|---|---|---|---|---|---|
| -3.00 | 3 | 3.52 | 4.03 | 2.32 | 114.4 |
| -4.00 | 3 | 13.12 | 12.37 | 7.14 | 94.3 |
| -4.48 | 1 | 20.45 | NC[b] | NC | NC |
| -4.52 | 1 | 55.70 | NC | NC | NC |
| -4.60 | 1 | 19.64 | NC | NC | NC |
| -5.00 | 4 | 40.75 | 12.39 | 6.20 | 30.4 |
| -5.30 | 1 | 49.42 | NC | NC | NC |
| -5.48 | 1 | 53.86 | NC | NC | NC |
| -5.52 | 1 | 81.53 | NC | NC | NC |
| -5.60 | 1 | 68.00 | NC | NC | NC |
| -6.00 | 4 | 82.86 | 4.90 | 2.45 | 5.9 |
| -7.00 | 4 | 95.67 | 3.80 | 1.90 | 4.0 |
| -8.00 | 4 | 97.15 | 6.16 | 3.08 | 6.3 |
| -9.00 | 4 | 95.16 | 2.72 | 1.36 | 2.9 |
| -10.00 | 4 | 95.59 | 4.36 | 2.18 | 4.6 |

a. Number of labs that conducted the assay at the cited reference chemical concentration. The run number varied because each laboratory used the results from their first run to select concentrations to be used in runs 2 and 3 that were believed would further (In Vitro) or better characterize the percent inhibition curve.

b. NC – Not Calculated. If the overall mean was calculated using an n ≤ 2, then the sd, SEM, and %CV were not calculated.

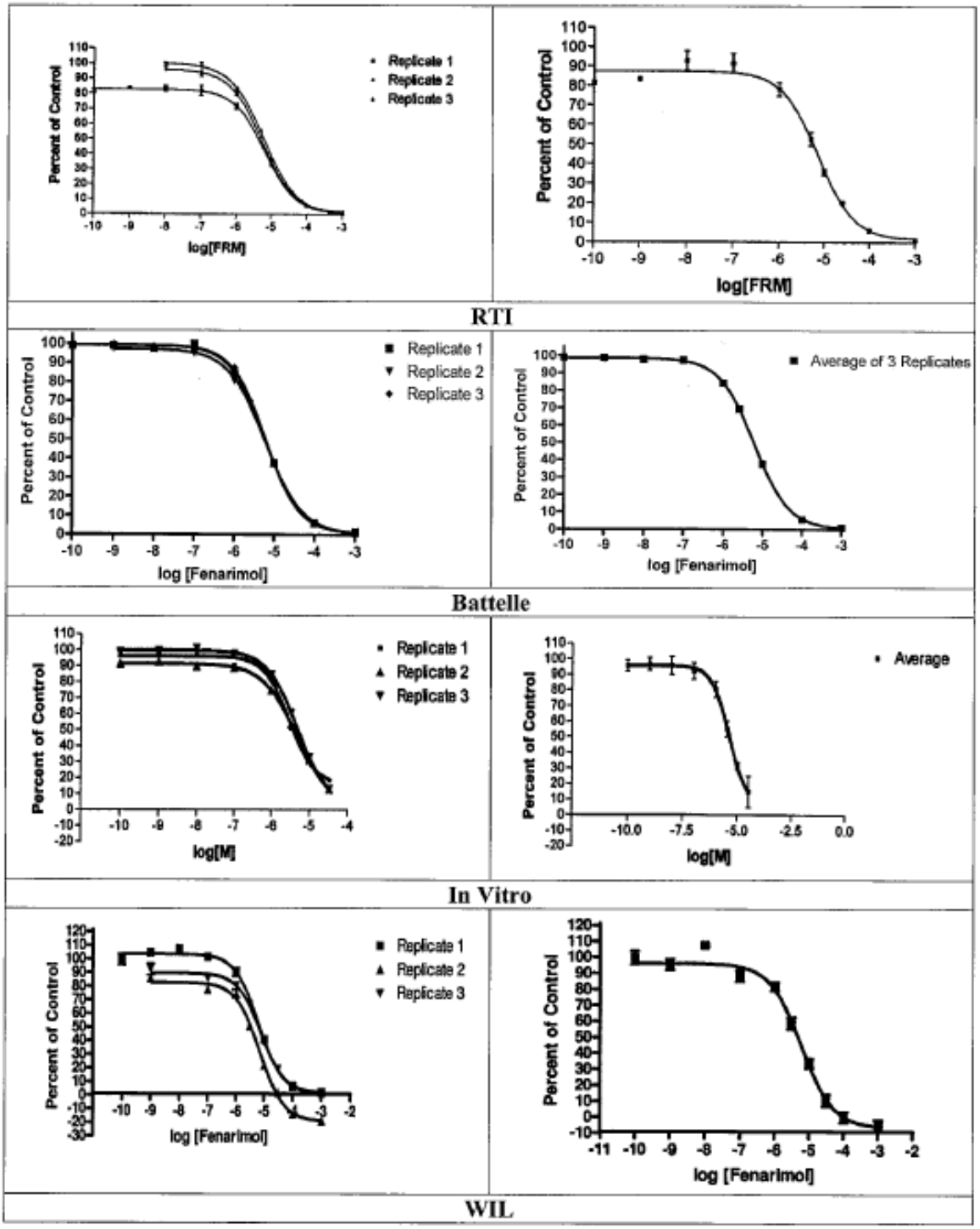



**Figure 9.7-1. Placental assay: individual run and average fenarimol inhibition response curves (percent of control) by laboratory**

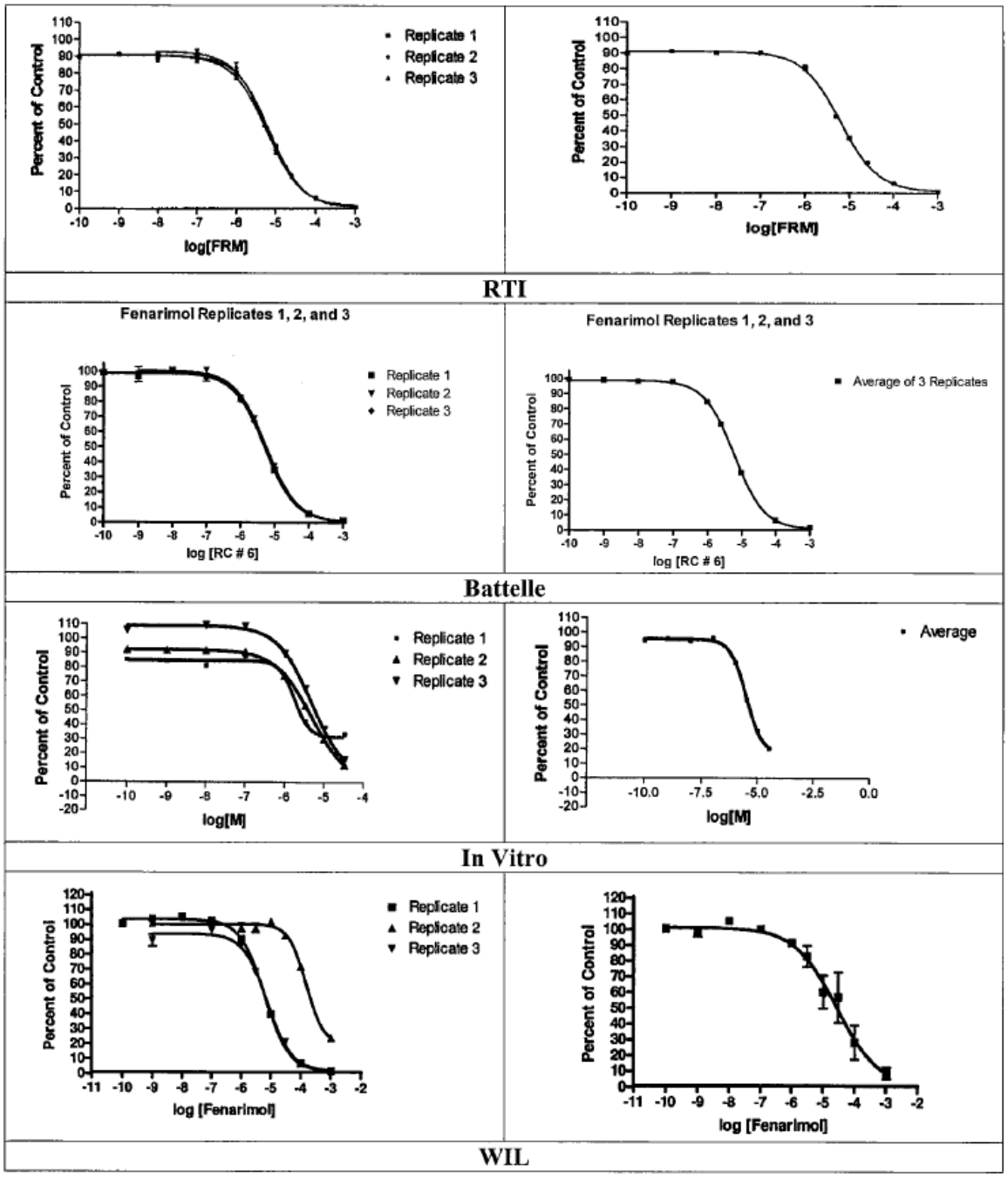



**Figure 9.7-2. Recombinant assay: individual run and average fenarimol inhibition response curves (percent of control) by laboratory**

**Fenarimol Placental $IC_{50}$:** The average ± SEM $IC_{50}$ values for RTI, Battelle, In Vitro, and WIL were 6.01 ± 0.31, 6.05 ± 0.04, 4.25 ± 0.65, and 7.05± 0.40 µM, respectively (Table 9.7-3). The percent CV values were 9.0, 1.2, 26.5, and 9.7 percent, respectively. The higher %CV value for In Vitro was attributed to the $IC_{50}$ value obtained from run 1. However, no modifications to the Draft Report 62 June 2006 concentrations tested were made by In Vitro after run 1. The results from RTI, Battelle, and WIL indicate that the $IC_{50}$ could be precisely estimated using the run 1 starting concentrations. However, three of four labs showed that modifications to the run 1 concentrations tested did improve the characterization of the steep portion of the inhibition cure and $IC_{50}$ estimate. The overall task group mean ± SEM $IC_{50}$ value was 5.84 ± 0.58 µM and the percent CV was 20.0 percent.

**Table 9.7-3. Placental $IC_{50}$ values by laboratory for fenarimol**

| Run | $IC_{50}$ Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Fenarimol (µM) | | | | |
| 1 | 6.49 | 5.97 | 2.95 | 6.35 |
| 2 | 5.42 | 6.06 | 4.93 | 7.10 |
| 3 | 6.12 | 6.11 | 4.86 | 7.72 |
| Mean | 6.01 | 6.05 | 4.25 | 7.05 |
| Sd | 0.54 | 0.07 | 1.12 | 0.69 |
| SEM | 0.31 | 0.04 | 0.65 | 0.40 |
| %CV | 9.0 | 1.2 | 26.5 | 9.7 |

Source: Reference 10

**Fenarimol Placental Slope:** The average ± SEM slope values for RTI, Battelle, In Vitro, and WIL were -0.9481 ± 0.0242, -0.9934 ± 0.0303, -1.0993 ± 0.1043, and -1.0314 ± 0.0076, respectively (Table 9.7-4). The percent CV values were 4.4, 5.3, 16.4, and 1.3 percent, respectively. The runs for a given lab and between labs were in close agreement. Modifications to concentrations or lack thereof did not seem to have any overt effect on slope values. The overall task group mean ± SEM slope value was -1.0180 ± 0.0320 and the percent CV was 6.3 percent.

**Table 9.7-4. Placental slope values by laboratory for fenarimol**

| Run | Slope Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Fenarimol | | | | |
| 1 | -0.9924 | -1.005 | -1.292 | -1.0253 |
| 2 | -0.9430 | -0.9361 | -0.9339 | -1.0224 |
| 3 | -0.9089 | -1.039 | -1.072 | -1.0465 |
| Mean | -0.9481 | -0.9934 | -1.0993 | -1.0314 |
| sd | 0.0420 | 0.0524 | 0.1806 | 0.0132 |
| SEM | 0.0242 | 0.0303 | 0.1043 | 0.0076 |
| %CV | 4.4 | 5.3 | 16.4 | 1.3 |

Source: Reference 10

**Fenarimol Recombinant $IC_{50}$:** The average ± SEM IC50 values for RTI, Battelle, In Vitro, and WIL were 6.38 ± 0.33, 5.42 ± 0.13, 3.79 ± 1.0l, and 6.95 ± 0.77 µM, respectively (Table 9.7-5). The percent CV values were 9.l, 4.3, 46.1, and l5.7 percent, respectively. The average reported for WIL was based on results only from runs 1 and 3 because WIL's percent of control curve for run 2 was determined using an aberrant full enzyme activity control value, which resulted in an $IC_{50}$ value of l50.5 (average ± SEM, %CV, n = 54.80 ± 47.85 µM, l51.3 %,3). The higher %CV value for In Vitro was attributed to the $IC_{50}$ value obtained from run 1, which did not have percent of control values that approached 0 or 100 percent at the higher and lower concentrations. Although In Vitro did not modify their concentrations after run l, the percent of control values obtained for runs 2 and 3 resulted in responses that were much closer to 0 and 100 percent, thereby resulting in better curves and parameter estimates. The results from RTI and Battelle indicated that the $IC_{50}$ could be precisely estimated using the run 1 starting concentrations. However, these two labs also showed that modifications to the run 1 concentrations tested did improve the characterization of the percent of control curve and $IC_{50}$ estimate. The overall task group mean ± SEM $IC_{50}$ value was 5.63 ± 0.69 µM and the percent CV was 24.6 percent.

**Table 9.7-5. Recombinant $IC_{50}$ values by laboratory for fenarimol**

| Run | $IC_{50}$ Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Fenarimol (µM) | | | | |
| 1 | 7.047 | 5.165 | 1.77 | 6.178 |
| 2 | 6.039 | 5.626 | 4.83 | 150.5 |
| 3 | 6.053 | 5.454 | 4.76 | 7.718 |
| Mean | 6.38 | 5.42 | 3.79 | 6.95 |
| Sd | 0.58 | 0.23 | 1.75 | 1.09 |
| SEM | 0.33 | 0.13 | 1.01 | 0.77 |
| %CV | 9.1 | 4.3 | 46.1 | 15.7 |

Source: Reference 12

**Fenarimol Recombinant Slope:** The average ± SEM slope values for RTI, Battelle, In Vitro, and WIL were -1.0283 ± 0.0169, -0.9463 ± 0.0158, -l.4016 ± 0.4341, and -1.2054 ±.0.1409, respectively (Table 9.7-6). The percent CV values were 2.8, 2.9, 53.6, and 20.3 percent, respectively. The low precision for In Vitro was attributed to the curve for run 1, which did not have a 0 and 100 percent of control responses at the high and low concentrations like were achieved for runs 2 and 3 (same concentrations were tested for all three runs). Although the precision for WIL was relatively high, the relatively slightly lower precision was attributed to the results from run 2, which had a low full enzyme activity control value and shifted the curve to the right. Since the shape of the curve was very similar to the other two runs, the aberrant full enzyme activity value had a greater affect on the IC50 value than on the slope. For RTI and Battelle, modifications to concentrations after run 1 did not seem to result in any appreciable improvement on estimating the slope values, thereby indicating that the initial concentrations tested produced a reliable estimate of the parameter. The overall task group mean ± SEM slope value was -1.1454 ± 0.1011 and the percent CV was 17.6 percent. Draft l 4.816 and 136.7 percent. The relatively high degree of precision achieved by RTI was attributed to their use of the same concentrations for all three runs. Regardless, it appears that the slope of the curve was precisely determined over the concentration range tested. The overall task group mean ± SEM

slope value was -1.240 ± 0.038 and the percent CV was 6.2 percent. (Note: overall values do not include the aberrant value for In Vitro.)

**Table 9.7-6. Recombinant slope values by laboratory for fenarimol**

| Run | Slope Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Fenarimol | | | | |
| 1 | -1.041 | -0.9738 | -2.269[a] | -1.0523 |
| 2 | -1.049 | -0.9460 | -0.9373 | -1.4869 |
| 3 | -0.9949 | -0.9191 | -0.9985 | -1.0769 |
| Mean | -1.0283 | -0.9463 | -1.4016 | -1.2054 |
| sd | 0.0292 | 0.0274 | 0.7518 | 0.2441 |
| SEM | 0.0169 | 0.0158 | 0.4341 | 0.1409 |
| %CV | 2.8 | 2.9 | 53.6 | 20.3 |

a. Considered an aberrant value. See source text for results without the value included.

Source: Reference 12.

## 9.8 Ketoconazole

**Ketoconazole Assay Overview.** The effect of increasing concentrations of ketoconazole on aromatase activity is reported by laboratory in Tables B.9.-15 and B.9-16 (Appendix B) and by overall task in Tables 9.8-1 and 9.8-2. In addition, the results are graphically presented by laboratory (individual runs and average) in Figures 9.8-1 and 9.8-2.

After the first run was completed, the study directors from all laboratories modified the ketoconazole concentrations selected for testing in runs 2 and 3 (Tables B.9-15 and B.9-16). In general, in the placental assay, the modifications involved including additional mid-concentrations between $10^{-4}$ and $10^{-7}$ M, which replaced the concentrations from $10^{-8}$ to $10^{-11}$ M (or $10^{-12}$ M for Battelle) that were tested in run 1. Battelle tested concentrations ranging from $10^{-5}$ M to $10^{-12}$ M rather than $10^{-4}$ M to $10^{-11}$ M due to a miscalculation. The error was identified and corrected prior to proceeding with runs 2 and 3. The laboratories did not test over the planned concentration range, i.e., $10^{-4}$ to $10^{-10}$ M, due to a miscommunication with the CR regarding the correct RC blind number and its corresponding concentration.

In general, in the recombinant assay, the modifications involved including additional mid-concentrations between $10^{-4}$ and $10^{-7}$ M, which replaced the concentrations tested in run 1 from $10^{-8}$ to $10^{-12}$ M, except for In Vitro that replaced 3 x $10^{-5}$ M. Battelle tested concentrations ranging from $10^{-5}$ M to $10^{-12}$ M rather than $10^{-4}$ M to $10^{-11}$ M due to a miscalculation. The error was identified and corrected prior to proceeding with runs 2 and 3. The laboratories did not test over the planned concentration range, i.e., $10^{-4}$ to $10^{-10}$ M, due to a miscommunication with the CR regarding the correct RC blind number and its corresponding concentration.

For all four laboratories, increasing concentrations of ketoconazole decreased the activity of the microsomal aromatase activity and the decrease was concentration-dependent (Tables 9.8-1 and 9.8-2). The shapes of the enzyme activity vs. ketoconazole curves were sigmoidal (Figures 9.8-1 and 9.8-2). At a ketoconazole concentration of $10^{-4}$ M, aromatase inhibition was complete.

In the placental assay, the laboratory percent of control values ranged from 5 to 6 percent for three of the labs and was 29 percent of control for In Vitro. In contrast, at a ketoconazole concentration of approximately $10^{-7}$ M, there was little to no aromatase inhibition; the laboratory percent of control values were greater than 95 percent for all labs. The overall task mean ± SEM percent of control values at $10^{-4}$ and $10^{-7}$ M were 11.17 ± 5.99 and 100.51 ± 2.35 percent, respectively (Table 9.8-1).

In the recombinant assay, the laboratory percent of control values ranged from 4 to 8 percent. In contrast, at a ketoconazole concentration of approximately $10^{-7}$ M, there was little to no aromatase inhibition; the laboratory percent of control values were greater than 92 percent for all labs. The overall task mean ± SEM percent of control values at $10^{-4}$ and $10^{-7}$ M were 6.01 ± 1.0l and 98.30 ± 2.l0 percent, respectively (Table 9.8-2).

**Table 9.8-1. Placental assay: effect of ketoconazole on aromatase activity (percent of control) by overall task**

| Reference Chemical Log Conc (M) | Number of Labs[a] | Overall Mean | Overall sd | Overall SEM | Overall %CV |
|---|---|---|---|---|---|
| -4.00 | 4 | 11.17 | 11.98 | 5.99 | 107.3 |
| -4.30 | 2 | 11.84 | NC[b] | NC | NC |
| -4.52 | 1 | 33.01 | NC | NC | NC |
| -4.60 | 2 | 20.41 | NC | NC | NC |
| -5.00 | 4 | 39.29 | 0.99 | 0.50 | 2.5 |
| -5.30 | 3 | 56.39 | 1.88 | 1.09 | 3.3 |
| -5.48 | 1 | 63.05 | NC | NC | NC |
| -5.60 | 1 | 70.19 | NC | NC | NC |
| -6.00 | 4 | 85.66 | 2.38 | 1.19 | 2.8 |
| -6.60 | 1 | 91.15 | NC | NC | NC |
| -7.00 | 4 | 100.51 | 4.70 | 2.35 | 4.7 |
| -8.00 | 4 | 101.08 | 3.37 | 1.69 | 3.3 |
| -9.00 | 4 | 100.31 | 4.17 | 2.09 | 4.2 |
| -10.00 | 4 | 100.62 | 4.68 | 2.34 | 4.6 |
| -11.00 | 3 | 99.99 | 5.12 | 2.95 | 5.1 |
| -12.00 | 1 | 98.71 | NC | NC | NC |

a. Number of labs that conducted the assay at the cited reference chemical concentration. The run number varied because each laboratory used the results from their first run to select concentrations to be used in runs 2 and 3 that were believed would better characterize the percent inhibition curve.
b. NC – Not Calculated. If the overall mean was calculated using an n ≤ 2, then the sd, SEM, and %CV were not calculated.

**Table 9.8-2. Recombinant assay: effect of ketoconazole on aromatase activity (percent of control) by overall task**

| Reference Chemical Log Conc (M) | Number of Labs[a] | Overall Mean | Overall sd | Overall SEM | Overall %CV |
|---|---|---|---|---|---|
| -4.00 | 4 | 6.01 | 2.02 | 1.01 | 33.6 |
| -4.30 | 2 | 13.12 | NC[b] | NC | NC |
| -4.52 | 1 | 17.88 | NC | NC | NC |
| -4.60 | 2 | 26.41 | NC | NC | NC |
| -5.00 | 4 | 39.84 | 7.25 | 3.63 | 18.2 |
| -5.30 | 3 | 59.60 | 4.58 | 2.64 | 7.7 |
| -5.48 | 1 | 37.41 | NC | NC | NC |
| -5.60 | 1 | 79.55 | NC | NC | NC |
| -6.00 | 4 | 87.23 | 0.80 | 0.40 | 0.9 |
| -6.60 | 1 | 101.75 | NC | NC | NC |
| -7.00 | 4 | 98.30 | 4.20 | 2.10 | 4.3 |
| -8.00 | 4 | 96.39 | 4.84 | 2.42 | 5.0 |
| -9.00 | 4 | 101.06 | 4.53 | 2.26 | 4.5 |
| -10.00 | 4 | 98.51 | 4.34 | 2.17 | 4.4 |
| -11.00 | 3 | 97.64 | 4.73 | 2.73 | 4.8 |
| -12.00 | 1 | 91.39 | NC | NC | NC |

a. Number of labs that conducted the assay at the cited reference chemical concentration. The run number varied because each laboratory used the results from their first run to select concentrations to be used in runs 2 and 3 that were believed would better characterize the percent inhibition curve.

b. NC – Not Calculated. If the overall mean was calculated using an $n \leq 2$, then the sd, SEM, and %CV were not calculated.

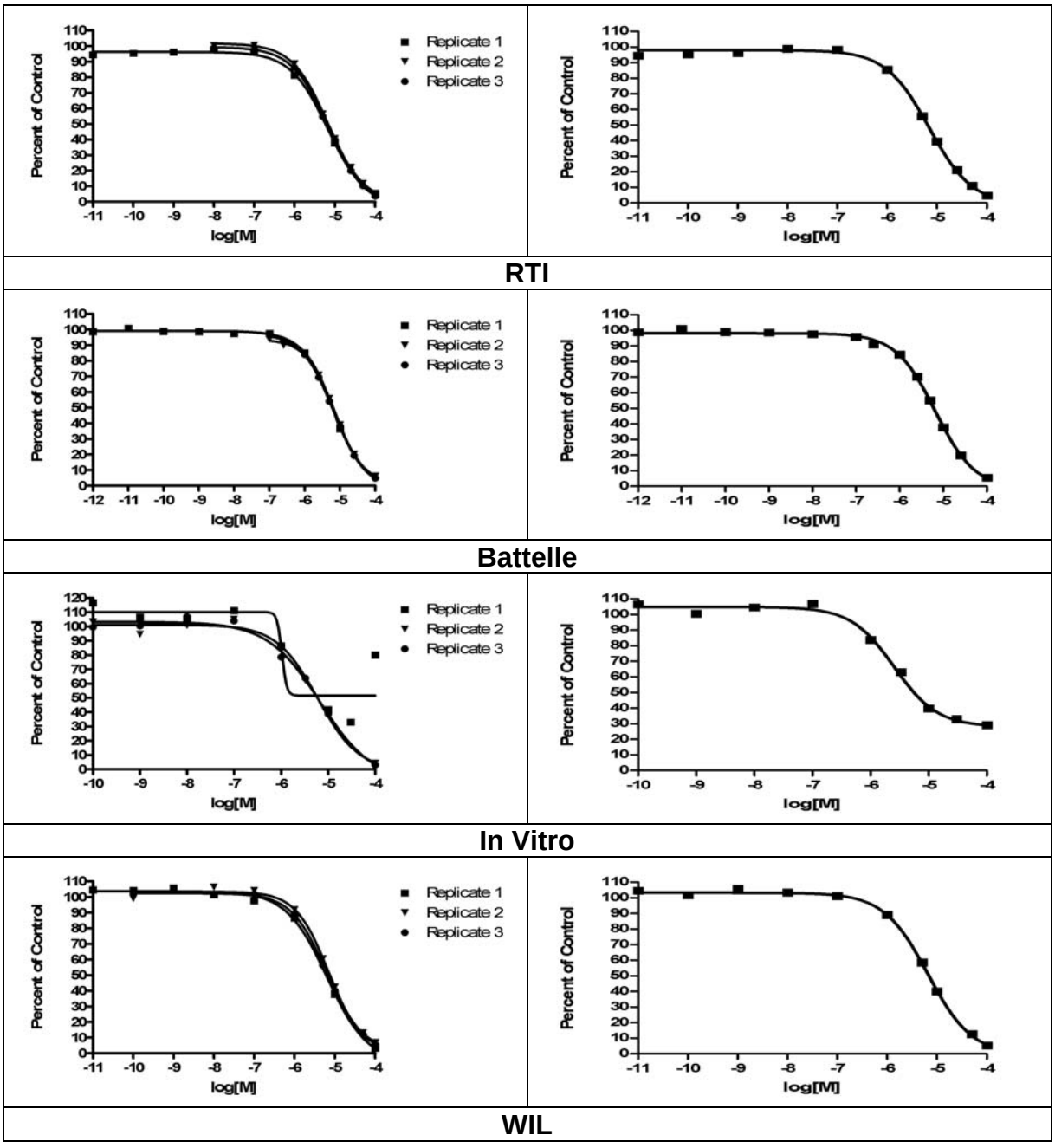



**Figure 9.8-1. Placental assay: individual run and average ketoconazole inhibition response curves (percent of control) by laboratory**

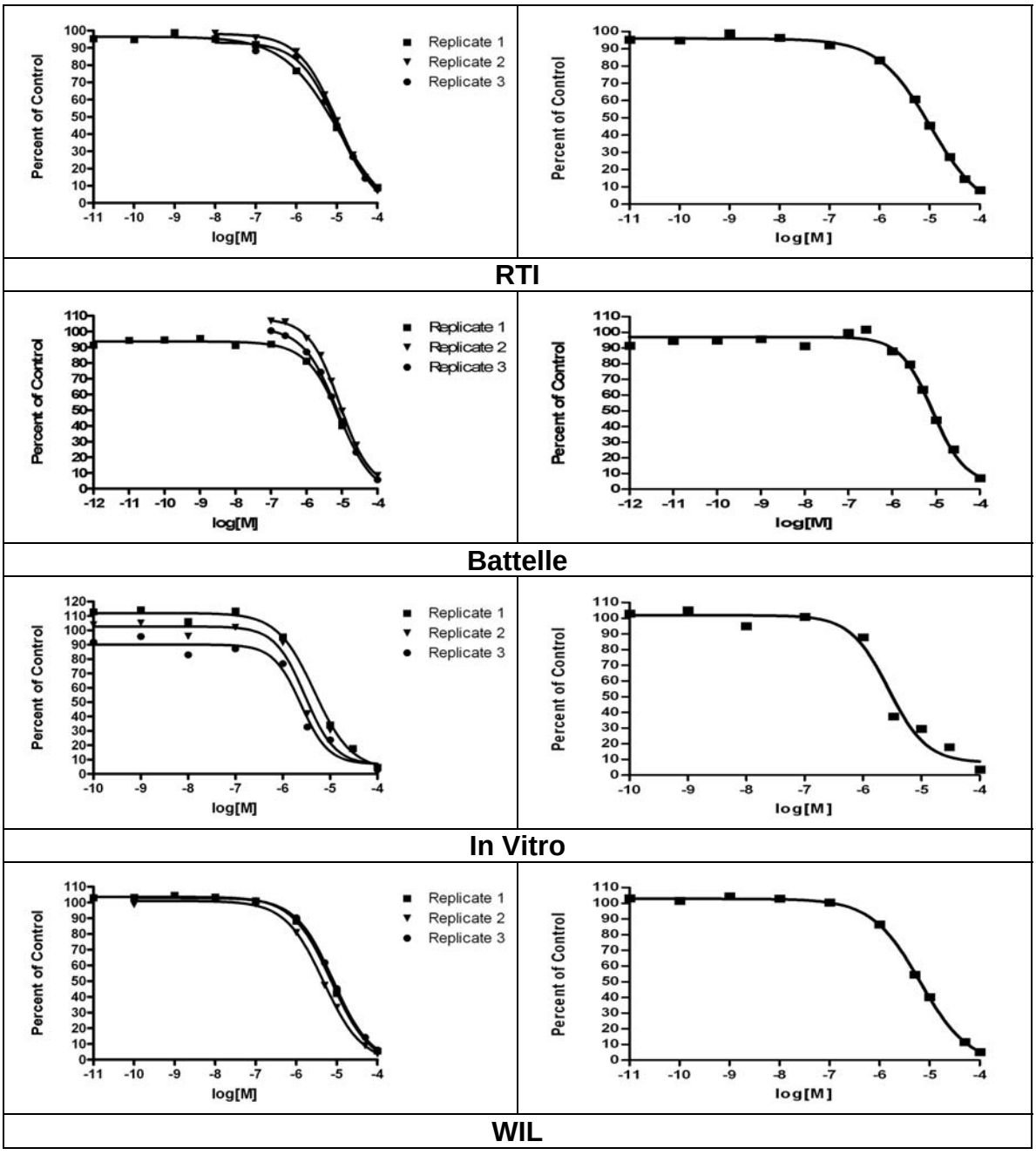



**Figure 9.8-2. Recombinant assay: individual run and average ketoconazole inhibition response curves (percent of control) by laboratory**

**Ketoconazole Placental $IC_{50}$:** The average ± SEM $IC_{50}$ values for RTI, Battelle, In Vitro, and WIL were 6.72 ± 0.11, 8.10 ± 1.26,4.58 ± 1.77, and 6.48 ± 0.15 µM, respectively (Table 9.8-3). The percent CV values were 2.8, 26.9, 67.0, and 4.1 percent, respectively. The higher %CV values for Battelle and In Vitro were attributed to the $IC_{50}$ value obtained from run 1. Modifications to the concentrations tested by Battelle and In Vitro after run 1 resulted in $IC_{50}$ values that were in better agreement with subsequent estimates, as well as estimates by the other labs. The results from RTI and WIL indicate that the $IC_{50}$ could be precisely estimated using the run 1 starting concentrations. However, three of four labs showed that modifications to the run 1 concentrations tested did improve the characterization of the steep portion of the inhibition curve and $IC_{50}$ estimate. The overall task group mean ± SEM $IC_{50}$ value was 6.47 ± 0.72 µM and the percent CV was 22.4 percent.

**Table 9.8-3. Placental $IC_{50}$ values by laboratory for ketoconazole**

| Run | $IC_{50}$ Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Ketoconazole (µM) | | | | |
| 1 | 6.58 | 10.6 | 1.07 | 6.37 |
| 2 | 6.64 | 7.11 | 5.91 | 6.78 |
| 3 | 6.93 | 6.59 | 6.76 | 6.28 |
| Mean | 6.72 | 8.10 | 4.58 | 6.48 |
| sd | 0.19 | 2.18 | 3.07 | 0.26 |
| SEM | 0.11 | 1.26 | 1.77 | 0.15 |
| %CV | 2.8 | 26.9 | 67.0 | 4.1 |

Source: Reference 10

**Ketoconazole Placental Slope:** The average ± SEM slope values for RTI, Battelle, In Vitro, and WIL were -0.9187 ± 0.0308, -0.9758 ± 0.0205, -0.9942 ± 0.0658, and -1.0354 ± 0.0527, respectively (Table 9.8-4). The percent CV values were 5.8, 3.6, 11.5, and 8.8 percent, respectively. The runs for a given lab and between labs were in close agreement. Modifications to concentrations or lack thereof did not seem to have any overt effect on slope values. The overall task group mean ± SEM slope value was -0.9810 ± 0.0242 and the percent CV was 4.9 percent.

**Table 9.8-4. Placental slope values by laboratory for ketoconazole**

| Run | Slope Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Ketoconazole | | | | |
| 1 | -0.9772 | -0.9384 | -0.8716 | -0.9736 |
| 2 | -0.8726 | -0.9801 | -1.014 | -0.9924 |
| 3 | -0.9062 | -1.009 | -1.097 | -1.1402 |
| Mean | -0.9187 | -0.9758 | -0.9942 | -1.0354 |
| sd | 0.0534 | 0.0355 | 0.1140 | 0.0912 |
| SEM | 0.0308 | 0.0205 | 0.0658 | 0.0527 |
| %CV | 5.8 | 3.6 | 11.5 | 8.8 |

Source: Reference 10

**Ketoconazole Recombinant $IC_{50}$:** The average ± SEM IC50 values for RTI, Battelle, In Vitro, and WIL were 9.38 ± 0.73, 11.07 ± 3.03, 3.34 ± 0.67, and 6.73  1.05 µM, respectively (9.8-5). The percent CV values were 13.5, 47.5, 34.7, and 27.0 percent, respectively. In general, the precision was low for most of the laboratories (RTI was the exception). The higher %CV values for Battelle and In Vitro were attributed to the $IC_{50}$ value obtained from run 1. Modifications to the concentrations tested by Battelle and In Vitro after run 1 resulted in $IC_{50}$ values that were in better agreement with subsequent estimates and, for Battelle, estimates by RTI and WIL. The results from RTI and WIL indicate that the $IC_{50}$ could be precisely estimated using the run 1 starting concentrations. However, RTI and Battelle showed that modifications to the run 1 concentrations tested did improve the characterization of the steep portion of the inhibition curve and IC50 estimate. The overall task group mean ± SEM $IC_{50}$ value was 7.63 ± 1.69 µM and the percent CV was 44.2 percent.

**Table 9.8-5. Recombinant $IC_{50}$ values by laboratory for ketoconazole**

| Run | $IC_{50}$ Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Ketoconazole (µM) | | | | |
| 1 | 8.017 | 17.12 | 4.64 | 7.272 |
| 2 | 10.52 | 8.503 | 2.95 | 4.703 |
| 3 | 9.594 | 7.599 | 2.42 | 8.215 |
| Mean | 9.38 | 11.07 | 3.34 | 6.73 |
| Sd | 1.27 | 5.26 | 1.16 | 1.82 |
| SEM | 0.73 | 3.03 | 0.67 | 1.05 |
| %CV | 13.5 | 47.5 | 34.7 | 27.0 |

Source: Reference 12

**Ketoconazole Recombinant Slope:** The average ± SEM slope values for RTI, Battelle, In Vitro, and WIL were -0.9593 ± 0.0397, -0.9026 ± 0.0582, -1.3210 ± 0.1126, and -0.9160 ± 0.0093, respectively (9.8-6). The percent CV values were 7.2, 11.2, 14.8, and 1.8 percent, respectively. The runs for a given lab and between labs were in close agreement. Modifications to concentrations did not seem to result in any appreciable improvement on estimating the slope values, thereby indicating that the initial concentrations tested produced a reliable estimate of the parameter. The overall task group mean ± SEM slope value was -1.0247 ± 0.0995 and the percent CV was 19.4 percent.

**Table 9.8-6. Recombinant slope values by laboratory for ketoconazole**

| Run | Slope Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Ketoconazole | | | | |
| 1 | -1.036 | -0.8087 | -1.108 | -0.8992 |
| 2 | -0.9035 | -1.009 | -1.364 | -0.9173 |
| 3 | -0.9384 | -0.8902 | -1.491 | -0.9315 |
| Mean | -0.9593 | -0.9026 | -1.3210 | -0.9160 |
| sd | 0.0687 | 0.1007 | 0.1951 | 0.0162 |
| SEM | 0.0397 | 0.0582 | 0.1126 | 0.0093 |
| %CV | 7.2 | 11.2 | 14.8 | 1.8 |

Source: Reference 12

## 9.9 4-Nonylphenol

**4-Nonylphenol Assay Overview.** The effect of increasing concentrations of 4-nonylphenol on aromatase activity is reported by laboratory in Tables B.9.-17 and B.9-18 (Appendix B) and by overall task in Tables 9.9-1 and 9.9-2. In addition, the results are graphically presented by laboratory (individual runs and average) in Figures 9.9-1 and 9.9-2.

After the first run was completed, the study directors from three of four laboratories modified the 4-nonylphenol concentrations selected for testing in runs 2 and 3 (Table 9.9-1). In Vitro used the same concentrations for all three runs and mistakenly did not test at a concentration of $10^{-3}$ M. In general, for the placental assay, the modifications involved including additional mid concentrations between $10^{-4}$ and $10^{-6}$ M for RTI and $10^{-4}$ M and $10^{-5}$ M for Battelle and WIL. The concentration(s) that were replaced by these modifications differed for the three labs but ranged from $10^{-8}$ to $10^{-10}$ M.

For the recombinant assay, in general, the modifications involved including additional mid-concentrations between $10^{-4}$ and $10^{-6}$ M. The concentration(s) that were replaced by these modifications ranged from $10^{-8}$ to $10^{-10}$M.

For all four laboratories, increasing concentrations of 4-nonylphenol decreased the activity of the microsomal aromatase activity and the decrease was concentration dependent (Tables 9.9-1 and 9.9-2). The shapes of the enzyme activity vs 4-nonylphenol curves were sigmoidal (Figures 9.9-1 and 9.9-2). At a 4-nonylphenol concentration of $10^{-3}$ M, aromatase inhibition was complete; the laboratory percent of control values were less than 1 percent for the three labs that tested at this concentration.

In the placental assay, for In Vitro, the percent of control at the highest concentration tested ($10^{-4}$ M) was 16 percent. In contrast, at a 4-nonylphenol concentration of approximately $10^{-6}$ M, there was little to no aromatase inhibition; the laboratory percent of control values were ~98 percent, except for In Vitro which reported a percent of control value of approximately 91 percent at this concentration. The overall task mean ± SEM percent of control values at $10^{-3}$ and $10^{-6}$ M were 0.26 ± 0.10 and 97.16 ± 2.24 percent, respectively (Table 9.9-1).

In the recombinant assay, for In Vitro, the percent of control at the highest concentration tested ($10^{-4}$ M) was 4 percent. In contrast, at a 4-nonylphenol concentration of approximately 10- 6 M, there was little to no aromatase inhibition; the laboratory percent of control values were ≥ 94 percent. The overall task mean ± SEM percent of control values at $10^{-3}$ and $10^{-6}$ M were 0.34 ± 0.22 and 97.03 ± 1.62 percent, respectively (Table 9.9-2).

**Table 9.9-1. Placental assay: effect of 4-nonylphenol on aromatase activity (percent of control) by laboratory**

| Reference Chemical Log Conc (M) | Number of Labs[a] | Overall Mean | Overall sd | Overall SEM | Overall %CV |
|---|---|---|---|---|---|
| -3.00 | 3 | 0.26 | 0.17 | 0.10 | 64.7 |
| -4.00 | 4 | 11.14 | 4.47 | 2.24 | 40.1 |
| -4.30 | 3 | 25.10 | 4.22 | 2.43 | 16.8 |
| -4.48 | 1 | 40.85 | NC[b] | NC | NC |
| -4.60 | 2 | 46.95 | NC | NC | NC |
| -5.00 | 4 | 86.81 | 2.39 | 1.19 | 2.8 |
| -5.30 | 1 | 95.10 | NC | NC | NC |
| -6.00 | 4 | 97.16 | 4.48 | 2.24 | 4.6 |
| -7.00 | 4 | 96.92 | 4.34 | 2.17 | 4.5 |
| -8.00 | 4 | 96.60 | 5.18 | 2.59 | 5.4 |
| -9.00 | 4 | 94.73 | 6.46 | 3.23 | 6.8 |
| -10.0 | 4 | 96.25 | 7.85 | 3.93 | 8.2 |

a. Number of labs that conducted the assay at the cited reference chemical concentration. The run number varied because each laboratory used the results from their first run to select concentrations to be used in runs 2 and 3 that were believed would further (In Vitro) or better characterize the percent inhibition curve.

b. NC – Not Calculated. If the overall mean was calculated using an n ≤ 2, then the sd, SEM, and %CV were not calculated.

**Table 9.9-2. Recombinant assay: effect of 4-nonylphenol on aromatase activity (percent of control) by overall task**

| Reference Chemical Log Conc (M) | Number of Labs[a] | Overall Mean | Overall sd | Overall SEM | Overall %CV |
|---|---|---|---|---|---|
| -3.00 | 3 | 0.34 | 0.38 | 0.22 | 114.1 |
| -4.00 | 4 | 3.45 | 1.15 | 0.58 | 33.4 |
| -4.30 | 3 | 14.55 | 5.34 | 3.08 | 36.7 |
| -4.48 | 1 | 27.11 | NC[b] | NC | NC |
| -4.60 | 2 | 38.19 | NC | NC | NC |
| -5.00 | 4 | 73.17 | 11.06 | 5.53 | 15.1 |
| -5.30 | 1 | 101.41 | NC | NC | NC |
| -6.00 | 4 | 97.03 | 3.24 | 1.62 | 3.3 |
| -7.00 | 4 | 97.87 | 2.62 | 1.31 | 2.7 |
| -8.00 | 4 | 96.38 | 4.78 | 2.39 | 5.0 |
| -9.00 | 4 | 98.51 | 6.43 | 3.21 | 6.5 |
| -10.00 | 4 | 98.95 | 6.80 | 3.40 | 6.9 |

a. Number of labs that conducted the assay at the cited reference chemical concentration. The run number varied because each laboratory used the results from their first run to select concentrations to be used in runs 2 and 3 that were believed would further (In Vitro) or better characterize the percent inhibition curve.

b. NC – Not Calculated. If the overall mean was calculated using an n ≤ 2, then the sd, SEM, and %CV were not calculated.

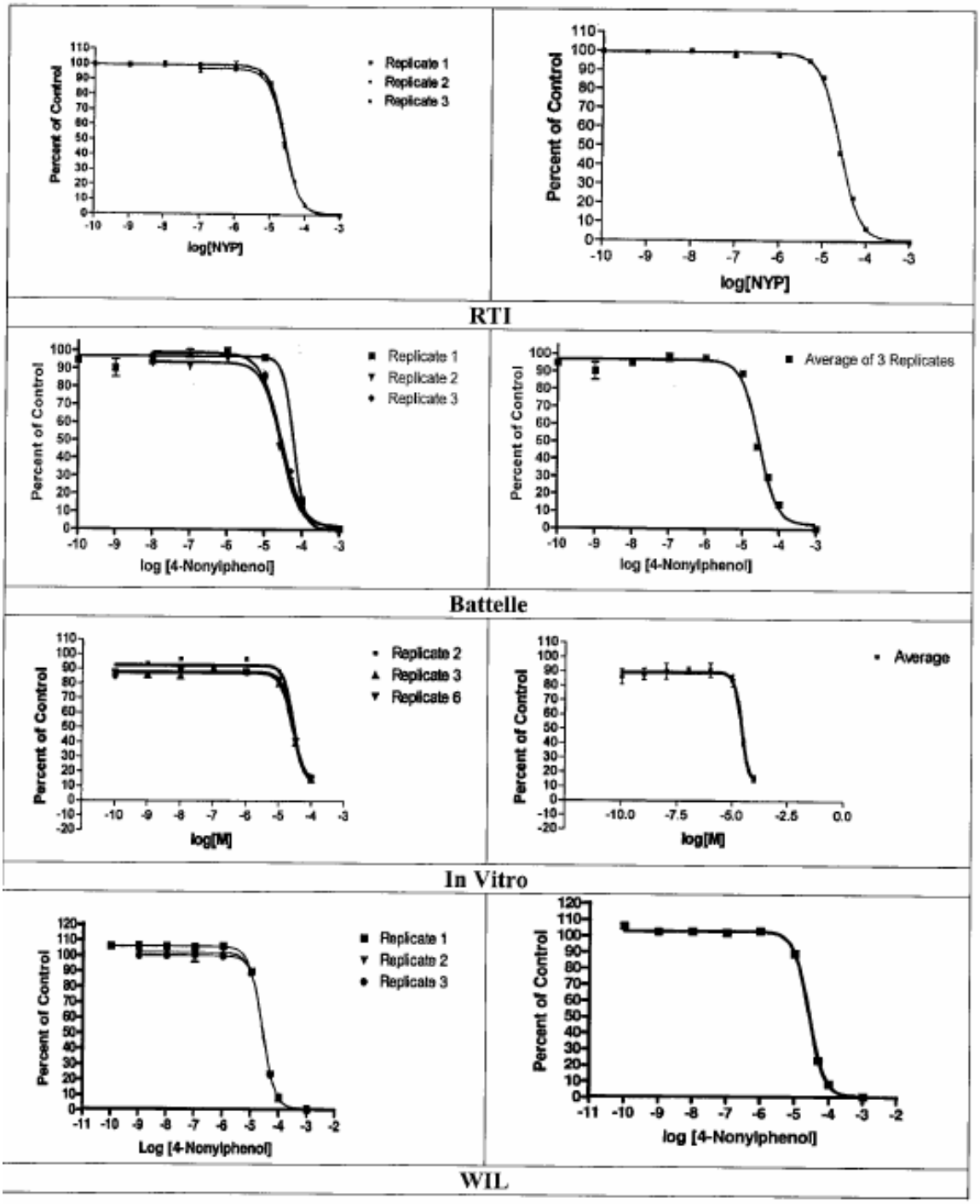



**Figure 9.9-1. Placental assay: individual run and average 4-nonylphenol inhibition response curves (percent of control) by laboratory**

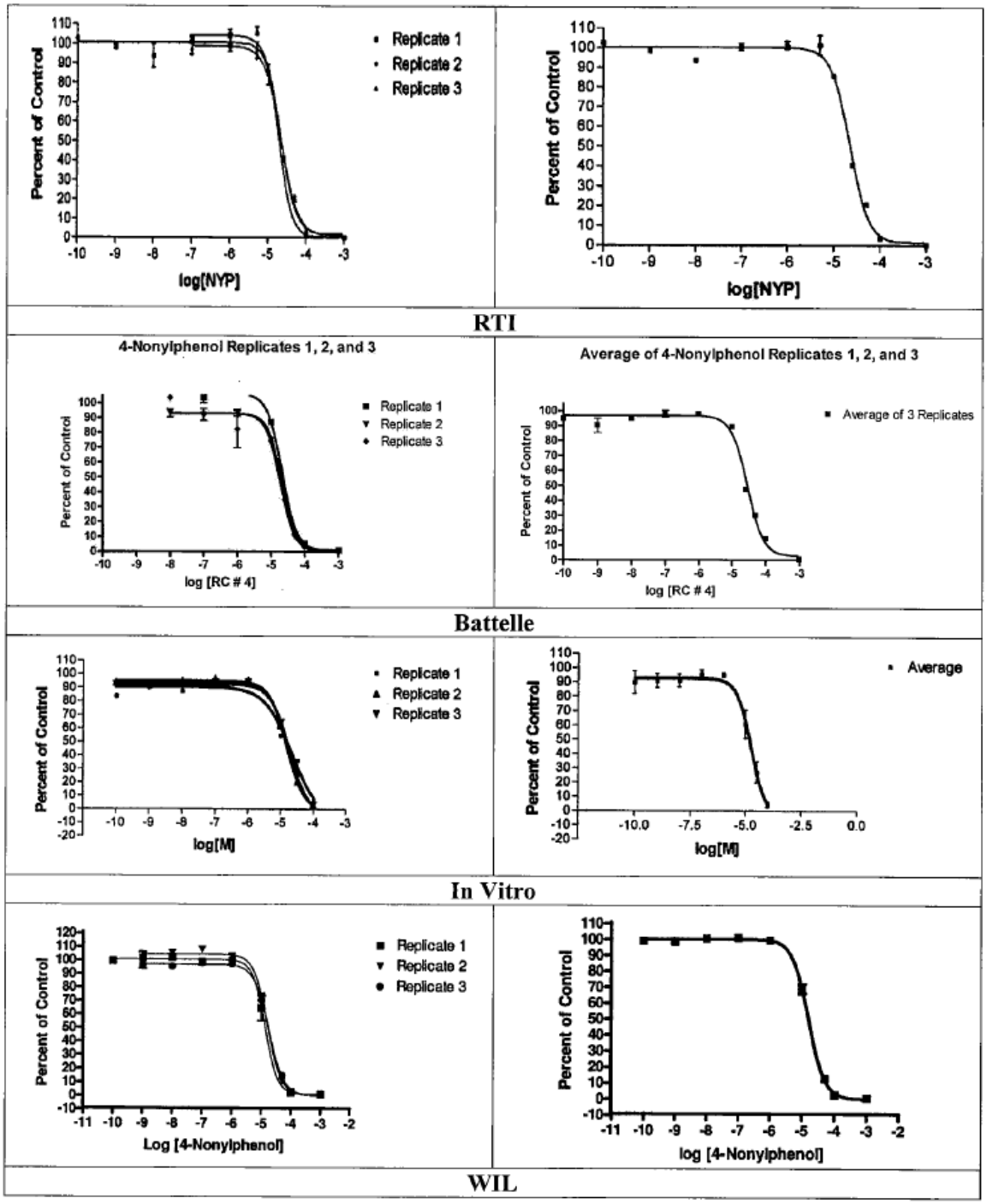



**Figure 9.9-2. Recombinant assay: individual run and average 4-nonylphenol inhibition response curves (percent of control) by laboratory**

**4-Nonylphenol Placental $IC_{50}$:** The average ± SEM IC50 values for RTI, Battelle, In Vitro, and WIL were 25.37 ± 0.65, 37.98 ± 10.57, 26.74 ± 0.23, and 26.31± 0.71µlM, respectively (Table 9.9-3). The percent CV values were 4.4, 48.2, 1.5, and 4.7 percent, respectively. The higher %CV value for Battelle was attributed to the $IC_{50}$ value obtained from run 1. Modifications to the concentrations tested by Battelle after run 1 resulted in $IC_{50}$ values that were in better agreement with subsequent estimates, as well as estimates by the other labs. The results from RTI, In Vitro, and WIL indicate that the $IC_{50}$ could be precisely estimated using the run 1 starting concentrations. However, three of four labs showed that modifications to the run 1 concentrations tested did improve the characterization of the steep portion of the inhibition curve and $IC_{50}$ estimate. The overall task group mean ± SEM IC50 value was 29.10 ± 2.97 µM and the percent CV was 20.4 percent.

**Table 9.9-3. Placental $IC_{50}$ values by laboratory for 4-nonylphenol**

| Run | $IC_{50}$ Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| 4-Nonylphenol (µM) | | | | |
| 1 | 26.67 | 59.11 | 26.82 | 25.03 |
| 2 | 24.66 | 26.84 | 26.31 | 26.43 |
| 3 | 24.77 | 27.98 | 27.10 | 27.48 |
| Mean | 25.37 | 37.98 | 26.74 | 26.31 |
| Sd | 1.13 | 18.31 | 0.40 | 1.23 |
| SEM | 0.65 | 10.57 | 0.23 | 0.71 |
| %CV | 4.4 | 48.2 | 1.5 | 4.7 |

Source: Reference 10

**4-Nonylphenol Placental Slope:** For 4-nonylphenol, the average ± SEM slope values for RTI, Battelle, In Vitro, and WIL were -1.9577 ± 0.0318, -2.1220 ± 0.4639, -2.627 1.734, and -1.9541 ± 0.0508, respectively (Table 9.9-4). The percent CV values were 2.8, 37.9, 11.4, and 4.5 percent, respectively. The runs for a given lab and between labs were in close agreement. Modifications to concentrations or lack thereof did not seem to have any overt effect on slope values. The overall task group mean ± SEM slope value was -2.1651 ± 0.1588 and the percent CV was 14.7 percent.

**Table 9.9-4. Placental slope values by laboratory for 4-nonylphenol**

| Run | Slope Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| 4-Nonylphenol | | | | |
| 1 | -2.017 | -3.042 | -2.939 | -1.8580 |
| 2 | -1.948 | -1.766 | -2.340 | -1.9735 |
| 3 | -1.908 | -1.558 | -2.601 | -2.0307 |
| Mean | -1.9577 | -2.1220 | -2.627 | -1.9541 |
| sd | 0.0551 | 0.8035 | 0.3003 | 0.0880 |
| SEM | 0.0318 | 0.4639 | 0.1734 | 0.0508 |
| %CV | 2.8 | 37.9 | 11.4 | 4.5 |

Source: Reference 10

**4-Nonylphenol Recombinant $IC_{50}$:** The average ± SEM IC50 values for RTI, Battelle, In Vitro, and WIL were 20.98 ± 0.77, 20.2l ± 0.84, 18.7 ± 2.06, and l5.59 ± 1.33 µM, respectively (Table 9.9-5). The percent CV values were 6.4, 7.2, 19.1, and 14.8 percent, respectively. A review of the percent of control curves for each laboratory indicated that there were no overt aberrant curves. Modifications to the concentrations tested run 1 did not appreciably alter the initially estimated $IC_{50}$ values. The results from all of the laboratories indicated that the $IC_{50}$ could be precisely estimated using the run 1 starting concentrations. The overall task group mean ± SEM $IC_{50}$ value was l8.87 ± l.l9 µM and the percent CV was 12.6 percent.

**Table 9.9-5. Recombinant $IC_{50}$ values by laboratory for 4-nonylphenol**

| Run | $IC_{50}$ Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| 4-Nonylphenol (µM) | | | | |
| 1 | 19.54 | 21.11 | 22.5 | 13.20 |
| 2 | 22.18 | 18.52 | 15.4 | 15.79 |
| 3 | 21.23 | 20.99 | 18.2 | 17.79 |
| Mean | 20.98 | 20.21 | 18.7 | 15.59 |
| Sd | 1.34 | 1.46 | 3.58 | 2.30 |
| SEM | 0.77 | 0.84 | 2.06 | 1.33 |
| %CV | 6.4 | 7.2 | 19.1 | 14.8 |

Source: Reference 12

**4-Nonylphenol Recombinant Slope:** The average ± SEM slope values for RTI, Battelle, In Vitro, and WIL were -2.264 ± 0.1691, -2.0740 ± 0.0304, -1.394 ± 0.1798, and -1.8823 ± 0.0930, respectively (Table 9.9-6). The percent CV values were 12.9, 2.5, 22.3, and 8.6 percent, respectively. The runs for a given lab and between labs were in close agreement. Modifications to concentrations or lack thereof did not seem to result in any appreciable improvement on estimating the slope values, thereby indicating that the initial concentrations tested produced a reliable estimate of the parameter. The overall task group mean ± SEM slope value was -1.9035 ± 0.1870 and the percent CV was 19.6 percent.

**Table 9.9-6. Recombinant slope values by laboratory for 4-nonylphenol**

| Run | Slope Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| 4-Nonylphenol | | | | |
| 1 | -2.588 | -2.014 | -1.039 | -2.0442 |
| 2 | -2.018 | -2.096 | -1.622 | -1.8805 |
| 3 | -2.186 | -2.112 | -1.520 | -1.7222 |
| Mean | -2.264 | -2.074 | -1.394 | -1.8823 |
| sd | 0.2929 | 0.0526 | 0.3114 | 0.1610 |
| SEM | 0.1691 | 0.0304 | 0.1798 | 0.0930 |
| %CV | 12.9 | 2.5 | 22.3 | 8.6 |

Source: Reference 12

## 9.10 Prochloraz

**Prochloraz Assay Overview.** The effect of increasing concentrations of prochloraz on aromatase activity is reported by laboratory in Tables B.9-19 and B.9-20 (Appendix B) and by overall task in Tables 9.10-1 and 9.10-2. In addition, the results are graphically presented by laboratory (individual runs and average) in Figures 9.10-1 and 9.10-2.

For all four laboratories, increasing concentrations of prochloraz decreased the activity of the microsomal aromatase and the decrease was concentration-dependent (Tables 9.10-1 and 9.10-2). The shapes of the enzyme activity vs prochloraz curves were sigmoidal (Figures 9.10-1 and 9.10-2).

In the placental assay, after the first run was completed, the study directors from three of four laboratories modified the prochloraz concentrations selected for testing in runs 2 and 3 (Table B.9-19). For both assays, in general, the modifications involved including additional mid-concentrations between $10^{-7}$ and$10^{-9}$ M, which replaced the concentrations from $10^{-3}$ and $10^{-4}$ M that were tested in run 1 for RTI and WIL, whereas Battelle replaced concentrations at $10^{-4}$ and $10^{-11}$ M. Battelle tested concentrations ranging from $10^{-4}$ M to $10^{-11}$ M rather than $10^{-3}$ M to $10^{-10}$ M due to a miscalculation. The error was identified and corrected prior to proceeding with runs 2 and 3. In Vitro used the same concentrations for the first two runs, but for its last run, used a concentration of 3.3 x $10^{-8}$ M rather than what it used in the previous two runs, i.e.3.3 x $10^{-9}$ M. Changing the concentration after the second rather than the first run was an unplanned change but was done so believing that it might result in a more well-defined IC50.

In the placental assay, at a prochloraz concentration of $10^{-6}$ M, aromatase inhibition was almost complete; the laboratory percent of control values ranged from 2 to 3 percent for three of the labs; the exception was WIL, which reported a negative percent of control value at this concentration. In contrast, at a prochloraz concentration of approximately $10^{-9}$ M, there was little to no aromatase inhibition; the laboratory percent of control values were ~95 percent for three of the four labs, and for In Vitro the percent of control value was 88 percent at this concentration. The overall task mean ± SEM percent of control values at $10^{-6}$ and $10^{-9}$ M were 0.76 ± 1.64 and 94.46 ± 2.44 percent, respectively (Table 9.10-1).

In the recombinant assay, at a prochloraz concentration of $10^{-6}$ M, aromatase inhibition was almost complete; the laboratory percent of control values ranged from 2 to 3 percent for three of the labs; the exception was WIL, which due to a low enzyme activity value for run 2 had a high percent of control value for this one run, i.e., 53 percent, but values of approximately 3 to 4 percent for the other two runs, thereby resulting in an average percent of control value of 20 percent. In contrast, at a prochloraz concentration of approximately $10^{-9}$ M, there was little to no aromatase inhibition; the laboratory percent of control values were ~93 percent for all four labs. The overall task mean ± SEM percent of control values at $10^{-6}$ and $10^{-9}$ M were 7.26 ± 4.27 and 96.33 ± 2.77 percent, respectively (Table 9.10-2).

**Table 9.10-1. Placental assay: effect of prochloraz on aromatase activity (percent of control) by overall task**

| Reference Chemical Log Conc (M) | Number of Labs[a] | Overall Mean | Overall sd | Overall SEM | Overall %CV |
|---|---|---|---|---|---|
| -3.00 | 2 | 0.04 | NC[b] | NC | NC |
| -4.00 | 4 | 0.23 | 0.61 | 0.31 | 265.6 |
| -5.00 | 4 | -1.60 | 3.47 | 1.74 | 217.6 |
| -6.00 | 4 | 0.76 | 3.27 | 1.64 | 433.5 |
| -7.00 | 4 | 17.68 | 2.74 | 1.37 | 15.5 |
| -7.48 | 1 | 78.51 | NC | NC | NC |
| -7.52 | 1 | 42.22 | NC | NC | NC |
| -7.60 | 2 | 49.98 | NC | NC | NC |
| -8.00 | 4 | 68.74 | 3.59 | 1.80 | 5.2 |
| -8.30 | 1 | 84.11 | NC | NC | NC |
| -8.48 | 1 | 82.65 | NC | NC | NC |
| -8.52 | 1 | 90.14 | NC | NC | NC |
| -8.60 | 1 | 88.90 | NC | NC | NC |
| -9.00 | 4 | 94.46 | 4.88 | 2.44 | 5.2 |
| -10.00 | 4 | 99.45 | 3.17 | 1.59 | 3.2 |
| -11.00 | 1 | 96.71 | NC | NC | NC |

a. Number of labs that conducted the assay at the cited reference chemical concentration. The run number varied because each laboratory used the results from their first run to select concentrations to be used in runs 2 and 3 that were believed would better characterize the percent inhibition curve. (In Vitro modified their concentrations between runs 3 and 4).
b. NC – Not Calculated. If the overall mean was calculated using an n ≤ 2, then the sd, SEM, and %CV were not calculated.

**Table 9.10-2. Recombinant assay: effect of prochloraz on aromatase activity (percent of control) by overall task**

| Reference Chemical Log Conc (M) | Number of Labs[a] | Overall Mean | Overall sd | Overall SEM | Overall %CV |
|---|---|---|---|---|---|
| -3.00 | 3 | 0.05 | 0.09 | 0.05 | 177.8 |
| -4.00 | 4 | 2.64 | 4.99 | 2.49 | 188.7 |
| -5.00 | 4 | 1.33 | 1.86 | 0.93 | 140.3 |
| -6.00 | 4 | 7.26 | 8.54 | 4.27 | 117.7 |
| -7.00 | 4 | 29.08 | 13.28 | 6.64 | 45.7 |
| -7.48 | 1 | 82.69 | NC[b] | NC | NC |
| -7.52 | 1 | 76.98 | NC | NC | NC |
| -7.60 | 2 | 55.80 | NC | NC | NC |
| -8.00 | 4 | 75.17 | 8.31 | 4.15 | 11.1 |
| -8.30 | 1 | 83.06 | NC | NC | NC |
| -8.48 | 1 | 87.75 | NC | NC | NC |
| -8.52 | 1 | 100.92 | NC | NC | NC |
| -8.60 | 1 | 92.89 | NC | NC | NC |
| -9.00 | 4 | 96.33 | 5.54 | 2.77 | 5.8 |
| -10.00 | 4 | 98.77 | 4.40 | 2.20 | 4.5 |
| -11.00 | 1 | 100.41 | NC | NC | NC |

a. Number of labs that conducted the assay at the cited reference chemical concentration. The run number varied because each laboratory used the results from their first run to select concentrations to be used in runs 2 and 3 that were believed would better characterize the percent inhibition curve. (In Vitro modified their concentrations between runs 3 and 4).
b. NC – Not Calculated. If the overall mean was calculated using an n ≤ 2, then the sd, SEM, and %CV were not calculated.

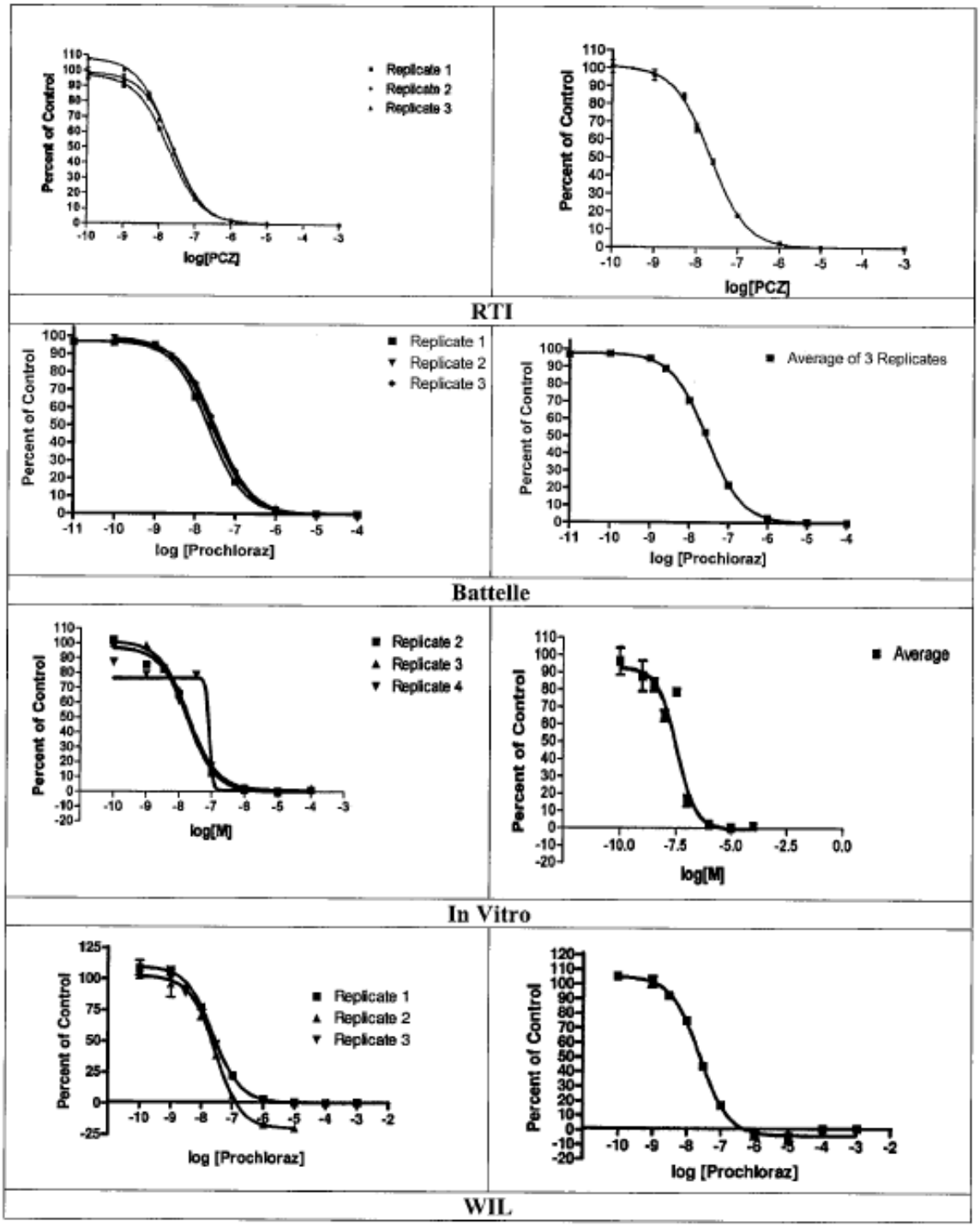



**Figure 9.10-1. Placental assay: individual run and average prochloraz inhibition response curves (percent of control) by laboratory**

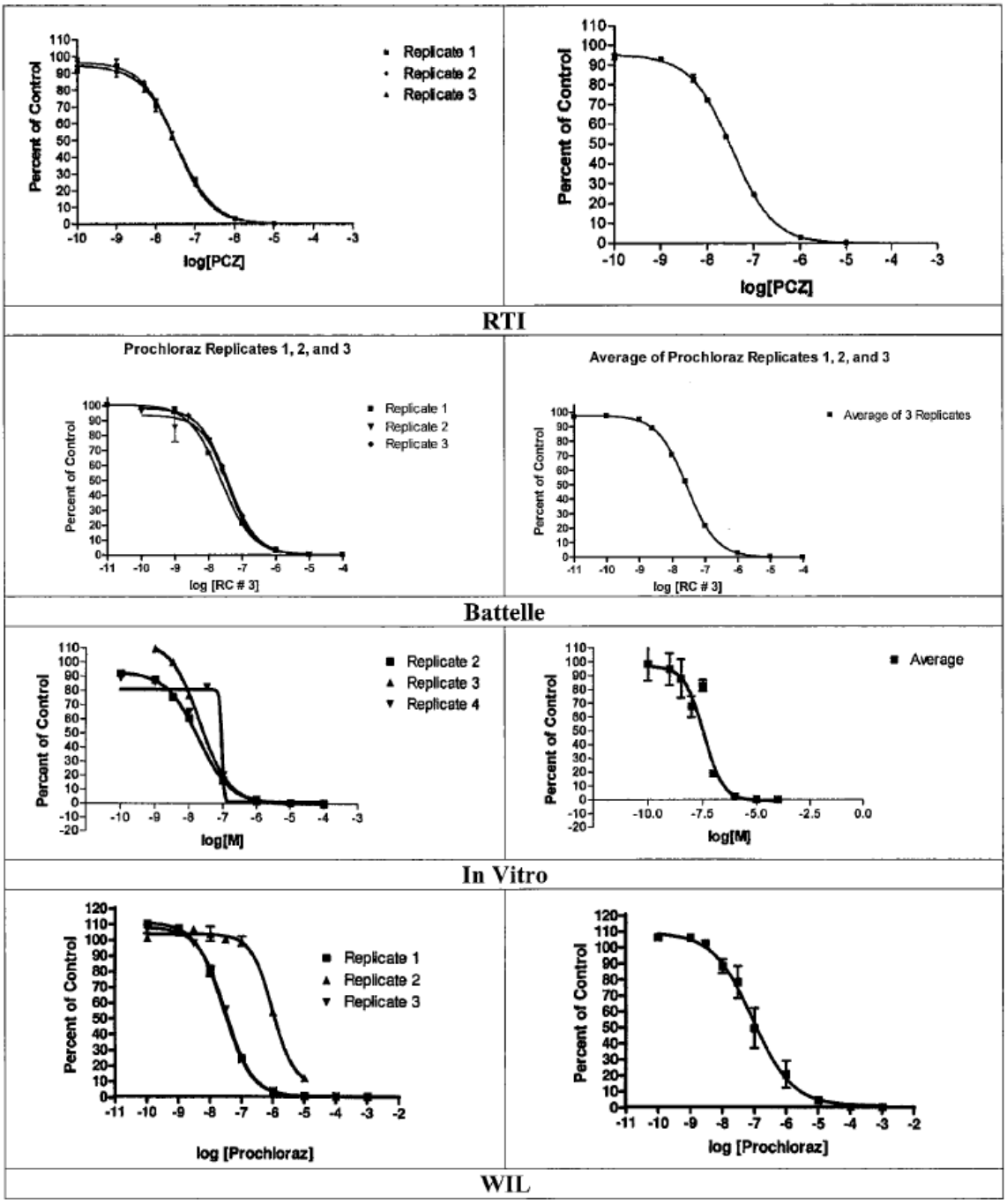



**Figure 9.10-2. Recombinant assay: individual run and average prochloraz inhibition response curves (percent of control) by laboratory**

**Prochloraz Placental $IC_{50}$:** The average ± SEM $IC_{50}$ values for RTI, Battelle, In Vitro, and WIL were 0.0202 ± 0.0018, 0.0269 ± 0.0031, 0.0408 ± 0.0220, and 0.0258 ± 0.0013 µM, respectively (Table 9.10-3). The percent CV values were 15.8, 20.3, 93.2, and 8.9 percent, respectively. The higher %CV value for In Vitro was attributed to the $IC_{50}$ value obtained from run 3. The results from RTI, Battelle, and WIL indicate that the $IC_{50}$ could be precisely estimated using the run 1 staring concentrations. However, three of four labs showed that modifications to the run 1 concentrations tested did improve the characterization of the steep portion of the inhibition curve and $IC_{50}$ estimate. The overall task group mean ± SEM $IC_{50}$ value was 0.0284 ± 0.0044 µM and the percent CV was 30.8 percent.

**Table 9.10-3. Placental $IC_{50}$ values by laboratory for prochloraz**

| Run | $IC_{50}$ Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Prochloraz (µM) | | | | |
| 1 | 0.0180 | 0.0218 | 0.0186 | 0.0240 |
| 2 | 0.0239 | 0.0263 | 0.0191 | 0.0284 |
| 3 | 0.0188 | 0.0326 | 0.0848 | 0.0251 |
| Mean | 0.0202 | 0.0269 | 0.0408 | 0.0258 |
| sd | 0.0032 | 0.0054 | 0.0381 | 0.0023 |
| SEM | 0.0018 | 0.0031 | 0.0220 | 0.0013 |
| %CV | 15.8 | 20.3 | 93.2 | 8.9 |

Source: Reference 10

**Prochloraz Placental Slope:** For prochloraz, the average ± SEM slope values for RTI, Battelle, In Vitro, and WIL were -0.9687 ± 0.0167, -0.9627 ± 0.0135, -3.3020 ± 2.3110 and -0.9995 ± 0.0327, respectively (Table 9.10-4). The percent CV values were 3.0, 2.4, 121.2, and 5.7 percent, respectively. The higher %CV value for In Vitro was attributed to a slope obtained from run 3. The results from RTI, Battelle, and WIL indicate that the slope could be precisely estimated using the run 1 starting concentrations. However, three of four labs showed that modifications to the run 1 concentrations tested did improve the characterization of the steep portion of the inhibition curve and slope estimate. The run 3 slope values reported by In Vitro was not included in the calculation used to determine the overall task values. The overall task group mean if SEM slope value was -0.9805 ± 0.0088 and the percent CV was18 percent

**Table 9.10-4. Placental slope values by laboratory for prochloraz**

| Run | Slope Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Prochloraz | | | | |
| 1 | -0.9392 | -0.9853 | -1.005 | -0.9841 |
| 2 | -0.9971 | -0.9386 | -0.9771 | -1.0622 |
| 3 | -0.9697 | -0.9641 | -7.924 | -0.9521 |
| Mean | -0.9687 | -0.9627 | -3.3020 | -0.9995 |
| Sd | 0.0290 | 0.0234 | 4.0028 | 0.0566 |
| SEM | 0.0167 | 0.0135 | 2.3110 | 0.0327 |
| %CV | 3.0 | 2.4 | 121.2 | 5.7 |

Source: Reference 10

**Prochloraz Recombinant $IC_{50}$:** The average ± SEM IC50 values for RTI, Battelle, In Vitro, and WIL were 0.0334 ± 0.0007, 0.0325 ± 0.0047, 0.0198 ± 0.0009, and 0.0284 ± 0.0021 µM, respectively (Table 9.10-5). The percent CV values were 3.5, 25.2, 6.1, and 10.5 percent, respectively. The average reported for In Vitro and WIL were based on results from only 2 of 3 runs because In Vitro's third run (run 4) had a very skewed percent of control curve and WIL's percent of control curve for run 2 was determined using an aberrant full enzyme activity control value. If all three runs were used, then the average ± SEM and %CV for In Vitro were 0.0436 ± 0.0239 µM and 94.8 percent and for WIL were 0.3353 ± 0.3069 µM and 158.5 percent. The slightly higher %CV value for Battelle was attributed to the $IC_{50}$ value obtained from run l. The results from all the labs indicate that the $IC_{50}$ could be precisely estimated using the run 1 starting concentrations. However, the results also showed that modifications to the run 1 concentrations, as was made by Battelle, could improve the characterization of the steep portion of the inhibition curve and $IC_{50}$ estimate. The overall task group mean ± SEM $IC_{50}$ value was 0.0285 ± 0.0031 µM and the percent CV was 21.9 percent (does not include the aberrant values for In Vitro and WIL).

**Table 9.10-5. Recombinant $IC_{50}$ values by laboratory for prochloraz**

| Run | $IC_{50}$ Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| Prochloraz (µM) | | | | |
| 1 | 0.03475 | 0.02307 | 0.0189 | 0.0263 |
| 2 | 0.03258 | 0.03697 | 0.0206 | 0.9492* |
| 3 | 0.03296 | 0.03749 | 0.0913 | 0.0305 |
| Mean | 0.0334 | 0.0325 | 0.0198 | 0.0284 |
| Sd | 0.0012 | 0.0082 | 0.0012 | 0.0030 |
| SEM | 0.0007 | 0.0047 | 0.0009 | 0.0021 |
| %CV | 3.5 | 25.2 | 6.1 | 10.5 |

Source: Reference 12

**Prochloraz Recombinant Slope:** The average ± SEM slope values for RTI, Battelle, In Vitro, and WIL were -0.9683 ± 0.0236, -1.016 ± 0.0503, -0.9587 ± 0.0534 and -1.0831 ± 0.0953, respectively (Table 9.10-6). The percent CV values were 4.2, 8.6, 7.9, and l5.2 percent, respectively. For In Vitro, the values reported above were calculated only using runs land 2 because the shape of the curve for the third run was very skewed, i.e., the slope was virtually perpendicular to the x-axis. If all three runs were used, then the average ± SEM slope and %CV values for In Vitro were -4.896 ± 3.937 and 139.3 percent. Although the precision for WIL was relatively high, the relatively slightly lower precision was attributed to the results from run 2, which had a low full enzyme activity control value and shifted the curve to the right. Since the shape of the cure was very similar to the other two runs, the aberrant full enzyme activity value had a greater affect on the $IC_{50}$ value than on the slope. For RTI and Battelle, modifications to concentrations after run 1 did not seem to result in any appreciable improvement on estimating the slope values, thereby indicating that the initial concentrations tested produced a reliable estimate of the parameter. The overall task group mean ± SEM slope value was -l.0066 ± 0.0569 and the percent CV was 5.7 percent.

**Table 9.10-6. Recombinant slope values by laboratory for prochloraz**

| Run | Slope Values | | | |
|---|---|---|---|---|
| | RTI | Battelle | In Vitro | WIL |
| **Prochloraz** | | | | |
| 1 | -0.9214 | -0.9271 | -0.9053 | -0.9744 |
| 2 | -0.9868 | -1.101 | -1.012 | -1.2731 |
| 3 | -0.9968 | -1.021 | -12.77[a] | -1.0019 |
| Mean | -0.9683 | -1.016 | -4.896 | -1.0831 |
| sd | 0.0410 | 0.0870 | 6.819 | 0.1651 |
| SEM | 0.0236 | 0.0503 | 3.937 | 0.0953 |
| %CV | 4.2 | 8.6 | 139.3 | 15.2 |

a. Considered an aberrant value. See source text for results without the value included.

Source: Reference 12

# 10.0 Assessment of the Interlaboratory Validation Study

## 10.1 Intralaboratory Statistical Analysis (Placental)

The intralaboratory analyses from each laboratory for background activity, full enzyme activity, and negative and positive controls, $IC_{50}$s, and slopes are provided in the appendices of the individual laboratory reports. Due to the integrated manner in which the intralaboratory statistical analysis reports (References 11 and 12) were presented, as well as the interrelationships among the endpoints, it was decided to present the salient information from the intralaboratory statistical analysis reports in a single section rather than attempt to extract information about individual endpoints and incorporate them into their previously presented respective sections.

### 10.1.1 RTI lntralaboratory Statistical Analysis

The full statistical analysis report and corresponding tables and graphs can be found in the appendices of the RTI report (Reference 10-A). Since the RTI report did not include a summary of the intralaboratory statistical analysis results, Battelle prepared the following summary from the information that was presented by RTI:

Dose response model curve fits were not achievable for dibenz(a,h)anthracene (DBA) and atrazine (ATZ) due to the low aromatase inhibition and thus these two chemical were categorized as non-inhibitors. Among the remaining eight chemicals, the estimated values for the $Log_{10}IC_{50}$ were -8.823 for econazole and -7.698 for prochloraz, and ranged from -6.395 to -4.1 71 for the rest. Thus econazole and prochloraz were relatively strong inhibitors. The steepest slope was observed in nonylphenol (-1.949) and the shallowest slope was observed in dicofol (-0.658). The slopes for the rest of the chemicals were around -1.0. The bottom thresholds were around zero for seven of the eight chemicals, with the exception of chrysin (20.631). The top thresholds were around 100 for all eight chemicals. There was very little variance in the $IC_{50}$s, slope, top and bottom estimates over the three runs for any of the eight chemicals. Significantly decreasing control activities between the beginnings and the ends of runs were observed for the full enzyme activity control, the negative control, and the positive control.

### 10.1.2 Battelle Intralaboratory Statistical Analysis

The full statistical analysis report and corresponding tables and graphs can be found in the appendices of the Battelle report (Reference 10-B). The following information was taken from the Summary and Conclusion section of the statistical analysis report.

Reference chemicals dibenz(a,h)anthracene and atrazine are non-inhibitors. The majority of concentration responses were above 80 percent of control.

For chrysin, the low threshold of responses was in the range of 17 percent and 25 percent. Bottom parameter estimates ranged from 17.370 to 21.550 for the three runs.

Among the four parameters of the concentration response curves (the baseline response (bottom), the maximum response (top), $IC_{50}$, and the slope), the top parameter had the most run-to-run variation. The other three parameters all had zero, close to zero, or minimal run-to-run variations.

Except for 4-nonylphenol (Run 1), the within-run variations were all close to zero for $\log_{10}IC_{50}$ and slope.

Except for background controls, the differences between the beginning and the end portions for the other three control types (full enzyme activity and positive and negative controls), when averaged across runs, were significant. The end portion was significantly lower than the beginning portion, which implied a reduction in aromatase activity between the beginning and the end of a run. For all the control types, chemical main effect and chemical by portion interaction effect were not significant.

Source of microsomes did not have a significant effect on the activities of the four controls (full enzyme activity, background activity, and positive and negative controls).

For econazole, the variation in run 1 for the full enzyme activity controls, the positive controls, and the negative controls was substantially larger and out of line with that for any run for all the chemicals.

For positive controls, the majority of variation was from run-to-run within chemicals.

### 10.1.3 In Vitro Intralaboratory Statistical Analysis

The full statistical analysis report and corresponding tables and graphs can be found in the appendices of the In Vitro report (Reference 10-C). The following information was taken from the Summary and Conclusion section of the statistical analysis report.

The reference chemicals dibenz(a,h)anthracene and atrazine are non-inhibitors. With only a few exceptions, the majority of concentration responses were close to or above 80 percent of control.

Most of the reference chemicals exhibited run-to-run variation in one or more characteristics of the concentration response curve fits. These included:

- Aminoglutethimide**:** The lower end of the concentration response curve for run 1 did not exhibit a threshold. This resulted in inflation in the estimate and variability of the average bottom parameter.
- Chrysin**:** The top, bottom, and slope parameters varied somewhat across runs.
- Dicofol: The bottom threshold was particularly variable among runs and to a lesser extent the top threshold. This resulted in variation of the $IC_{50}$ estimates across runs since they estimated different percentiles ((B+ T)I2) of the response distributions and introduced bias into their comparisons.
- Econazole: The run 1 concentration response curve was out of line with those from the other runs.

- Ketoconazole: The top threshold from run 1 differed somewhat from those of the other runs. This inflated the variability of the average top threshold.
- 4-Nonylphenol: The top threshold for run 2 was out of line with and higher than those from the other two runs. This inflated the variability of the overall average estimate.
- Prochloraz: The concentration response curve for run 4 was well out of line with the response curves for the other runs. The top threshold and the slope were particularly different.

Econazole is a strong aromatase inhibitor. The bottom thresholds of the concentration response distributions extended below a concentration $10^{-7}$ M. The concentration response distributions exhibit inhibition and do not attain their top thresholds even at concentration $10^{-10}$ M.

Dicofol is a weak inhibitor. The concentration response curves maintain their top thresholds to approximately a concentration of $10^{-6}$ M and do not approach their bottom threshold even at a concentration of $10^{-4}$ M. The average percent of control at a concentration of $10^{-4}$ M varies from 33 percent to 52 percent across runs.

The behavior of the full enzyme activity controls and the background activity controls was for the most part (except for a couple of isolated runs) consistent across chemicals and runs. The activity at the beginning of the run was greater than the activity at the end of the run. The full enzyme activity control portion effect (end minus beginning) averaged across runs was significant.

The behavior of the positive controls was for the most part (except for an isolated run, fenarimol run 1) consistent across chemicals and runs. For the microsomes prepared at Battelle the activity at the end of the run was greater than the activity at the beginning of the run. For the microsomes prepared at In Vitro the activity at the end and at the beginning of the run was more similar.

The behavior of the negative controls was for the most part (except for an isolated run, fenarimol run 1) consistent across chemicals and runs. For the microsomes prepared at Battelle the activity at the end of the run was considerably greater than the activity at the beginning of the run. For the microsomes prepared at In Vitro, the activity at the end of the run was greater than the activity at the beginning of the run but the average difference was less than for the microsomes prepared at Battelle.

The source of microsomes fixed effect and the chemical by portion interaction fixed effect were not significant for full enzyme activity, background activity, and positive controls. However, the source of microsomes fixed effect and the chemical by portion interaction fixed effect were significant for the negative controls. Test substances whose microsomes were prepared at In Vitro had significantly higher average negative control values than test substances whose microsomes were prepared at Battelle. This is due to the considerably lower activity at the beginning of each run for the microsomes prepared at Battelle, as compared to the microsomes prepared at In Vitro.

### 10.1.4 WIL Intralaboratory Statistical Analysis

The full statistical analysis report and corresponding tables and graphs can be found in the appendices of the WIL report (Reference10-D). Since the WIL report did not include a summary of the intralaboratory statistical analysis results, Battelle prepared the following summary from the information that was presented by WIL:

The results for WIL were similar to those for RTI in most part. The dose response model curve fits were not achievable for, chemicals 5 and 10, dibenz(a,h)anthracene (DBA) and atrazine (ATZ) due to their low aromatase inhibition. ECZ and PCZ (chemicals 7 and 3) were relatively strong inhibitors. The estimated values for the loglOIC50 were -8.78 for ECZ and -7.60 for PCZ, and ranged from -6.390 to -4.193 for the six remaining chemicals. The deepest slopes were observed in NYP (-1.952) and CYN (-1.258), and the slowest slope was observed in DCF (-0.639). The slopes for the rest of the chemicals were around -1.0. Chrysin (chemical 8) had a relatively high bottom threshold. The bottoms were -10.38, -6.70 and -6.47 for DCF, PCZ and FRM respectively, 13.30 for CYN, and around zero (between -1.11 to 0.44) for the other four chemicals. The top were all around 100 (ranging from 90.23 to 103.82). There was very little variance in the $IC_{50}$, slope, top and bottom estimates over the three runs for any of the eight positive reference chemicals. Significantly decreasing control activity between the beginnings and the ends of runs were observed for the full enzyme activity controls (P=0.002), the negative controls (P=0.001) and the positive controls (P=0.012).

## 10.2 Intralaboratory Statistics (Recombinant)

The intralaboratory analyses from each laboratory for background activity, full enzyme activity, and negative and positive controls, $IC_{50}$s, and slopes are provided in the appendices of the individual laboratory reports. Due to the integrated manner in which the intralaboratory statistical analysis reports were presented (References 12, 12-A through 12-D), as well as the interrelationships among the endpoints, it was decided to present the salient information from the intralaboratory statistical analysis reports in a single section rather than attempt to extract information about individual endpoints and incorporate them into their previously presented respective sections.

### 10.2.1 RTI Intralaboratory Statistical Analysis.

The full statistical analysis report and corresponding tables and graphs can be found in the appendices of the RTI report (Reference 12-A). Since the RTI report did not include a summary of the intralaboratory statistical analysis results, Battelle prepared the following summary from the information that was presented by RTI:

Concentration response relations could not be determined for dibenz(a,h)anthracene and for atrazine due to the low aromatase inhibition. These two chemicals were categorized as non-inhibitors. Among the other eight chemicals, the estimated logioIC50s were: -8.60 for econazole, -7.48 for prochloraz, -6.28 for aminoglutethimide, and from -5.66 to -4.03 for the remainder. Thus econazole, prochloraz, and to a lesser extent aminoglutethimide were relatively strong inhibitors. The most negative slope was observed for 4-nonylphenol (-2.26). The slopes for the

other chemicals were about - 1.0. The bottom thresholds were close to zero, except for chrysin (22.58). The top thresholds were about 91 to 100 for all eight chemicals. There was little variance in the $IC_{50}$, slope, top and bottom estimates over the three runs for any of the eight chemicals for which concentration response relations were fitted. Aromatase activity decreased significantly between the beginnings and the ends of runs for the full enzyme activity, the negative, and the positive controls.

### 10.2.2 Battelle Intralaboratory Statistical Analysis

The full statistical analysis report and corresponding tables and graphs can be found in the appendices of the Battelle report (Reference 12-B). The following information was taken from the Summary and Conclusion section of the statistical analysis report.

Dibenz(a,h)anthracene and atrazine were non-inhibitors. For dibenz(a,h) anthracene all responses were above 90 percent of control. For atrazine all but two responses were above 80 percent of control. The other two responses were at 78.65 and 77.95 percent of control.

For chrysin the low threshold of responses was in the range of 18 percent to 26 percent.

Among the four parameters, the top parameter had the most run-to-run variation. The other three parameters had run-to-run variation that was small or close to zero.

The overall standard errors of the mean of the weighted average estimates of the $\log_{10}IC_{50}$ and the slope were close to zero.

For 4-nonylphenol - slope; chrysin $-\log_{10}IC_{50}$ and slope; and dicofol - slope; the standard errors were several times larger than for the other chemicals.

For the full enzyme activity, positive, and negative controls the differences between the beginning and the end portions, when averaged across runs, were significant. The end portion was significantly lower than the beginning portion. This implies a reduction in aromatase activity between the beginning and the end of a run.

For all the control types, the control averages did not differ significantly across chemicals and the portion effects (i.e., end minus beginning) did not differ significantly across chemicals.

For econazole the variation in run 1 for the full enzyme activity controls, the positive controls, and the negative controls was substantially larger and out of line with that for any run for all the chemicals.

For the positive control the majority of variation was from run-to-run within chemicals.

There is potential for ambiguity in the $\log_{10}IC_{50}$ estimate. The $\log_{10}IC_{50}$ concentration from the standard nonlinear regression model, in fact, is the $EC_{50}$ that corresponds to the (B+ T)/2 percentile, which for some chemicals (e.g., chrysin) deviate somewhat from 50 percent. Such variation in percent inhibition can inflate the run-to-run variation among the $\log_{10}IC_{50}$ estimates.

An alternative (nonstandard) parameterization of the model should be adopted in which the $\log_{10}IC_{50}$ concentration corresponds to the 50th percentile, irrespective of the top and bottom thresholds.

### 10.2.3 In Vitro Intralaboratory Statistical Analysis

The full statistical analysis report and corresponding tables and graphs can be found in the appendices of the In Vitro report (Reference 12-C). The following information was taken from the Summary and Conclusion section of the statistical analysis report.

Dibenz(a,h)anthracene and atrazine were non-inhibitors. For dibenz(a,h)anthracene all responses but one (59.4 percent) were above 79 percent of control. For atrazine all responses were above 80 percent of control.

Among the four concentration response curve parameters the top threshold had the most run-to-run variation within chemicals.

For most of the reference chemicals there was considerable run-to-run
variation within chemicals for at least some of the concentration response curve parameters:

- Aminoglutethimide - Top
- Ketoconazole - Top
- Prochloraz - Top, $IC_{50}$, Slope
- Fenarimol- Bottom, Top
- Econazole - Top
- Chrysin - Bottom, Top, Slope
- Dicofol - Bottom, Top

For dicofol the bottom threshold was in the range 28 percent to 50 percent.
The overall standard errors of the mean of the weighted average estimates of the $\log_{10}IC_{50}$ and the slope were small.

For the full enzyme activity controls the differences between the beginning and the end portions, when averaged across runs, were significant. The end portion was significantly lower than the beginning portion. This implies a reduction in aromatase activity between the beginning and the end of a run. For the negative control the differences between the beginning and the end portions, when averaged across runs, approached significance ($p=0.054$). The end portion was significantly higher than the beginning portion. This implies an increase in aromatase activity between the beginning and the end of a run. It is not understood why there is a difference in the direction of change of activity between the negative controls and the full enzyme activity controls.

For the background activity controls and the positive controls the differences between the beginning and the end portions, when averaged across runs, were not significant.
For all control types the average control results did not vary significantly across chemicals. The portion effects within chemicals also did not vary significantly across chemicals.

There is some ambiguity in the $log_{10}IC_{50}$ estimate. The $log_{10}IC_{50}$ concentration from the standard nonlinear regression model, in fact, is the $EC_{50}$ that corresponds to the (B+ T)/2 percentile, which for some chemicals (e.g., fenarimol- (B+T)/2 = 58.l, 45.5, 55.7) deviates somewhat from 50 percent. Such variation in percent inhibition can inflate the run-to-run variation among the $log_{10}IC_{50}$ estimates. An alternative (nonstandard) parameterization of the model should be adopted in which the $log_{10}IC_{50}$ concentration corresponds to the 50th percentile, irrespective of the top and bottom thresholds.

Variation in aromatase activity during the course of a run can result in potential bias or potential loss of precision due to decrease or increase of aromatase activity within runs. If the inhibition concentration tubes are tested in order of inhibitor concentration, the change in aromatase activity across the run can accentuate or can lessen the apparent inhibition, depending on the ordering of the reference chemical concentrations. If the 24 tubes (8 inhibitor concentrations x 3 replicates per concentration) were tested in randomized order then the change in aromatase activity within the run would increase the residual variation about the fitted concentration response curves for each run but would avoid resulting in biased response curves.

### 10.2.4 WIL Intralaboratory Statistical Analysis

The full statistical analysis report and corresponding tables and graphs can be found in the appendices of the WIL report (Reference 12-D). Since the WIL report did not include a summary of the intralaboratory statistical analysis results, Battelle prepared the following summary from the information that was presented by WIL:

Concentration response relations could not be determined for dibenz(a,h) anthracene and atrazine due to their low aromatase inhibition. Econazole, prochloraz, and ketoconazole were relatively strong inhibitors. The estimated logio$IC_{50}$s for these chemicals were -8.63 for econazole, -7.04 for prochloraz, and -6.39 for ketoconazole. For the other chemicals the logioIC50s ranged from -5.48 to -4.19. The most negative slope was observed for 4-nonylphenol (-1.87) and the least negative slope was observed for dicofol (-0.73). The slopes for the other chemicals were distributed around -1.0. Chrysin had a relatively high bottom threshold, l2.79. For the other chemicals the bottom thresholds varied between -6.00 and 5.73. The top thresholds ranged from 94.l9 to l06.25.

Prochloraz showed some variation among the three runs for the $log_{10}IC_{50}$ and the bottom threshold parameters. Fenarimol showed some variation among the three runs for the $log_{10}IC_{50}$, the top threshold, and the bottom threshold parameters. For the other chemicals there was little variation in the $IC_{50}$, slope, top and bottom threshold parameter estimates over the three runs.

Aromatase activity decreased significantly between the beginnings and the ends of runs for the full enzyme activity, the negative, and the positive controls.

### 10.3 Placental Assay: Concentration Response Relations of $Log_{10}IC_{50}$, Slope, Top and Bottom

Convergence problems prevented successful concentration response curve fits for the two non-inhibitor chemicals dibenz(a,h)anthracene (DBA) and atrazine (ATZ) in all four intralaboratory analyses of the individual laboratory data. Therefore, the interlaboratory analyses of the concentration response curve parameters were performed only for the eight positive chemicals.

The mixed model analysis of variance comparisons among the laboratories accounted for potential systematic differences between the two sources of microsomes. Table 10.3-1 displays the estimated $\log_{10}IC_{50}$ and slope within each laboratory and the associated 95 percent confidence intervals about the $\log_{10}IC_{50}$ and slope for each chemical.

Table 10.3-2 also displays the mean difference between the two sources of microsomes and the overall mean values across laboratories and their associated 95 percent confidence intervals, incorporating among laboratory variation based on the random effects analysis of variance and heterogeneous within laboratory variation. The estimated CV s and their associated 95 percent confidence intervals for the overall means for the $\log_{10}IC_{50}$ and the slope parameters are also presented in Table 10.3-1.

Table 10.3-2 displays the within-laboratory variances and their associated degrees of freedom for each laboratory for the $\log_{10}IC_{50}$ and the slope parameters. These are the squares of the within laboratory standard errors associated with the estimated parameter values. Table 10.3-2 also displays the among laboratory component of random variation, the p-values associated with the tests that the laboratory variation is zero, and the squares of the standard errors of the overall mean values, as well as their associated degrees of freedom. The ratios of the among laboratory variances to the unweighted average within laboratory variances are also displayed, with their associated 95 percent confidence intervals.

Similar statistics for the top and the bottom parameters are displayed in the Interlaboratory Statistical Analysis Report Appendix to Reference 10 (Tables A-1 and A-2 and Figures A-1 though A-16). Note that the CV was not calculated for the bottom parameter since its distribution straddles zero.

The estimated $\log_{10}IC_{50}$ with the Battelle microsomes was significantly lower than that with In Vitro microsomes for 4-nonylphenol (NYP), but this difference was small (-0.028) compared to the mean estimated $\log_{10}IC_{50}$ (-4.583). No other significant differences between the two sources of microsomes were observed for either the $\log_{10}IC_{50}$ or the slope (Table 10.3-1). The estimated top threshold for ketoconazole was significantly lower for the Battelle microsomes that for the In Vitro microsomes. No other significant differences between the two sources of microsomes were observed for either the top or bottom thresholds.

The estimates for $\log_{10}IC_{50}$ (Table 10.3-1) were similar among the four laboratories for most chemicals except for econazole (ECZ). For econazole, the estimated $\log_{10}IC_{50}$ for In Vitro was smaller than for any of the other three laboratories and the associated 95 percent confidence interval for In Vitro did not overlap with those for the other three laboratories. The CVs for $IC_{50}$

exceeded 22.5 percent for econazole and for chrysin and 47.3 percent for dicofol. They were less than 14.7 percent for the $IC_{50}$s for the other chemicals (Table 10.3-1). The ratios of the among laboratory variance to the average within laboratory variance were 6.8 for econazole, 4.6 for chrysin, 2.3 for prochloraz, and less than 0.05 for the remaining five chemicals (Table 10.3-2). The large CV for dicofol (47.4 percent, Table 10.3-1) was caused by the relatively large within laboratory variance. The pooled within laboratory variance was 0.045 for dicofol, compared to less than or equal to 0.002 for the other seven chemicals. For chrysin, the estimated $\log_{10}IC_{50}$ for RTI was smaller than those for the other three laboratories, which was the principal reason for the relatively large CV and among laboratory variance for this chemical.

The estimates for slope (Table 10.3-1) were similar among the four laboratories for most chemicals except for econazole (ECZ). For econazole, the estimated slope for In Vitro was less negative than the other three laboratories and the associated 95 percent confidence interval did not overlap with those for the other three laboratories. For 4-nonylphenol the In Vitro slope was more negative than those at the other laboratories. The ratios of the among laboratory variances to the pooled within laboratory variance were 9.0 for econazole and zero for the seven other chemicals (Table 10.3-2). The CVs for the slope were 10.2 percent for chrysin, 15.2 percent for dicofol, and less than or equal to 8.8 percent for the other chemicals (Table 10.3-1). The relatively large CVs for chrysin and for dicofol were due to the relatively large within laboratory variances for these chemicals. The pooled within laboratory variances were 0.024 for dicofol, 0.021 for chrysin, 0.013 for 4-nonylphenol, and less than or equal to 0.004 for the other chemicals. Note that for 4-nonylphenol (NP), the estimated slope for In Vitro was more negative than for the other three laboratories (-2.48 compared to the closest one at -1.95) (Table 10.3-1). However for 4-nonylphenol (N), the within-laboratory variance for In Vitro was at least 3 times of the other three laboratories, and therefore less weight was given to In Vitro results when combining the intralaboratory results (Table 10.3-2).

The estimates for the top were similar for the four laboratories. The CVs for the top were less than 3 percent for each of the chemicals. The among laboratory variances to the pooled within laboratory variance ratios were 2.3 for 4-nonylphenol, 0.7 for dicofol, and zero for the rest. Some laboratories had very large within laboratory variances relatively to the others for some chemicals, however there were no consistent patterns for any laboratory. The estimated bottom thresholds for In Vitro were higher than those for the other three laboratories for 4-nonylphenol and for dicofol. The associated 95 percent 4-nonylphenol confidence interval for In Vitro did not overlap with those for the other three laboratories. The ratios of the among laboratory variances to the pooled within laboratory variance ratios were 12.6 for 4-nonylphenol and 5.6 for chrysin, and zero for the other chemicals. Some laboratories had very large within laboratory variances relatively to the others for some chemicals, however there were no consistent patterns for any laboratory.

**Control Activity: Differences Between the Beginning and the End Portions and Differences Between the Two Sources of Microsomes.** Table 10.3-3 displays the estimated parameter values and the associated within laboratory 95 percent confidence intervals for the differences between the beginning and the end, and for the differences between the two sources of microsomes. It also displays the overall mean differences across laboratories and their

associated 95 percent confidence intervals, incorporating among laboratory variation based on the random effects analysis of variance.

Table 10.3-4 displays the within laboratory variances and their associated degrees of freedom for each laboratory. These are the squares of the within laboratory standard errors associated with the estimated parameter values. Table 10.3-4 also displays the laboratory to laboratory random variation and the p-values, and the squares of the standard errors of the overall mean values, as well as their associated degrees of freedom. The ratios of the among laboratory variances to the unweighted average within laboratory variances are also displayed, with their associated 95 percent confidence intervals.

The following results were observed: For the background activity controls, there were no significant differences between the beginnings and the ends of runs. For the full enzyme activity control, the end portion was statistically significantly lower than the beginning portion for each individual laboratory and on average across laboratories.

The estimated variance among the laboratories was zero. For the negative controls, the result for In Vitro was different than those from any of the other three laboratories. The end portion was significantly higher than the beginning portion for In Vitro, but significantly lower than the beginning for each of the other three laboratories. As a result of this inconsistency, the estimated variance among the laboratories was more than 16 times higher than the average within-laboratory variance. The two portions on average across laboratories did not differ significantly. For the positive controls, the result for In Vitro was different from the other three laboratories. The end portion was (nearly significantly) higher than the beginning portion for In Vitro, but significantly lower than the beginning for each of the other three laboratories. As a result of this disagreement, the estimated variance among the laboratories was more than six times higher than the average within laboratory variance. The two portions did not differ significantly on average across laboratories.

Comparisons between the microsomes prepared at Battelle and those prepared at In Vitro were carried out for the negative and the positive controls. For the negative controls the microsomes prepared at Battelle were significantly lower than the microsomes prepared at In Vitro for RTI and In Vitro and on average across laboratories. For the positive controls the microsomes prepared at Battelle were not significantly different than the microsomes prepared at In Vitro for any of the individual laboratories or on average across all the laboratories. The estimated variance among the laboratories was zero for the negative and positive controls. Note that the difference between the two sources of microsomes at In Vitro was larger in absolute value for both the negative and the positive controls.

**Table 10.3-1. Parameter Estimates and the 95 percent Confidence Intervals for $Log_{10}IC_{50}$ and Slope Parameter of Concentration Response Curves for Placental Assay. Estimated by Chemical**

| Chemical[5] | Param | Estimate and 95 percent Confidence Interval | | | | | | CV( percent) and 95 percent CI[4] |
|---|---|---|---|---|---|---|---|---|
| | | RTI[1] | Battelle[1] | WIL[1] | In Vitro[1] | Difference Between Two Microsome Sources[3] (Battelle – In Vitro) | Overall[2] | |
| AG | Log_IC50 | -5.395(-5.429,-5.361) | -5.310(-5.501,-5.119) | -5.390(-5.506,-5.274) | -5.407(-5.651,-5.163) | 0.014(-0.050,0.078) | -5.391(-5.422,-5.359) | 5.765(3.621,13.865) |
| AG | Slope | -0.913(-0.977,-0.850) | -0.971(-1.006,-0.936) | -1.026(-1.237,-0.815) | -1.007(-1.650,-0.364) | 0.059(-0.042,0.160) | -0.987(-1.037,-0.936) | 3.751(2.268,10.388) |
| KCZ | Log_IC50 | -5.171(-5.200,-5.141) | -5.166(-5.199,-5.133) | -5.193(-5.270,-5.116) | -5.205(-5.316,-5.094) | 0.026(-0.015,0.067) | -5.181(-5.202,-5.161) | 4.616(3.667,6.230) |
| KCZ | Slope | -0.966(-1.029,-0.904) | -1.011(-1.113,-0.909) | -0.963(-1.221,-0.705) | -0.858(-1.073,-0.643) | -0.042(-0.161,0.077) | -0.957(-1.017,-0.898) | 6.017(4.680,8.439) |
| PCZ | Log_IC50 | -7.698(-7.776,-7.621) | -7.577(-7.794,-7.360) | -7.597(-7.683,-7.511) | -7.723(-7.779,-7.667) | -0.012(-0.183,0.159) | -7.651(-7.736,-7.566) | 14.682(8.903,41.008) |
| PCZ | Slope | -0.966(-1.006,-0.926) | -0.961(-1.013,-0.909) | -0.971(-1.139,-0.803) | -0.983(-1.079,-0.887) | -0.013(-0.083,0.057) | -0.970(-1.004,-0.935) | 3.317(2.398,5.381) |
| NYP | Log_IC50 | -4.604(-4.621,-4.586) | -4.562(-4.595,-4.529) | -4.579(-4.639,-4.519) | -4.574(-4.600,-4.548) | -0.028(-0.054,-0.002) | -4.583(-4.596,-4.570) | 2.845(2.181,4.092) |
| NYP | Slope | -1.949(-2.064,-1.833) | -1.655(-1.872,-1.438) | -1.952(-2.219,-1.685) | -2.479(-2.855,-2.103) | -0.096(-0.295,0.104) | -1.902(-2.002,-1.803) | 5.225(4.357,6.534) |
| FRM | Log_IC50 | -5.227(-5.275,-5.180) | -5.218(-5.240,-5.196) | -5.145(-5.244,-5.046) | -5.373(-5.716,-5.030) | 0.051(-0.031,0.133) | -5.194(-5.235,-5.153) | 5.504(3.038,23.906) |
| FRM | Slope | -0.931(-0.986,-0.877) | -0.990(-1.112,-0.868) | -1.034(-1.241,-0.827) | -1.021(-1.158,-0.884) | -0.076(-0.168,0.016) | -0.992(-1.038,-0.946) | 4.434(3.363,6.516) |
| ECZ | Log_IC50 | -8.823(-8.859,-8.788) | -8.624(-8.835,-8.413) | -8.776(-8.823,-8.729) | -9.055(-9.150,-8.960) | -0.219(-0.490,0.052) | -8.818(-8.953,-8.682) | 22.545(13.342,72.698) |
| ECZ | Slope | -1.054(-1.090,-1.018) | -1.039(-1.091,-0.987) | -1.094(-1.150,-1.038) | -0.797(-0.887,-0.707) | 0.128(-0.127,0.384) | -1.003(-1.131,-0.875) | 8.824(5.181,27.943) |
| CYN | Log_IC50 | -5.767(-5.839,-5.695) | -5.670(-5.739,-5.601) | -5.503(-5.645,-5.361) | -5.543(-5.724,-5.362) | -0.081(-0.354,0.191) | -5.627(-5.763,-5.490) | 22.583(13.335,73.684) |
| CYN | Slope | -1.394(-1.533,-1.255) | -1.386(-2.369,-0.403) | -1.258(-1.934,-0.582) | -1.082(-1.430,-0.734) | -0.028(-0.356,0.299) | -1.324(-1.487,-1.160) | 10.199(6.580,22.526) |
| DCF | Log_IC50 | -4.379(-4.824,-3.934) | -4.626(-4.927,-4.325) | -4.334(-4.954,-3.714) | -4.935(-5.626,-4.244) | -0.015(-0.477,0.447) | -4.549(-4.780,-4.318) | 47.367(30.420,114.548) |
| DCF | Slope | -0.658(-0.882,-0.434) | -0.983(-1.904,-0.062) | -0.639(-0.820,-0.458) | -0.830(-1.404,-0.256) | 0.039(-0.203,0.282) | -0.673(-0.795,-0.552) | 15.242(9.950,32.692) |

1. The estimates and 95 percent confidence intervals are based on the intralaboratory analyses for the four participating laboratories. The intralaboratory analyses were performed by individual chemical.
2. The overall estimates and confidence intervals in this table were estimated using a mixed effects analysis of variance, with microsome source as a fixed effect and laboratory as a random effect, and with heterogeneous variances among the four laboratories. The variances for each laboratory were specified as the squares of the within laboratory standard errors. The degrees of freedom were reported in Table 10.3-2.
3. Microsomes were prepared by Battelle and In Vitro laboratories and distributed to the four laboratories. The differences in average responses between the two sources of microsomes were estimated based on the model fit, accounting for among laboratory variation and heterogeneous within laboratory variation. The degrees of freedom are same as those for the overall means.
4. CV was calculated for $IC_{50}$ and the slope parameters based on the average results. CVs associated with $\log_{10}IC_{50}$ are actually CVs on the $IC_{50}$s.
5. Concentration response relations were not fitted for dibenz [a,h]anthracene and for atrazine, since they resulted in no aromatase inhibition.

**Table 10.3-2. Variance Components and Ratio of Between and Within Laboratories Variances for $Log_{10}IC_{50}$ and Slope Parameter of Concentration Response Curves for Placental Assay. Estimated by Chemical**

| Chemical[7] | Param | Within Laboratory Variance[1] | | | | | Among Laboratory Variance[3] and (p-value) (df=3) | Mean Variance[4, 5] | Ratio and 95 percent CI[6] |
|---|---|---|---|---|---|---|---|---|---|
| | | RTI | Battelle | WIL | In Vitro | Pooled Results[2] | | | |
| AG | Log_IC50 | 0.000/df=16.0 | 0.002/df=1.8 | 0.001/df=2.0 | 0.001/df=1.2 | 0.001/df=5.2 | 0.000 (1) | 0.000/df=5.2 | 0.000 (0.000, 0.000) |
| AG | Slope | 0.001/df=16.0 | 0.000/df=32.4 | 0.002/df=2.0 | 0.002/df=1.0 | 0.001/df=4.2 | 0.000 (1) | 0.000/df=4.2 | 0.000 (0.000, 0.000) |
| KCZ | Log_IC50 | 0.000/df=16.0 | 0.000/df=20.1 | 0.000/df=2.0 | 0.003/df=20.0 | 0.001/df=28.4 | 0.000 (1) | 0.000/df=28.4 | 0.000 (0.000, 0.000) |
| KCZ | Slope | 0.001/df=16.0 | 0.002/df=14.7 | 0.004/df=2.0 | 0.011/df=20.0 | 0.004/df=24.1 | 0.000 (1) | 0.001/df=24.1 | 0.000 (0.000, 0.000) |
| PCZ | Log_IC50 | 0.001/df=16.0 | 0.003/df=2.0 | 0.000/df=2.0 | 0.001/df=20.0 | 0.001/df=7.3 | 0.003 (0.14617) | 0.001/df=4.3 | 2.272 (0.397, 33.164) |
| PCZ | Slope | 0.000/df=16.0 | 0.000/df=2.9 | 0.002/df=2.0 | 0.002/df=20.0 | 0.001/df=12.8 | 0.000 (1) | 0.000/df=12.8 | 0.000 (0.000, 0.000) |
| NYP | Log_IC50 | 0.000/df=16.0 | 0.000/df=20.6 | 0.000/df=2.0 | 0.000/df=44.3 | 0.000/df=20.4 | 0.000 (1) | 0.000/df=20.4 | 0.000 (0.000, 0.000) |
| NYP | Slope | 0.003/df=16.0 | 0.011/df=20.2 | 0.004/df=2.0 | 0.034/df=30.1 | 0.013/df=51.2 | 0.000 (1) | 0.002/df=51.2 | 0.000 (0.000, 0.000) |
| FRM | Log_IC50 | 0.000/df=16.0 | 0.000/df=56.9 | 0.001/df=2.0 | 0.004/df=1.7 | 0.001/df=2.6 | 0.000 (1) | 0.000/df=2.6 | 0.000 (0.000, 0.000) |
| FRM | Slope | 0.001/df=16.0 | 0.001/df=2.4 | 0.002/df=2.0 | 0.004/df=29.5 | 0.002/df=18.9 | 0.000 (1) | 0.000/df=18.9 | 0.000 (0.000, 0.000) |
| ECZ | Log_IC50 | 0.000/df=16.0 | 0.002/df=1.9 | 0.000/df=2.0 | 0.002/df=43.5 | 0.001/df=8.7 | 0.008 (0.11037) | 0.002/df=3.9 | 6.843 (1.324, 99.158) |
| ECZ | Slope | 0.000/df=16.0 | 0.001/df=49.9 | 0.000/df=2.0 | 0.002/df=56.8 | 0.001/df=99.1 | 0.007 (0.11215) | 0.002/df=3.6 | 8.965 (2.758, 125.120) |
| CYN | Log_IC50 | 0.001/df=16.0 | 0.001/df=42.9 | 0.001/df=2.0 | 0.003/df=3.1 | 0.002/df=10.6 | 0.008 (0.12552) | 0.002/df=3.9 | 4.576 (0.972, 65.867) |
| CYN | Slope | 0.004/df=16.0 | 0.036/df=1.7 | 0.025/df=2.0 | 0.020/df=5.6 | 0.021/df=6.2 | 0.000 (1) | 0.005/df=6.2 | 0.000 (0.000, 0.000) |
| DCF | Log_IC50 | 0.044/df=16.0 | 0.005/df=2.0 | 0.021/df=2.0 | 0.110/df=20.8 | 0.045/df=34.8 | 0.002 (0.46330) | 0.010/df=7.0 | 0.047 (0.013, 0.654) |
| DCF | Slope | 0.011/df=16.0 | 0.039/df=1.9 | 0.002/df=2.0 | 0.043/df=4.1 | 0.024/df=7.0 | 0.000 (1) | 0.003/df=7.0 | 0.000 (0.000, 0.000) |

1. The within laboratory variance for each laboratory is the square of the standard error associated with parameter estimate, which was reported in the intralaboratory analyses for each of the four participating laboratories.
2. Pooled average for the within laboratory variances is the unweighted average of the within laboratory variances among the four laboratories. Associated degrees of freedom were based on Satterthwaite's approximation
3. Variance among laboratories is based on a mixed effects analysis of variance model with heterogeneous variances among the four laboratories, equal to the squares of the within laboratory standard errors.
4. Mean variance is the square of the standard error of the pooled weighted mean value. It includes both within and among laboratory variation.
5. Degrees of freedom for the (mean) overall effect variance were estimated as $2*((1/K)*\sum(S_L^2 + S_i^2))^2/(var(S_L^2)+(2/K^2)*\sum(S_i^4/df_i))$, where $S_L^2$ is the among laboratory variance, $S_i^2$ and $df_i$ are the reported variance and degrees of freedom for laboratory i, $var(S_L^2)$ is the variance of $S_L^2$, and K is the number of laboratories (Hartung and Makambi, 2001).
6. Ratio of the among-laboratory variance and the pooled average within laboratory variance.
7. Concentration response relations were not fitted for dibenz [a,h]anthracene and for atrazine, since they resulted in no aromatase inhibition.

**Table 10.3-3. Parameter Estimate and the 95 percent Confidence Interval for Differences Between Beginning and End (End Minus Beginning) and Between the Two Sources of Microsomes (Battelle minus In Vitro) for the Percent of Control Responses in the Placental Aromatase Assay.**

| Parameter | Estimate and 95 percent Confidence Interval | | | | |
|---|---|---|---|---|---|
| | RTI[1,2] | Battelle[1,2] | WIL[1,2] | In Vitro[1,2] | Mean[3] |
| **End – Beginning** | | | | | |
| Background Activity Control | 0.172 (-0.034, 0.378) | 0.062 (-0.118, 0.242) | 0.056 (-0.918, 1.030) | -0.407 (-0.921, 0.107) | 0.071 (-0.061, 0.203) |
| Full Enzyme Activity Control | -9.873 (-11.092, -8.654) | -10.444 (-14.975, -5.913) | -6.062 (-9.399, -2.725) | -10.016 (-12.854, -7.178) | -9.543 (-10.552, -8.535) |
| Negative Control | -11.212 (-12.541, -9.883) | -8.294 (-13.226, -3.362) | -9.995 (-15.102, -4.888) | 17.384 (7.898, 26.870) | -3.611 (-19.896, 12.674) |
| Positive Control | -4.913 (-6.122, -3.704) | -2.233 (-4.100, -0.366) | -3.740 (-6.484, -0.996) | 3.581 (-0.269, 7.431) | -2.086 (-6.538, 2.366) |
| **Battelle Microsomes – In Vitro Microsomes[4]** | | | | | |
| Negative Control | -1.446 (-2.775, -0.117) | -0.782 (-6.031, 4.467) | -2.216 (-8.358, 3.926) | -12.063 (-21.609, -2.517) | -1.636 (-2.858, -0.413) |
| Positive Control | 1.210 (-0.979, 3.399) | 1.109 (-1.949, 4.168) | -2.318 (-6.793, 2.158) | -4.905 (-13.509, 3.699) | 0.343 (-1.311, 1.996) |

1. The estimates and 95 percent confidence intervals are based on the intralaboratory analyses for the four participating laboratories.
2. The results from the four laboratories are listed in Appendix E in the individual laboratory report.
3. The overall (mean) effects and confidence intervals in this table were estimated using a one-way random effects analysis of variance, with laboratory as a random effect, and with heterogeneous variances among the four laboratories. The variances for each laboratory were specified as the squares of the within laboratory standard errors.
4. Two microsomes were prepared by Battelle and In Vitro laboratories and distributed to the four laboratories. Results for the difference between the two sources of microsomes within each laboratory were listed in Appendix E in the individual laboratory report.

**Table 10.3-4. Variance Components and Ratio of among and Within Laboratories Variances for Differences between Beginning and End and between the Two Sources of Microsomes by Battelle and In Vitro for the Percent of Control Responses for Placental Aromatase Assay**

| Parameter | Within Laboratory Variance[1] | | | | | Among Laboratory Variance[3] and (p-value) (df=3) | Mean Variance[4,5] | Ratio and 95 percent CI[6] |
|---|---|---|---|---|---|---|---|---|
| | RTI | Battelle | WIL | In Vitro | Pooled Results[2] | | | |
| **End – Beginning** | | | | | | | | |
| Background Activity Control | 0.010/df=20.0 | 0.008/df=61.8 | 0.200/df=12.0 | 0.052/df=9.3 | 0.067/df=20.1 | 0.000 (1) | 0.004/df=20.1 | 0.000 (0.000, 0.000) |
| Full Enzyme Activity Control | 0.341/df=20.0 | 3.375/df=5.8 | 2.346/df=12.0 | 1.880/df=22.7 | 1.985/df=24.4 | 0.000 (1) | 0.239/df=24.4 | 0.000 (0.000, 0.000) |
| Negative Control | 0.406/df=20.0 | 4.360/df=7.0 | 5.495/df=12.0 | 18.173/df=10.1 | 7.108/df=21.3 | 117.867 (0.10411) | 31.159/df=3.6 | 16.581 (4.356, 234.648) |
| Positive Control | 0.336/df=20.0 | 0.585/df=6.1 | 1.586/df=12.0 | 2.676/df=7.2 | 1.296/df=21.1 | 7.813 (0.12999) | 2.255/df=3.4 | 6.029 (1.581, 85.332) |
| **Battelle Microsome – In Vitro Microsome[7]** | | | | | | | | |
| Negative Control | 0.406/df=20.0 | 5.328/df=8.7 | 7.946/df=12.0 | 18.770/df=10.9 | 8.112/df=25.8 | 0.000 (1) | 0.353/df=25.8 | 0.000 (0.000, 0.000) |
| Positive Control | 1.101/df=20.0 | 1.981/df=12.3 | 4.219/df=12.0 | 9.641/df=4.0 | 4.235/df=11.5 | 0.000 (1) | 0.570/df=11.5 | 0.000 (0.000, 0.000) |

1. The within laboratory variance for each laboratory is the square of the standard error associated with the parameter estimate, as reported in the intralaboratory analyses for the four participating laboratories (Appendix E of the individual laboratory report).
2. Pooled average for the within laboratory variances is the unweighted average of the within laboratory variances among the four laboratories. Associated degrees of freedom were based on Satterthwaite's approximation
3. Among laboratories variance is based on a one-way random effects analysis of variance model with heterogeneous within laboratory variances, equal to the squares of the within laboratory standard errors.
4. Mean variance is the square of the standard error of the pooled weighted mean value. It includes both within and among laboratory variation.
5. Degrees of freedom for the (mean) overall effect variance were estimated as $2*((1/K)*\sum(S_L^2 + S_i^2))^2/(var(S_L^2)+(2/K^2)*\sum(S_i^4/df_i))$, where $S_L^2$ is the among laboratory variance, $S_i^2$ and $df_i$ are the reported variance and degrees of freedom for laboratory i, $var(S_L^2)$ is the variance of $S_L^2$, and K is the number of laboratories (Hartung and Makambi, 2001).
6. Ratio of the among-laboratory variance and the pooled average within laboratory variance.

Microsomes were prepared by Battelle and In Vitro laboratories and distributed to the four laboratories. Results for the difference between the two sources of microsomes within each laboratory were listed in Appendix E of the individual laboratory report.

## 10.4 Recombinant Assay: Concentration Response Relations of $Log_{10}IC_{50}$, Slope, Top and Bottom

Convergence problems prevented successful concentration response relation fits for the two non-inhibitor chemicals dibenz(a,h)anthracene and atrazine in all four intralaboratory analyses of the individual laboratory data. Therefore the interlaboratory analyses for the concentration response relation parameters were restricted to the other eight chemicals.

Table 10.4.-1 displays the estimated $\log_{10}IC_{50}$ and the slope within each laboratory and the associated 95 percent confidence intervals for each chemical[2]. This table also displays the overall mean values across laboratories and their associated 95 percent confidence intervals, incorporating among laboratory variation based on the random effects analysis of variance. Each figure includes a reference line corresponding to the overall average. The estimated CV s and their associated 95 percent confidence intervals for the overall means for the $\log_{10}IC_{50}$ and the slope parameters are also presented in Table 10.4.-1.

Table 10.4.-2 displays the within laboratory variances and their associated degrees of freedom for each laboratory for the logioIC50 and the slope parameters[3]. These are the squares of the within laboratory standard errors associated with the estimated parameter values. Table 10.4.-2 also displays the laboratory-to-laboratory random variations, the p-values associated with the test that the among-laboratory variation is zero, and the squares of the standard errors of the overall mean values, as well as their associated degrees of freedom. The ratios of the among laboratory variances to the unweighted average within laboratory variances are also displayed, with their associated 95 percent confidence intervals.

Similar statistics for the top and the bottom parameters are displayed in the appendix of the Interlaboratory Statistical Analysis Report (Appendix E of Reference 12-E). Notice that CV was not calculated for the bottom parameter since its distribution straddles zero.

The estimates for $\log_{10}IC_{50}$ (Table 10.4.-1) were generally consistent among the four laboratories for most chemicals. The In Vitro estimates were slightly lower than those for the other three laboratories for ketoconazole, econazole, and dicofol. The ratios of the among-laboratory variance to the pooled within-laboratory variance were relatively large for dicofol (9.5), ketoconazole (6.4), and econazole (4.9) and were less than 2.7 for the other chemicals (Table 10.4.-2). The CVs for the IC50 were 39.5 percent for ketoconazole, 70.6 percent for dicofol, 26.9 percent for chrysin, 29.2 for prochloraz, and between 11.4 and 18.3 percent for the other chemicals (Table 10.4.-1). All the CVs exceeded 10 percent.

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[2] The confidence intervals in Table 10.3-1 were calculated for the interlaboratory analysis based on the least squares means, standard errors, and degrees of freedom reported in the intra laboratory analyses within each laboratory. The confidence intervals in Table 10.3-1 thus may differ in the low significant digits from those displayed in the intralaboratory analysis reports due to round off error in intermediate calculations.

[3] Degrees of freedom within laboratories (Table 10.3-2) for Battelle and In Vitro were based on those specified in the intralaboratory analysis reports. Degrees of freedom for WIL Laboratories were based on the number of replicates (3) minus 1. Two degrees of freedom was assigned. The degrees of freedom for R TI were based on an analysis of variance model for all four chemicals combined. There were 24 observations (8 chemicals x 3 replicates per chemical) and 8 effects estimated, leaving 16 degrees of freedom for residual.

The slope estimates (Table 10.4-1) were similar among the four laboratories for most chemicals. The In Vitro slope estimates were more negative than those for the other three laboratories for ketoconazole and dicofol and were less negative than those for the other three laboratories for 4-nonylphenol and econazole. The slope CVs were 28.l percent for dicofol, 14.5 percent for 4-nonylphenol, 7.4 percent for econazole, and less than 6.l percent for the other five chemicals (Table 10.4-1). The ratios of the among laboratory variance to the pooled within laboratory variance were 2.5 or less for all of the chemicals (Table 10.3-2).

The estimates for the top thresholds varied between 85 and 111 percent. They were, in general, consistent among the four laboratories for most chemicals except for aminoglutethimide. The estimated top threshold for In Vitro was higher than the other three laboratories for aminoglutethimide (110.3 compared to 99 percent or less). The top threshold CVs were 6.0 percent for aminoglutethimide and less than 5.4 percent for the other seven chemicals. The ratios of the among laboratory variances to the pooled within laboratory variance ratios were 7.L for aminoglutethimide and 3.9 or less for the other seven chemicals.

The estimated bottom thresholds were between approximately -2.5 and 11 for all chemicals except for chrysin and dicofol. The bottom thresholds were between 12.8 and 22.6 percent for chrysin, and consistent among laboratories. In Vitro had a bottom threshold of 4 1.1 percent for dicofol, which was inconsistent with those of the other three laboratories (-6.0 to 2.1 percent). The 95 percent confidence interval for In Vitro did not overlap with those for the other three laboratories. The ratios of the among laboratory variances to the pooled within laboratory variance ratios were 21.4 for dicofol and less than 1.4 for any of the other seven chemicals.

**Control Activity: Differences Between the Beginning and the End Portions.** Table 10.4-3 displays the estimated parameter values and the associated within laboratory 95 percent confidence intervals for the differences between the beginning and the end portions[4]. It also displays the overall mean differences across laboratories and their associated 95 percent confidence intervals, incorporating among laboratory variation based on the random effects analysis of variance.

Table 10.4-3 displays the within laboratory variances and their associated degrees of freedom for each laboratory. These are the squares of the within laboratory standard errors associated with the estimated parameter values. Table 10.4-3 also displays the laboratory-to-laboratory random variation and the p-values associated with their significance, and the squares of the standard errors of the overall mean values, as well as their associated degrees of freedom. The ratios of the among laboratory variances to the unweighted average within laboratory variances are also displayed, with their associated 95 percent confidence intervals.

The following results were observed:

- For the background activity controls, there were no differences between the end and the beginning of runs. The among laboratory variance was zero.

[4] The confidence intervals are based on the least squares means, standard errors, and degrees of freedom shown in Appendix E of Reference 12, which in turn are based on those reported in the intralaboratory analyses

- For the full enzyme activity controls, the end portion was statistically significantly lower than the beginning portion for each individual laboratory and on average across laboratories. The difference between the two portions for RTI was more than two times those for the other laboratories. The estimated variance among the laboratories was about eight times the pooled within laboratory variance.

- For the negative controls, the result for In Vitro was different from the other three laboratories. The end portion was (nearly significantly) higher than the beginning portion for In Vitro, but statistically significantly lower than the beginning for the other three laboratories. As a result of this disagreement, the estimated variance among the laboratories was about 4.6 times of the average within laboratory variance. The two portions did not differ significantly on average across laboratories.

- For the positive controls, the result for In Vitro differed slightly from the other three laboratories. The end portion was slightly higher than the beginning portion for In Vitro, but statistically significantly lower than the beginning portion for the other laboratories, and on average across laboratories. The estimated variance among laboratories was about 1.6 times that of the pooled average within laboratory variance.

**Table 10.4-1. Parameter estimates and 95 percent confidence intervals for the $Log_{10}IC_{50}$ and slope parameters of the concentration response relations for the recombinant aromatase assay. Estimated by chemical.**

| Chemical[4] | Parameter | Estimate and 95 Percent Confidence Interval | | | | | CV( percent) and 95 percent CI[3] |
|---|---|---|---|---|---|---|---|
| | | RTI[1] | Battelle[1] | WIL[1] | In Vitro[2] | Overall[2] | |
| AG | Log_IC50 | -5.279(-5.358,-5.201) | -5.250(-5.449,-5.051) | -5.385(-5.759,-5.011) | -5.393(-5.441,-5.345) | -5.325(-5.411,-5.239) | 15.647(9.763,39.077) |
| AG | Slope | -0.980(-1.108,-0.853) | -0.987(-1.039,-0.935) | -0.970(-1.086,-0.854) | -0.939(-1.017,-0.861) | -0.972(-1.005,-0.939) | 3.345(2.726,4.329) |
| KCZ | Log_IC50 | -5.031(-5.111,-4.952) | -5.097(-5.147,-5.047) | -5.185(-5.503,-4.867) | -5.492(-5.852,-5.132) | -5.186(-5.428,-4.943) | 39.467(22.510,185.087) |
| KCZ | Slope | -0.937(-1.037,-0.836) | -0.943(-1.064,-0.822) | -0.916(-1.032,-0.800) | -1.248(-1.641,-0.855) | -0.934(-0.986,-0.881) | 4.592(2.955,10.168) |
| PCZ | Log_IC50 | -7.481(-7.520,-7.443) | -7.503(-7.787,-7.219) | -7.040(-9.226,-4.854) | -7.704(-7.742,-7.666) | -7.562(-7.772,-7.353) | 29.237(16.024,174.277) |
| PCZ | Slope | -0.973(-1.048,-0.899) | -0.981(-1.079,-0.883) | -0.991(-1.137,-0.845) | -0.957(-1.068,-0.846) | -0.979(-1.019,-0.938) | 4.029(3.231,5.354) |
| NYP | Log_IC50 | -4.666(-4.702,-4.630) | -4.721(-4.772,-4.670) | -4.782(-4.902,-4.662) | -4.781(-4.854,-4.708) | -4.731(-4.798,-4.665) | 11.409(6.913,31.719) |
| NYP | Slope | -2.264(-2.620,-1.907) | -2.095(-2.355,-1.835) | -1.874(-2.747,-1.001) | -1.576(-1.738,-1.414) | -1.930(-2.295,-1.565) | 14.548(8.941,38.504) |
| FRM | Log_IC50 | -5.195(-5.243,-5.147) | -5.269(-5.297,-5.241) | -4.717(-6.636,-2.798) | -5.452(-6.101,-4.803) | -5.242(-5.334,-5.150) | 11.832(6.432,58.984) |
| FRM | Slope | -1.023(-1.094,-0.951) | -0.954(-1.008,-0.900) | -1.069(-1.280,-0.858) | -0.952(-1.031,-0.873) | -0.990(-1.053,-0.926) | 4.320(2.514,14.136) |
| ECZ | Log_IC50 | -8.604(-8.641,-8.566) | -8.630(-8.888,-8.372) | -8.625(-8.694,-8.556) | -8.786(-8.818,-8.754) | -8.664(-8.768,-8.561) | 18.347(11.300,48.541) |
| ECZ | Slope | -1.174(-1.262,-1.085) | -1.152(-1.475,-0.829) | -1.177(-1.272,-1.082) | -0.998(-1.056,-0.940) | -1.121(-1.228,-1.014) | 7.418(4.622,18.391) |
| CYN | Log_IC50 | -5.663(-5.736,-5.590) | -5.862(-6.340,-5.384) | -5.481(-5.593,-5.369) | -5.599(-5.751,-5.447) | -5.616(-5.773,-5.458) | 26.852(15.982,85.968) |
| CYN | Slope | -1.304(-1.485,-1.123) | -1.074(-1.648,-0.500) | -1.132(-1.304,-0.960) | -1.209(-2.029,-0.389) | -1.158(-1.231,-1.086) | 6.052(4.711,8.475) |
| DCF | Log_IC50 | -4.446(-4.516,-4.376) | -4.762(-4.958,-4.566) | -4.421(-4.778,-4.064) | -5.151(-5.582,-4.720) | -4.676(-5.081,-4.272) | 70.615(38.533,776.709) |
| DCF | Slope | -0.974(-1.158,-0.791) | -1.113(-1.565,-0.661) | -0.728(-0.883,-0.573) | -2.179(-3.062,-1.296) | -1.024(-1.511,-0.536) | 28.065(15.240,159.525) |

1. The estimates and 95 percent confidence intervals are based on the intralaboratory analyses for the four participating laboratories. The intralaboratory analyses were performed by individual chemical.
2. The overall estimates and confidence intervals were estimated using a random effects analysis of variance, with laboratory as a random effect and with heterogeneous variances among the four laboratories. The variance for each laboratory was specified as the square of the within laboratory standard error. The degrees of freedom are given in Table 36.
3. CV was calculated for the $IC_{50}$ and the slope parameters based on average results. CVs shown as associated with $log_{10}IC_{50}$ are actually CVs of the $IC_{50}$s.
4. Concentration response relations were not fitted for dibenz[a,h]anthracene and for atrazine, since they resulted in very little aromatase inhibition (i.e. they were noninhibitors).

**Table 10.4-2. Variance components and ratio of between and within laboratories variances for the $Log_{10}IC_{50}$ and slope parameter of the concentration response relations for the recombinant aromatase assay. Estimated by chemical.**

| Chemical[7] | Parameter | Within Laboratory Variance[1] | | | | | Among Laboratory Variance[3] and (p-value) (df=3) | Mean Variance[4,5] | Ratio and 95 percent CI[6] |
|---|---|---|---|---|---|---|---|---|---|
| | | RTI | Battelle | WIL | In Vitro | Pooled Results[2] | | | |
| AG | Log_IC50 | 0.001/df=16.0 | 0.002/df=2.0 | 0.008/df=2.0 | 0.001/df=44.3 | 0.003/df=4.4 | 0.003 (0.17921) | 0.001/df=5.1 | 0.896 (0.100, 13.458) |
| AG | Slope | 0.004/df=16.0 | 0.001/df=56.1 | 0.001/df=2.0 | 0.002/df=53.2 | 0.002/cf=37.7 | 0.000 (1.000) | 0.000/df=37.7 | 0.000 (0.000, 0.000) |
| KCZ | Log_IC50 | 0.001/df=16.0 | 0.001/df=20.0 | 0.005/df=2.0 | 0.007/df=2.1 | 0.004/df=5.3 | 0.024 (0.12436) | 0.007/df=3.5 | 6.431 (0.874, 95.409) |
| KCZ | Slope | 0.002/df=16.0 | 0.003/df=20.6 | 0.001/df=2.0 | 0.016/df=3.1 | 0.005/df=6.0 | 0.000 (1.000) | 0.000/df=6.0 | 0.000 (0.000, 0.000) |
| PCZ | Log_IC50 | 0.000/df=16.0 | 0.005/df=2.2 | 0.258/df=2.0 | 0.000/df=20.0 | 0.066/df=2.1 | 0.010 (0.12884) | 0.004/df=2.7 | 0.155 (0.004, 2.471) |
| PCZ | Slope | 0.001/df=16.0 | 0.002/df=8.6 | 0.001/df=2.0 | 0.003/df=20.0 | 0.002/cf=31.9 | 0.000 (1.000) | 0.000/df=31.9 | 0.000 (0.000, 0.000) |
| NYP | Log_IC50 | 0.000/df=16.0 | 0.000/df=5.1 | 0.001/df=2.0 | 0.001/df=19.8 | 0.001/cf=17.3 | 0.002 (0.14043) | 0.001/df=4.3 | 2.694 (0.675, 38.267) |
| NYP | Slope | 0.028/df=16.0 | 0.017/df=44.2 | 0.041/df=2.0 | 0.006/df=26.8 | 0.023/df=9.4 | 0.058 (0.12870) | 0.020/df=4.8 | 2.505 (0.504, 36.191) |
| FRM | Log_IC50 | 0.001/df=16.0 | 0.000/df=44.9 | 0.199/df=2.0 | 0.019/df=1.8 | 0.055/df=2.4 | 0.001 (0.24440) | 0.001/df=2.5 | 0.019 (0.001, 0.295) |
| FRM | Slope | 0.001/df=16.0 | 0.001/df=46.2 | 0.002/df=2.0 | 0.001/df=20.6 | 0.001/cf=10.6 | 0.001 (0.34020) | 0.000/df=3.5 | 0.401 (0.085, 5.776) |
| ECZ | Log_IC50 | 0.000/df=16.0 | 0.004/df=2.0 | 0.000/df=2.0 | 0.000/df=47.2 | 0.001/df=3.0 | 0.005 (0.09468) | 0.002/df=4.8 | 4.873 (0.317, 75.225) |
| ECZ | Slope | 0.002/df=16.0 | 0.006/df=2.0 | 0.000/df=2.0 | 0.001/df=43.5 | 0.002/df=4.7 | 0.005 (0.12045) | 0.002/df=5.0 | 2.389 (0.287, 35.709) |
| CYN | Log_IC50 | 0.001/df=16.0 | 0.015/df=2.2 | 0.001/df=2.0 | 0.005/df=20.0 | 0.006/df=4.8 | 0.009 (0.16991) | 0.003/df=4.1 | 1.670 (0.206, 24.922) |
| CYN | Slope | 0.007/df=16.0 | 0.022/df=2.3 | 0.002/df=2.0 | 0.154/df=20.0 | 0.046/cf=24.3 | 0.000 (1.000) | 0.001/df=24.3 | 0.000 (0.000, 0.000) |
| DCF | Log_IC50 | 0.001/df=16.0 | 0.003/df=2.5 | 0.007/df=2.0 | 0.018/df=3.0 | 0.007/df=6.2 | 0.070 (0.11404) | 0.019/df=3.5 | 9.519 (1.473, 140.080) |
| DCF | Slope | 0.007/df=16.0 | 0.024/df=3.6 | 0.001/df=2.0 | 0.190/df=37.0 | 0.056/cf=43.4 | 0.058 (0.27558) | 0.021/df=2.7 | 1.038 (0.302, 14.554) |

1. The within laboratory variance for each laboratory is the square of the standard error associated with the parameter estimate, which was reported in the intralaboratory analyses for each of the four participating laboratories.
2. Pooled average for the within laboratory variances is the unweighted average of the within laboratory variances among the four laboratories. Associated degrees of freedom were based on Satterthwaite's approximation
3. Variance among laboratories is based on a random effects analysis of variance model with heterogeneous variances among the individual laboratories equal to the squares of the within laboratory standard errors. P-values are associated with the test that the among laboratory variation is zero.
4. Mean variance is the square of the standard error of the pooled weighted mean value. It includes both within and among laboratory variation.
5. Degrees of freedom for the (mean) overall effect variance were estimated as $2*((1/K)*\sum(S_L^2 + S_i^2)^2/(var(S_L^2)+(2/K^2)*\sum(S_i^4/df_i))$, where $S_L^2$ is the among laboratory variance, $S_i^2$ and $df_i$ are the reported variance and degrees of freedom for laboratory i, $var(S_L^2)$ is the variance of $S_L^2$, and K is the number of laboratories (Hartung and Makambi, 2001).
6. Ratio of the among-laboratory variance to the pooled average within laboratory variance.
7. Concentration response relations were not fitted for dibenz[a,h]anthracene and for atrazine, since they resulted in very little aromatase inhibition (i.e. they were noninhibitors).

**Table 10.4-3. Variance components and ratio of among and within laboratories variances for differences between the beginning and the end portions for the percent of control responses for the recombinant aromatase assay.**

| Parameter | Estimate and 95 Percent Confidence Interval | | | | |
|---|---|---|---|---|---|
| | RTI[1,2] | Battelle[1,2] | WIL[1,2] | In Vitro[1,2] | Mean[3] |
| Background Activity Control | 0.007 (-0.017, 0.031) | -0.159 (-0.405, 0.087) | 0.040 (-0.083, 0.163) | -0.049 (-0.193, 0.095) | 0.005 (-0.017, 0.027) |
| Full Enzyme Activity Control | -14.225 (-16.008, -12.442) | -5.878 (-8.381, -3.375) | -5.244 (-7.189, -3.299) | -6.354 (-10.330, -2.378) | -8.032 (-13.160, -2.903) |
| Negative Control | -8.049 (-10.727, -5.372) | -4.115 (-6.300, -1.930) | -6.467 (-9.558, -3.376) | 7.479 (-0.152, 15.110) | -3.732 (-12.321, 4.858) |
| Positive Control | -4.407 (-5.527, -3.287) | -2.795 (-4.246, -1.344) | -4.214 (-5.366, -3.061) | 0.619 (-2.587, 3.825) | -3.250 (-5.945, -0.555) |

1. The estimates and 95 percent confidence intervals are based on the intralaboratory analyses for the four participating laboratories.
2. The results from the four laboratories are given in Appendix E.
3. The overall (mean) effects and confidence intervals were estimated using a one-way random effects analysis of variance, with laboratory as a random effect, and with heterogeneous variances among the individual laboratories. The variances for each laboratory were specified as the squares of the within laboratory standard errors.

## 10.5 Comparison of the Performance of the Placental and Recombinant Assays

The data generated in the interlaboratory validation studies of the placental and recombinant versions of the microsomal aromatase assays shows that the two assays are comparable for the purpose of identifying chemicals that inhibit aromatase and measuring their log $IC_{50}$s. The agreement between the two methods gives added confidence in their relevance and reliability. For the 10 reference chemicals in the interlaboratory validation studies, both assays correctly identified the inhibitors and non-inhibitors in all laboratories.

**Table 10.5-1. Placental Aromatase Assay: Classification Based on 3 Runs for Each Chemical**

| Chemical | RTI | Battelle | In Vitro | WIL |
|---|---|---|---|---|
| **Aminoglutethimide** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Ketoconazole** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Prochloraz** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **4-Nonylphenol** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Dibenz[a,h]anthracene** | Non-Inhibitor | Non-Inhibitor | Non-Inhibitor | Non-Inhibitor |
| **Fenarimol** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Econazole** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Chrysin** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Dicofol** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Atrazine** | Non-Inhibitor | Non-inhibitor | Non-inhibitor | Non-inhibitor |

**Table 10.5-2. Recombinant Aromatase Assay: Classification Based on 3 Runs for Each Chemical**

| Chemical | RTI | Battelle | In Vitro | WIL |
|---|---|---|---|---|
| **Aminoglutethimide** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Ketoconazole** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Prochloraz** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **4-Nonylphenol** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Dibenz[a,h]anthracene** | Non-Inhibitor | Non-Inhibitor | Non-Inhibitor | Non-Inhibitor |
| **Fenarimol** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Econazole** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Chrysin** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Dicofol** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Atrazine** | Non-inhibitor | Non-inhibitor | Non-inhibitor | Non-inhibitor |

For the 10 reference chemicals, the recombinant assay showed slightly greater variability and, therefore, broader 95 percent confidence intervals of the mean log IC50s than the placental assay. As shown in Table 10.5-3, in all but one case, the 95 percent confidence bands of the log IC50s overlapped, and in five cases the 95% confidence intervals of the log IC50s of the placental assay lay within the 95% confidence intervals of the recombinant assay. For nonylphenol the

mean log $IC_{50}$ of the recombinant assay (-4.731) was only slightly lower than that of the placental assay (-4.583).

**Table 10.5-3. 95% Confidence Limits for Log $IC_{50}$s**

| | Placental | | Recombinant | | Relative Position |
|---|---|---|---|---|---|
| **Chemical** | Lower Limit | Upper Limit | Lower Limit | Upper Limit | |
| Aminoglutethimide | -5.422 | -5.359 | -5.411 | -5.239 | Overlap |
| Ketoconazole | -5.202 | -5.161 | -5.428 | -4.943 | Placental Within |
| Prochloraz | -7.736 | -7.566 | -7.772 | -7.353 | Placental Within |
| Nonylphenol | -4.596 | -4.570 | -4.798 | -4.665 | Outside |
| Fenarimol | -5.235 | -5.153 | -5.334 | -5.150 | Placental Within |
| Econazole | -8.953 | -8.682 | -8.768 | -8.561 | Overlap |
| Chrysin | -5.763 | -5.490 | -5.773 | -5.458 | Placental Within |
| Dicofol | -4.549 | -4.318 | -5.081 | -4.272 | Placental Within |

## 10.6 Investigation of the Binding Characteristics of Nonylphenol

Nonylphenol was tested in early pre-validation studies in the range of $10^{-9}$ to $10^{-5}$ M. At this concentration range, nonylphenol did not inhibit aromatase and was regarded as a non-inhibitor which agreed with the results obtained in the KGN cell assay (Morinaga, 2004). During the later interlaboratory studies (Section 9.9), the concentration range was extended to $10^{-3}$ M. At these higher concentrations nonylphenol appeared to be an inhibitor but exhibited a steeper than normal slope. Because of these unexpected results, EPA decided to investigate whether or not nonylphenol was a true competitive inhibitor or denatured the enzyme at the higher concentrations used in the interlaboratory studies. The investigative approach was to conduct Ki determinations with recombinant aromatase in which the concentration of the substrate is varied in each of a series of tests, each test using different fixed concentration of the inhibitor. In addition to nonylphenol, aminoglutethimide was used as a positive control since it is a known competitive inhibitor of aromatase.

A pilot and four additional runs of the assay were conducted using aminoglutethimide as the inhibitor at concentrations of 0, 25, 50 and 100 μM. The study design, measured aromatase activity, and calculations are presented in the report "Characterization of the Inhibition of Aromatase by Nonylphenol" (Reference 13). These studies demonstrated the ability of the laboratory to conduct the assays. The mean calculated $K_m$ was 50.6 nM, which is in the range of values reported in the literature, and the mean $V_{max}$ was 0.320 nmol/mg/min. The $K_i$ for AG calculated using the SNLR method was 1.62 μM, which falls within the range of values (ca. 0.7 – 2.7 μM) reported in the literature (Brueggemeier, et al., 2005; Kao et al, 2001).

A pilot and four additional runs of the assay were conducted using nonylphenol as the inhibitor at concentrations of 0, 7.5, 15 and 20 μM. There were errors in the preparation of the inhibitor dilutions in run 1, so these data were excluded from the summary data. The results for the SNLR analysis are summarized in Table 10.6-1 and are presented in Figures 10.6-1 and 10.6-2. The full data are presented in Reference 13. The mean calculated $K_m$ was 37.1 nM, which is in the

range of values reported in the literature, and the mean $V_{max}$ was 0.334 nmol/mg/min. The $K_i$ for nonylphenol calculated using this method was 6.83 μM, which is near the estimate for $K_i$ of 10 μM determined based on $IC_{50}$ data from WA 4-17, Task 4.

**Table 10.6-1. Nonylphenol: kinetic parameters calculated by SNLR**

| Run | $K_m$[a] | $V_{max}$[b] | $K_i$[c] |
|---|---|---|---|
| 2 | 39.1 | 0.374 | 6.10 |
| 3 | 42.4 | 0.383 | 11.79 |
| 4 | 35.1 | 0.296 | 8.01 |
| **Mean** | **38.9** | **0.351** | **8.63** |
| **SEM** | **2.1** | **0.028** | **1.67** |

[a]nM
[b]nmol ASDN metabolized/mg protein/min
[c]μM

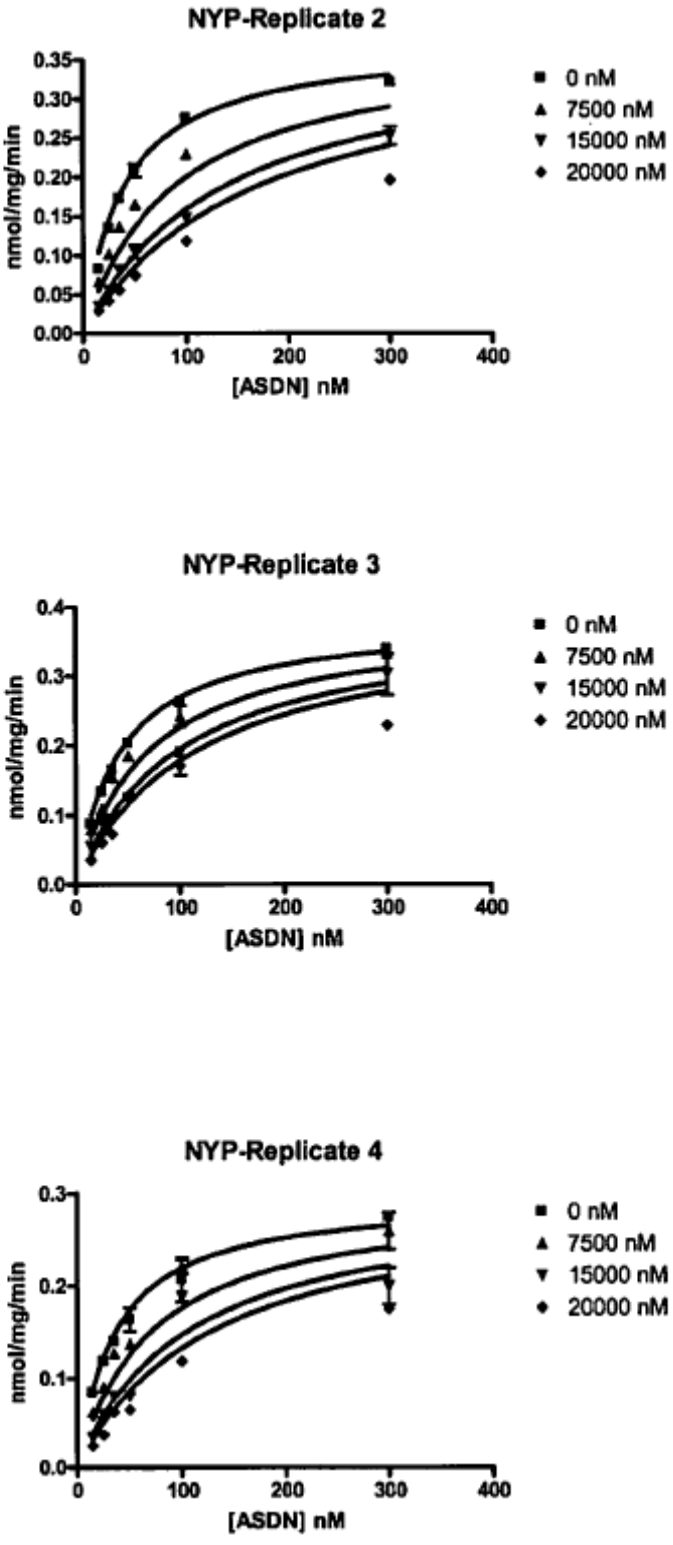


**Figure 10.6-1. Runs 1-4 of reaction velocity curves-- ASDN substrate in the presence of nonylphenol**

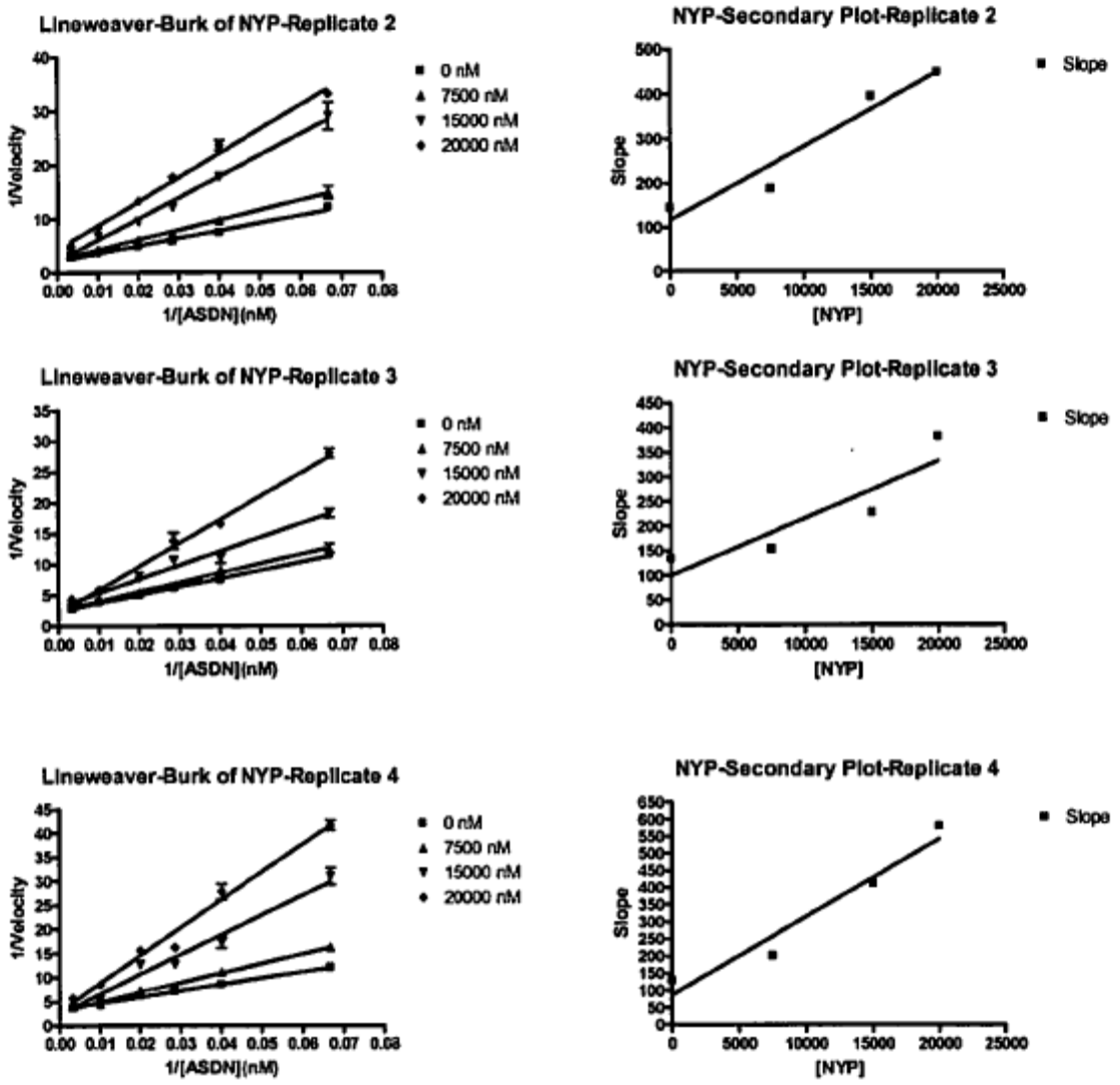


**Figure 10.6-2. Lineweaver Burke plots and secondary plots of nonylphenol**

Visual examination of the Lineweaver-Burk and secondary plots (Figure 10.6-2) indicates that the inhibition is primarily competitive, as evidenced by the common y-intercept for the lines on the Lineweaver-Burk plots and the linear relationship between the slopes of the Lineweaver-Burk plot and the inhibitor concentration (shown graphically on the secondary plots). The correlation coefficients for the secondary plots range from 0.817 to 0.994 and may be indicative of a small contribution of another inhibition type to the interaction of NYP and aromatase.

The mean $K_m$ and $V_{max}$ estimated (Table 10.6-1) from the Lineweaver-Burk plot (from the inverses of the x- and y-intercepts, respectively, of the control runs) were 51.35 nM and 0.376 nmol/mg/min. The mean $K_i$ (extrapolated from the secondary plot as the negative of the x-intercept), was 5.11 μM. The values for $K_m$, $V_{max}$ and $K_i$ estimated from the plots are in good agreement with those found through SNLR methods.

In conclusion, the data obtained indicate that NYP acts primarily as a competitive inhibitor of recombinant aromatase although the relationship between the slopes of the Lineweaver-Burk plot and the inhibitor concentration (shown graphically on the secondary plots) may not be strictly linear which may be indicative of a small contribution of another inhibition type to the interaction of NYP and aromatase

# 11.0 Performance Criteria and Data Interpretation Procedure

## 11.1 Performance Criteria for Controls

The development of performance criteria is one of the key outputs of a validation program. Performance criteria provide feedback to the testing laboratory and ensure reviewers of the data that the assay was conducted properly.

Based on activity levels reported by the laboratories in the validation study, EPA has established performance criteria for the full activity control and the background controls as shown in Table 11.1-1. These values will guarantee that the assay will have an acceptable dynamic range to detect both strong and weak inhibitors.

**Table 11.1-1. Full Activity and Background Control Criteria**

| | Parameter | Value |
|---|---|---|
| **Full Activity Control** | Minimum Activity | 0.100 nmol/mg/min |
| **Background Control** | Maximum Activity | 1% of full activity control |

For the positive control, 4-hydroxyandrostenedione, EPA considered setting performance criteria on several different variables including top, bottom, log $IC_{50}$, slope, $R^2$, width of confidence intervals, variance, and standard error. After considerable analysis, EPA chose to specify performance criteria for four parameters: top, bottom, slope, and log $IC_{50}$. EPA did not consider a precision criterion for the positive control as adding any value.

EPA proposed a set of performance criteria on the basis of professional judgment. These were used in the testing of the 16 supplementary chemicals discussed in Chapter 12. Although these criteria proved to be appropriate and readily achievable in the lead laboratory's testing of the 16 supplementary chemicals, EPA decided that the performance criteria should probably be set on a more rigorous statistical basis.

From a statistical standpoint, the determination of performance criteria is a tolerance interval problem. A tolerance interval differs from a confidence interval in that the confidence interval bounds a population parameter (e.g. the mean) with specified confidence, whereas a tolerance interval bounds a percentile range that represents a specified proportion of the population. A confidence interval characterizes, for example, the average laboratory performance; whereas, a tolerance interval characterizes a range of laboratory responses from within the population.

Tolerance bounds (i.e. the upper and lower limits of the tolerance intervals) are based on a reference set of laboratories—the set of acceptable laboratories is considered to be a sample from the population of laboratories that can perform the assay satisfactorily, in this case the laboratories participating in the validation study. The variation observed among results within this sample represents the inherent assay variation expected in the future results of laboratories drawn from the population of "acceptable" laboratories, represented by the reference sample. Future results with variation within the observed range will be considered to be acceptable.

Variation of results in excess of the observed range will be considered excessive and an indicator of an unacceptable run. The range of acceptable variation is reflected in the tolerance bounds.

It is desired that results from a large proportion of laboratories from the population of acceptable laboratories be included within the intervals and that this probability be controlled. For this assay, tolerance intervals to contain 80 percent of the population with 95 percent confidence have been selected.[5] That is, using tolerance interval methodology, we are choosing performance criteria that we expect with 95 percent confidence that 80 percent of the laboratories will meet. These are rather commonly used

Tolerance intervals may either be two-sided or one-sided. Two-sided tolerance intervals are appropriate for "accuracy parameters" such as slope, top, bottom and $\log IC_{50}$ of the response curve fits. One-sided tolerance intervals (upper bounds) are appropriate for "precision parameters" such as residual variation from the response curve fits.

The determination of the tolerance intervals for the aromatase assay involves several "components of variation." Each laboratory in the reference set produced results in multiple tasks separated in time, in multiple runs within each task that were more closely bunched, and in variation within each run. Thus there were four components of variation:

- Lab to lab
- Task to task within lab
- Run to run within task and lab
- Within run.

Each of these components of variation needs to be accounted for in the determination of the tolerance intervals.

A non-linear regression to fit the model, described in Chapter 5, was applied to the positive control data for the aromatase assay. The tolerance intervals shown in Table 11.1-2 reflect the results of the laboratories in the validation program. There was excellent agreement between the proposed criteria based on professional judgment and these that were based on tolerance intervals.

For a run to be considered acceptable, the output from the model should fall within the following tolerance intervals although small deviations should not disqualify a run since they are based on a limited sample of laboratories and runs and may not be fully representative of the performance expected in the field. If deviations are substantial, the cause of the problem should be determined and the run repeated.

[5] Technically, a tolerance interval to contain 100p percent (e.g. 80 percent) with 100(1-α) percent (e.g. 95 percent) confidence will, with probability 100(1-α) percent, include 100p percent of the population from which the reference set of "acceptable" laboratories was drawn.

**Table 11.1-2 Performance Criteria (Tolerance Intervals) for the Positive Control**

| | Parameter | Lower limit | Upper Limit |
|---|---|---|---|
| **Positive Control** | **Slope** | -1.2 | -0.8 |
| | **Top (%)** | 90 | 110 |
| | **Bottom (%)** | -5 | +6 |
| | **Log $IC_{50}$** | -7.3 | -7.0 |

## 11.2 Performance Criteria for Test Chemicals

Recognizing that satisfactory performance on controls is a good indictor of acceptable test results but does not ensure the quality of an individual run, EPA examined two test chemical parameters, top and variability. Variability was analyzed in three separate ways: residual variance, coefficients of variation (CVs) for the percent of control values at each concentration of the test chemicals, and the mean CV across all concentrations. Data from all studies were used in determining residual variance. Data for the CVs came from the testing of the 16 chemicals discussed in Chapter 12.

The tolerance levels to contain at least 80 percent of the population of test runs at 95 percent confidence was a lower limit of 86 percent and an upper limit of 110 percent. However, tolerance interval for top was not included as a performance criterion because, unless extreme, low or high values in the top did not have much influence over the log $IC_{50}$. On the other hand, bottom did affect the curve and frequently the log $IC_{50}$s in a substantial way. However, bottom cannot be specified as a performance criterion because non-inhibitors will not fit the model and weak inhibitors may legitimately give partial curves. Choosing the high test concentrations has the greatest effect on the curve bottom; thus, the guidance is for the highest test concentration to be at $10^{-3}$ M unless precluded by solubility constraints or denaturation of the microsome. If either of these occurs, the protocol requires that the chemical be tested at $10^{-3.3}$ M and $10^{-4}$ after that.

Variability in the data points along the curve did give rise to misshapen curves which was more likely to affect log $IC_{50}$. A one-sided tolerance upper limit on within run $\log_e$ residual variance to contain at least 80 percent of the population of test runs at 95 percent was calculated to be 3.45. In a simpler approach, experimental data in Chapter 12 showed that most within run CVs at each concentration are often less than 10 percent but could range higher. This suggested that laboratories should examine their data carefully if CVs exceeded 15 percent. Alternatively for data fitting the model, one could examine the 95 percent confidence limit to determine data quality. The 95 percent upper and lower bounds should smoothly hug the curve. Deviations from this indicate variability in the data that may lead to erroneous interpretation and misclassification of a chemical.

Based upon the experience gained in the validation studies, EPA would caution test laboratories to examine data for outliers that might indicate experimental error, but the Agency is not establishing specific performance criterion for variability of test data at this time.

### 11.3 Data Interpretation Procedure

The original focus of data interpretation was the calculation of an $IC_{50}$ for a test substance. For this purpose laboratories were asked to determine whether curves were "complete" or "incomplete" and, if incomplete whether the log $IC_{50}$ was interpolated or extrapolated from the model. The determination of the completeness of the curve was important because it determined whether the simple form of the regression equation ( i.e., the $EC_{50}$ and $IC_{50}$ are equal) could be used or the more complex form that provides an accurate $IC_{50}$ for partial curves must be used. The interpolation determination meant that data at at least one concentration demonstrated that the chemical caused at least 50% inhibition; extrapolation meant that all data was above the 50% inhibition level. Extrapolation to obtain an $IC_{50}$ was a concern to EPA as the Agency did not believe this to be a sufficiently robust determination of inhibition. This concern led to an emphasis on ensuring that the data at the highest concentration produced at least 50% inhibition. The focus on the highest concentration and the recognition that the purpose of the assay was largely met through the qualitative determination that a substance was or was not an inhibitor and not necessarily on the calculation of an $IC_{50}$ led to the exploration of two other data interpretation procedures.

The second approach explored by EPA involved the inhibition curve fit by the model judged against the following criteria:

- If the four-parameter model can be fit to the data from some runs but not others, the data interpretation criteria will be based only on the average of those runs for which the model can be fit.
- If the four-parameter model cannot be fit to the data from any runs, the data interpretation criteria will be based on the average of all the runs.
- If the best fit curve crosses 50 percent, the chemical is an inhibitor.
- If the best fit curve at the highest usable concentration has a value between 50 percent and 75 percent, the chemical is equivocal.
- If the best fit curve has a value higher than 75 percent at the highest usable concentration, the chemical is a non-inhibitor.
- If no curve can be fit and the means of the runs at the highest concentration are above 75 percent, the chemical is a non-inhibitor.
- If no curve can be fit and the mean of the runs at the highest concentration is below 75 percent, the chemical is equivocal.

The third approach studied by EPA utilizes the 95 percent confidence interval generated by the four-parameter Hill equation as the estimator of binding. In this approach the regression model calculated upper and lower 95 percent confidence limits of the dose-response curve generates a confidence interval to which the following criteria are applied. If the test results for a run cannot be fitted by the four-parameter concentration response model discussed in Chapter 5, then one should determine the model prediction and its associated standard error and 95 percent confidence interval based only on the three responses at the highest test concentration:

- If the four-parameter model can be fit to the data from some runs but not others, the data interpretation criteria will be based only on the average those runs for which the model can be fit.
- If the four-parameter model cannot be fit to the data from any runs, the data interpretation criteria will be based on the average value at the highest concentration of all the runs.
- If the upper confidence bound of the model fit at the highest concentration is less than 50 percent (i.e., the confidence interval lies below 50 percent), the chemical will be classified as an "inhibitor."
- If the lower confidence bound of the model fit at the highest concentration or of the highest concentration (if a model cannot be fit) is greater than 50 percent, the chemical will be classified as a "non-inhibitor."
- If the CI of the model fit at the highest concentration or of the highest concentration (if the model cannot be fit) includes 50 percent as an interior point, the chemical will be classified as "equivocal."

With the exception of a reformulated version of the equivocal and non-inhibitor criteria (an equivocal was defined as chemicals with a CI falling between 50 percent and 75 percent with non-inhibitors having a CI greater than 75 percent), this approach was used by the lead laboratory, RTI, for the interpretation of the test results on the 16 chemicals described in Chapter 12.

For all approaches a method for integrating the results of the three different runs needed to be established. In one case, termed the "voting method," the overall result was based on agreement of two of the three runs. In the other case, where different results were obtained across the three runs, an arithmetic average was taken. A comparison of the different approaches shows identical results except for the two test results shown in Table 11.3-1.

**Table 11.3-1 Discrepancies Between the 95% CI and Inhibition Curve Data Interpretation Procedures for Two Chemicals**

| **Lab** | | 95% CI Voting | 95% CI Average | Inhibition Curve Voting | Inhib Curve Average |
|---|---|---|---|---|---|
| **In Vitro RTI** | Dicofol Genestein | Equivocal Non-Inhibitor | Inhibitor Non-Inhibitor | Inhibitor Equivocal | Inhibitor Equivocal |

The discrepancy in dicofol can be traced to the lowest test concentration used by In Vitro. The other three laboratories tested dicofol up to 10-3 M and obtained complete or nearly complete inhibition curves which would yield classification as an inhibitor by any of the four methods. The highest concentrations tested by In Vitro were $10^{-4.48}$ and $10^{-4}$ M. In addition, the standard deviations at both of these concentrations were large (14.21 and 8.34, respectively) giving rise to a substantial uncertainty at the lowest part of the curve. The mean percent of control values of the three runs for dicofol were 44.15, 41.57, and 28.59. The CI's for runs one and two straddle 50 percent whereas that for run three is clearly below 50 percent. Runs one and two would be termed equivocal on this basis and run three classified as an inhibitor giving an equivocal classification overall using the voting method. The 95 percent CI average approach would result

in a classification of an inhibitor equivocal for this chemical because the mean upper confidence bound is less than 50 percent.

Model convergence was observed for genestein, but the fits were poor for all runs as noted by R2 values that ranged from 0.59 to 0.86. Run 1 was run up to 10-3 M but solubility problems caused the other two runs to be made at a maximum concentration of 10-3.3 M. The percent of control for the highest concentration in Runs 2 and 3 was 72.47 and 74.09 respectively for a mean of 73.28. This placed it just across the dividing line between non-inhibitor and equivocal using the inhibition curve criterion which explains the discrepancy observed in the two methods. Although there is considerable appeal to the use of the confidence limits and it worked well in the supplementary studies conducted by RTI, EPA concluded that, without constraints being specified on data variability, poor quality data would give broad confidence intervals which could result in chemicals that are inhibitors being misclassified as equivocal.

Since approaches #2 and #3 performed comparably enough on the data sets generated in the validation program, the Agency opted for the simpler criteria involving the best fit curve. The criteria that apply, therefore, are listed in Table 11.3-2.

**Table 11.3-2 Adopted Data Interpretation Criteria**

| Criteria | | Classification |
|---|---|---|
| Data fit 4-parameter nonlinear regression model | Curve crosses 50% | Inhibitor |
| | Lower portion of curve is between 50% and 75% activity | Equivocal |
| Data do not fit the model | Data points at highest concentration range below 75% | |
| | Data points at the highest concentration are above 75% | Non-inhibitor |

# 12.0 Testing of an Additional 16 Chemicals with Recombinant Microsomes

## 12.1 Tested Reference Chemical Concentration Ranges

Three independent run assays were conducted for each reference chemical and all assays for a given reference chemical were conducted by the same technician. In cases where evidence of insolubility of the reference chemical was noted in the assay tubes of Run 1 or where different concentrations would better describe the concentration response curve, changes were made to the reference chemical concentrations tested in succeeding runs. Information regarding assay dates, substrate specific activity, and tested reference chemical concentration ranges is presented in Table 12.1-1.

**Table 12.1-1 Reference chemical assay dates and concentration ranges**

| Chemical Code | Test Chemical ID | Technician | Run | Assay Date | Substrate Soln SA µCi/µg ASDN | Reference Chemical Concentration Range (M) |
|---|---|---|---|---|---|---|
| CR11601 | Vinclozolin | 1 | 1 | 2/22/07 | 1.461 | 1.00E-03 to 1.00E-10 |
| | | | 2 | 2/27/07 | 1.220 | 1.00E-04 to 1.00E-10 |
| | | | 3 | 2/28/07 | 1.521 | 1.00E-04 to 1.00E-10 |
| CR11602 | Bisphenol A | 4 | 1* | 3/1/07 | 1.599 | 1.00E-03 to 1.00E-10 |
| | | | 4 | 3/9/07 | 1.503 | 1.00E-03 to 1.00E-06 |
| | | | 5 | 3/13/07 | 1.603 | 1.00E-03 to 1.00E-06 |
| CR11603 | Tributyltin | 2 | 1 | 3/1/07 | 1.570 | 1.00E-03 to 1.00E-10 |
| | | | 2 | 3/6/07 | 1.480 | 1.00E-03 to 1.00E-10 |
| | | | 3 | 3/7/07 | 1.478 | 1.00E-03 to 1.00E-10 |
| CR11604 | Diethylhexyl phthalate | 2 | 1 | 2/22/07 | 1.454 | 1.00E-03 to 1.00E-10 |
| | | | 2 | 2/27/07 | 1.509 | 1.00E-04 to 1.00E-10 |
| | | | 3 | 2/28/07 | 1.599 | 1.00E-04 to 1.00E-10 |
| CR11605 | Methoxychlor | 1 | 1 | 3/9/07 | 1.528 | 1.00E-03 to 1.00E-10 |
| | | | 2 | 3/13/07 | 1.585 | 1.00E-05 to 1.00E-10 |
| | | | 3 | 3/14/07 | 1.472 | 1.00E-05 to 1.00E-10 |
| CR11606 | Aldicarb | 2 | 1 | 3/9/07 | 1.514 | 1.00E-03 to 1.00E-10 |
| | | | 2 | 3/13/07 | 1.578 | 1.00E-03 to 1.00E-10 |
| | | | 3 | 3/14/07 | 1.572 | 1.00E-03 to 1.00E-10 |
| CR11607 | Flavone | 3 | 1 | 3/1/07 | 1.579 | 1.00E-03 to 1.00E-10 |
| | | | 2 | 3/6/07 | 1.580 | 2.50E-04 to 1.00E-07 |
| | | | 3 | 3/7/07 | 1.503 | 2.50E-04 to 1.00E-07 |
| CR11608 | Triadimefon | 1 | 1 | 3/16/07 | 1.529 | 1.00E-03 to 1.00E-10 |
| | | | 2 | 3/20/07 | 1.526 | 1.00E-04 to 1.00E-08 |
| | | | 3 | 3/21/07 | 1.513 | 1.00E-04 to 1.00E-08 |
| CR11609 | Imazalil | 1 | 1 | 3/22/07 | 1.476 | 1.00E-03 to 1.00E-10 |
| | | | 2 | 3/27/07 | 1.472 | 1.00E-05 to 1.00E-10 |
| | | | 3 | 3/28/07 | 1.515 | 1.00E-05 to 1.00E-10 |
| CR11610 | Apigenin | 2 | 1 | 3/22/07 | 1.551 | 1.00E-03 to 1.00E-10 |

| Chemical Code | Test Chemical ID | Technician | Run | Assay Date | Substrate Soln SA µCi/µg ASDN | Reference Chemical Concentration Range (M) |
|---|---|---|---|---|---|---|
| | | | 2 | 3/27/07 | 1.563 | 5.00E-05 to 1.00E-08 |
| | | | 3 | 3/28/07 | 1.561 | 5.00E-05 to 1.00E-08 |
| CR11611 | Ronidazole | 4 | 1 | 3/16/07 | 1.612 | 1.00E-03 to 1.00E-10 |
| | | | 2 | 3/20/07 | 1.492 | 1.00E-05 to 1.00E-10 |
| | | | 3 | 3/21/07 | 1.536 | 1.00E-05 to 1.00E-10 |
| CR11612 | Genistein | 3 | 1 | 3/22/07 | 1.494 | 1.00E-03 to 1.00E-10 |
| | | | 2 | 3/27/07 | 1.542 | 5.00E-04 to 1.00E-09 |
| | | | 3 | 3/28/07 | 1.535 | 5.00E-04 to 1.00E-09 |
| CR11613 | p,p'-DDE | 3 | 1 | 2/22/07 | 1.482 | 1.00E-03 to 1.00E-10 |
| | | | 2 | 2/27/07 | 1.500 | 1.00E-05 to 1.00E-10 |
| | | | 3 | 2/28/07 | 1.547 | 1.00E-05 to 1.00E-10 |
| CR11614 | Alachlor | 3 | 1 | 3/9/07 | 1.513 | 1.00E-03 to 1.00E-10 |
| | | | 2 | 3/13/07 | 1.736 | 1.00E-04 to 1.00E-10 |
| | | | 3 | 3/14/07 | 1.703 | 1.00E-04 to 1.00E-10 |
| CR11615 | Nitrofen | 2 | 1 | 3/16/07 | 1.554 | 1.00E-03 to 1.00E-10 |
| | | | 2 | 3/20/07 | 1.511 | 5.00E-05 to 1.00E-08 |
| | | | 3 | 3/21/07 | 1.510 | 5.00E-05 to 1.00E-08 |
| CR11616 | Trifluralin | 3 | 1 | 3/16/07 | 1.675 | 1.00E-03 to 1.00E-10 |
| | | | 2 | 3/20/07 | 1.604 | 1.00E-04 to 1.00E-10 |
| | | | 3 | 3/21/07 | 1.566 | 1.00E-04 to 1.00E-10 |

*Runs 2 and 3 of Bisphenol A did not meet performance criteria and were excluded from analysis
**SA = specific activity**

## 12.2 Full Activity and Background Activity Control Results

Each run set for each reference chemical included two types of controls, each run in quadruplicate. The control types were full aromatase activity and background activity controls. The overall mean activities for full and background activity controls for each assay and the percent of full activity represented by the uncorrected background activity are presented in Table 12.2-1. These data were examined to determine if they met applicable performance criteria and those that did not are noted in Table 12.2-1. The performance criterion for full activity control value require that the minimum mean activity be 0.100 nmol/mg protein/min. The performance criterion for background activity controls required that they be ≤ 1 percent of the full activity control.

There were five failures of the background control criterion, four of which occurred in assays of a single chemical conducted by the same technician. Only two of the background control criterion failures occurred in assays that met all other performance criteria. The failures seem to be related to a lower full activity in those assays rather than to an elevated background. No technical issues were identified to explain these lower full activities. Prior to percent of control calculation, all activities are corrected by subtracting the mean background activity, and these low background activities have little effect on the magnitude of the corrected activities. Therefore, the data from CR11602, Runs 4 and 5 and CR11611 Run 1, were not removed from the data set for analysis. Data from Runs 2 and 3 for Chemical CR11602 were excluded from analysis; Run 2 because of reference chemical Top values >110 percent and Run 3 because of full aromatase activity < 0.100 nmol/mg/min.

**Table 12.2-1. Full activity and background activity control results for reference chemicals**

| Chemical Code | Chemical Name | Run | Full Activity | Background Activity (uncorrected) | Background Control Activity as Percentage of Full Activity |
|---|---|---|---|---|---|
| CR11601 | Vinclozolin | 1 | 0.419 | 0.003 | 0.6 |
| | | 2 | 0.417 | 0.003 | 0.7 |
| | | 3 | 0.358 | 0.002 | 0.4 |
| CR11602 | Bisphenol A | 1 | 0.293 | 0.003 | 1.0 |
| | | 2 | 0.145 | 0.003 | ***1.8*** |
| | | 3 | ***0.056*** | 0.001 | ***1.4*** |
| | | 4 | 0.171 | 0.002 | ***1.2*** |
| | | 5 | 0.173 | 0.003 | ***1.7*** |
| CR11603 | Tributyltin | 1 | 0.279 | 0.002 | 0.5 |
| | | 2 | 0.321 | 0.002 | 0.5 |
| | | 3 | 0.265 | 0.002 | 0.6 |
| CR11604 | Diethylhexyl phthalate | 1 | 0.297 | 0.002 | 0.6 |
| | | 2 | 0.304 | 0.002 | 0.5 |
| | | 3 | 0.297 | 0.002 | 0.6 |
| CR11605 | Methoxychlor | 1 | 0.276 | 0.002 | 0.6 |
| | | 2 | 0.195 | 0.002 | 0.9 |
| | | 3 | 0.222 | 0.002 | 0.8 |
| CR11606 | Aldicarb | 1 | 0.279 | 0.001 | 0.5 |

| Chemical Code | Chemical Name | Run | Full Activity | Background Activity (uncorrected) | Background Control Activity as Percentage of Full Activity |
|---|---|---|---|---|---|
| | | 2 | 0.204 | 0.002 | 0.8 |
| | | 3 | 0.216 | 0.001 | 0.6 |
| CR11607 | Flavone | 1 | 0.343 | 0.002 | 0.5 |
| | | 2 | 0.355 | 0.002 | 0.5 |
| | | 3 | 0.333 | 0.002 | 0.6 |
| CR11608 | Triadimefon | 1 | 0.431 | 0.002 | 0.4 |
| | | 2 | 0.400 | 0.001 | 0.3 |
| | | 3 | 0.393 | 0.002 | 0.5 |
| CR11609 | Imazalil | 1 | 0.405 | 0.001 | 0.4 |
| | | 2 | 0.382 | 0.002 | 0.5 |
| | | 3 | 0.397 | 0.002 | 0.6 |
| CR11610 | Apigenin | 1 | 0.334 | 0.002 | 0.5 |
| | | 2 | 0.361 | 0.002 | 0.4 |
| | | 3 | 0.218 | 0.002 | 0.7 |
| CR11611 | Ronidazole | 1 | 0.345 | 0.005 | ***1.4*** |
| | | 2 | 0.409 | 0.002 | 0.4 |
| | | 3 | 0.367 | 0.002 | 0.4 |
| CR11612 | Genistein | 1 | 0.444 | 0.002 | 0.4 |
| | | 2 | 0.399 | 0.002 | 0.4 |
| | | 3 | 0.358 | 0.002 | 0.5 |
| CR11613 | p,p'-DDE | 1 | 0.289 | 0.002 | 0.6 |
| | | 2 | 0.360 | 0.002 | 0.5 |
| | | 3 | 0.357 | 0.002 | 0.5 |
| CR11614 | Alachlor | 1 | 0.254 | 0.002 | 0.6 |
| | | 2 | 0.244 | 0.002 | 0.6 |
| | | 3 | 0.250 | 0.002 | 0.7 |
| CR11615 | Nitrofen | 1 | 0.262 | 0.002 | 0.7 |
| | | 2 | 0.341 | 0.003 | 0.8 |
| | | 3 | 0.351 | 0.002 | 0.4 |
| CR11616 | Trifluralin | 1 | 0.260 | 0.002 | 0.6 |
| | | 2 | 0.373 | 0.002 | 0.5 |
| | | 3 | 0.427 | 0.003 | 0.6 |
| | Mean | | 0.313 | 0.002 | 0.7 |
| | (SD) | | (0.085) | (0.001) | (0.3) |

[a]Units are nmol/mg protein/min

Bold = Failure to meet performance criteria

## 12.3 Positive Control Results

The positive control data were entered into spreadsheets for calculation of aromatase activity (Appendix E of the individual laboratory report). The aromatase activity measured at each concentration of the positive control (4-OH ASDN) assay was divided by the average full enzyme activity control activity for each chemical and run to calculate percent of control values. The percent of control values ranged approximately from 0 to 100 percent over the 4-OH ASDN concentration range of 1 x $10^{-5}$M to 1 x $10^{-10}$M. The percent of control values was fitted to the models described in Chapter 5 using Prism 4.03, and the results are presented in Appendix F of the original laboratory report. The fitted curves resulting from the four-parameter model are shown in Figure 12.3-1. These plots also include the 95 percent confidence intervals for each curve which are generally very narrow, indicating a good curve fit and low variance across all concentrations.

A mixed effects model using the 4-OH ASDN concentrations as fixed effects and the technicians, along with the runs within technicians, as random effects was fit to the percent of control values for the positive controls. Table 12.3-1 shows the variance components estimates and significance tests for the terms in the model.

The results in Table 12.3-1 show that there was not significant variation between technicians, meaning that results did not differ significantly overall from technician to technician. After removing the effects of the concentration levels for 4-OH ASDN from the model (as a fixed effect), there was still significant variation across runs for the technicians, meaning that the positive control results vary from run to run for each technician. Figure 12.3-2 shows the percent of control means with error bars corresponding to one standard deviation above and below the means for each technician.

**Table 12.3-1. Results of random effects ANOVA in mixed effects model**

| Term | Parameter Estimate | Test Statistic | Degrees of Freedom | P-value |
|---|---|---|---|---|
| Technician | 0.1239 | 1.20 | 92 | 0.3158 |
| Run (Technician) | 12.0506 | 5.55 | 665 | <.0001 |
| Residual | 21.1849 | - | - | - |

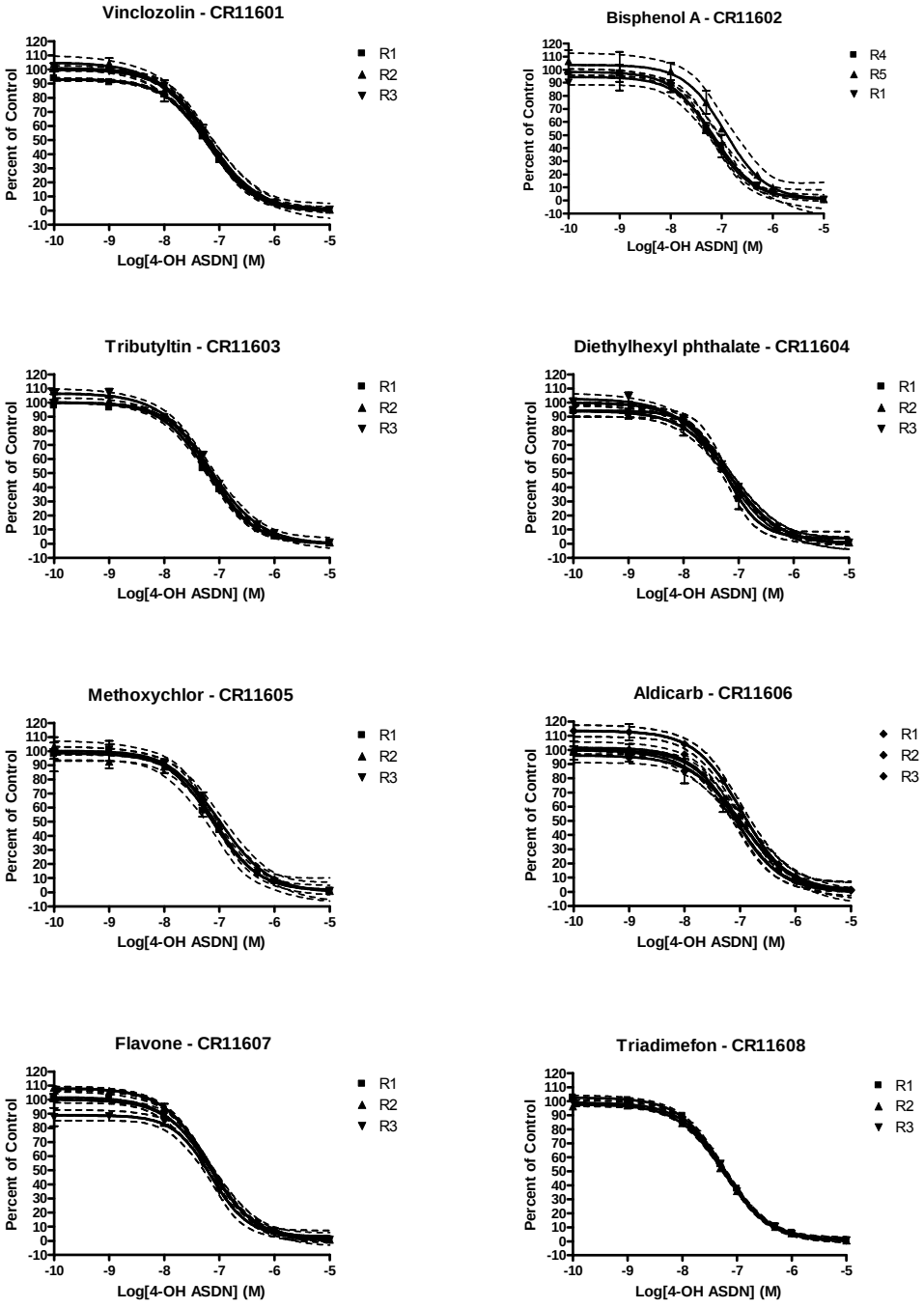


**Figure 12.3-1. Concentration response curves for 4-OH ASDN**

Chemical codes indicate the reference chemical assay conducted in conjunction with the 4-OH ASDN assay.

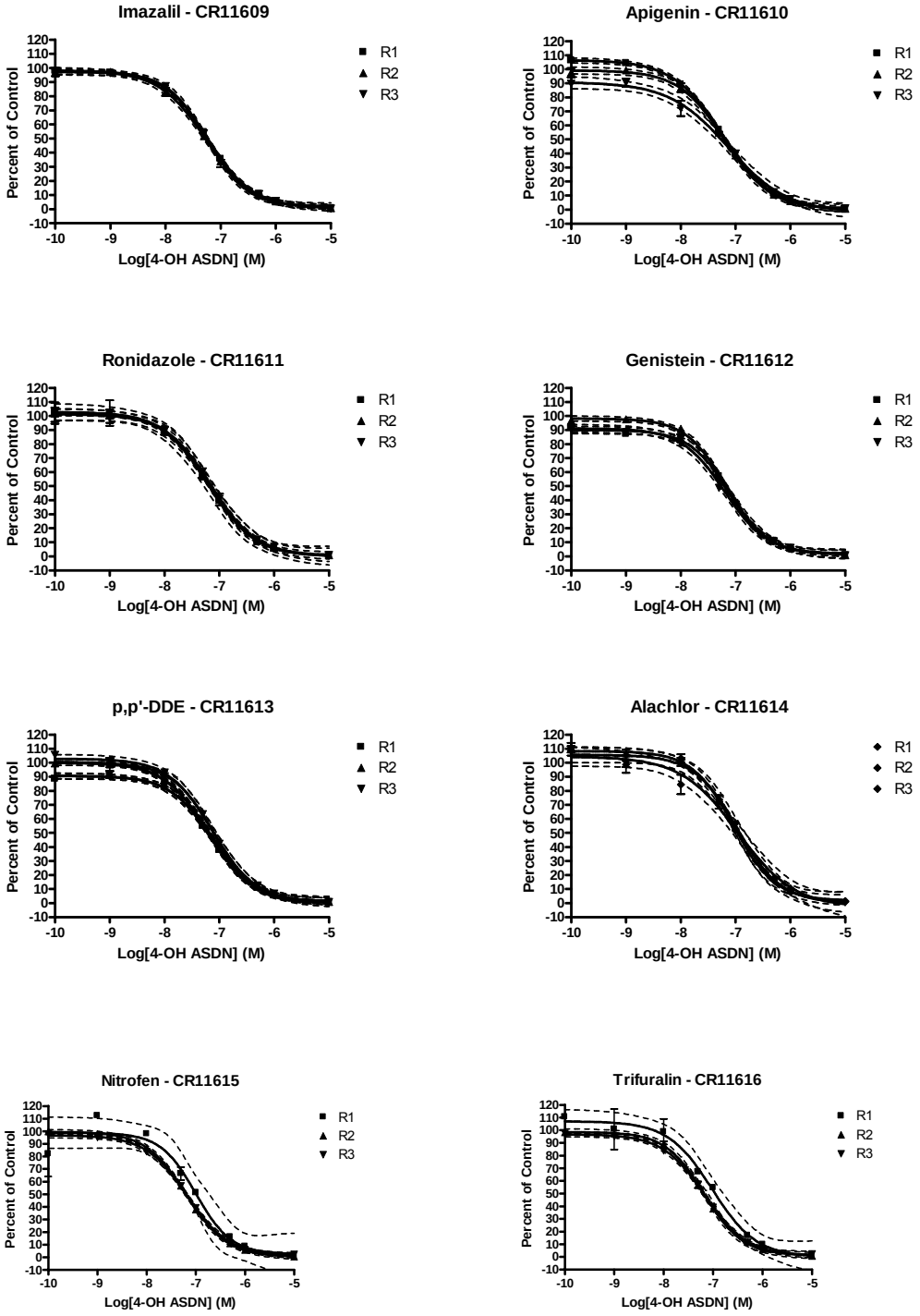


**Figure 12.3-1. Concentration response curves for 4-OH ASDN (continued)**

Chemical codes indicate the reference chemical assay conducted in conjunction with the 4-OH ASDN assay.

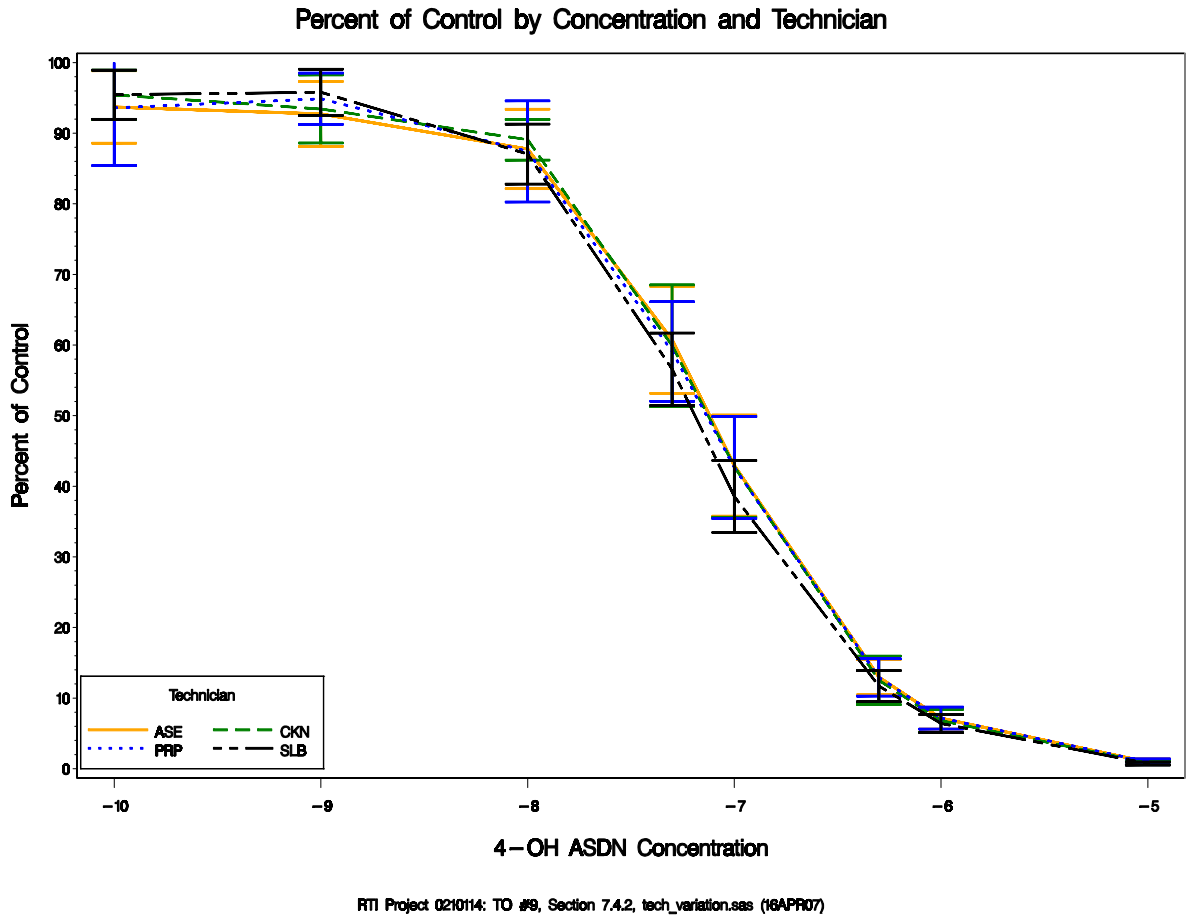


**Figure 12.3-2. Mean concentration response curves for 4-OH ASDN by technician**

The parameters (Top, Bottom, Log$IC_{50}$, and Hillslope) for each fitted curve (Table 12.3-2) were examined to determine if they met the performance criteria described in Table 12.3-3. There were 9 positive control assays (of 48) with at least one criterion failure, and these were distributed among the 4 participating technicians. Four of the assays with failures were conducted on the same day and shared positive control chemical dilutions which points to a possible problem with the dilutions as the cause of the failures. These assays included those conducted in conjunction with the second runs of CR11605, CR11606, CR11614 and the fifth run of CR11602, and each had Log[$IC_{50}$] values between -6.93 and -6.97. Excluding those runs, only 5 other runs had any failed criteria, and those failures were just outside the acceptable range, and in only one case were there multiple failures within a run.

The utility of the performance criteria ranges can be assessed by comparing them with the 95 percent confidence intervals bands for the parameters. The 95 percent confidence interval ranges for the curve parameters are presented in Table 12.3-3. The parameters with the highest failure (either the lower or upper bound out of range) rates are Bottom and Hillslope with 20 and 32 (of 48), respectively. Bottom derives primarily from LSS data which are near background levels and where variance is near 15 percent, so it is not unexpected that many of the 95 percent confidence interval bounds for Bottom fall outside of the 0 ± 5 percent performance criterion range. HillSlope is highly dependent on the defined Bottom value, so when there are a large

number of failures in Bottom, a similar finding is expected in HillSlope. There are fewer 95 percent confidence interval bound values for Log[$IC_{50}$] and Top (14 in each case) that fall outside the criteria ranges, and many of these values are barely outside the ranges. The data show that in most cases, even the bounds of the 95 percent confidence intervals fall within the performance criteria ranges, indicating a high degree of certainty that the correct estimate falls within that range and that the performance criteria ranges are appropriate.

**Table 12.3-2. Positive control assay model fit parameters***

| | Vinclozolin CR11601 | | | Flavone CR11607 | | |
|---|---|---|---|---|---|---|
| | Rep 1 | Rep 2 | Rep 3 | Rep 1 | Rep 2 | Rep 3 |
| BOTTOM | 0.4 | -1.1 | 0.2 | 0.7 | 0.5 | 3.0 |
| TOP | 93.1 | 105 | 101 | 102 | 108 | ***89*** |
| LOGIC50 | -7.20 | -7.23 | -7.20 | -7.17 | -7.19 | -7.15 |
| HILLSLOPE | -1.1 | -0.9 | -1.1 | -1.0 | -1.0 | ***-1.3*** |
| | **Bisphenol A CR11602** | | | **Triadimefon CR11608** | | |
| | Rep 1 | Rep 4 | Rep 5 | Rep 1 | Rep 2 | Rep 3 |
| BOTTOM | 0.7 | 1.7 | 0.7 | 0.9 | 0.8 | 0.6 |
| TOP | 94.5 | 98.3 | 104 | 103 | 97.7 | 99 |
| LOGIC50 | -7.13 | -7.21 | ***-6.93*** | -7.24 | -7.24 | -7.22 |
| HILLSLOPE | -1.1 | -1.1 | -1.1 | -1.1 | -1.0 | -1.1 |
| | **Tributyltin CR11603** | | | **Imazalil CR11609** | | |
| | Rep 1 | Rep 2 | Rep 3 | Rep 1 | Rep 2 | Rep 3 |
| BOTTOM | 1.7 | 0.1 | -0.3 | 0.9 | 0.7 | 2.0 |
| TOP | 98.3 | 103 | 107 | 98.1 | 98 | 97.2 |
| LOGIC50 | -7.21 | -7.18 | -7.17 | -7.22 | -7.27 | -7.25 |
| HILLSLOPE | -1.1 | -1.0 | -0.9 | -1.1 | -1.0 | -1.2 |
| | **Diethylhexyl phthalate CR11604** | | | **Apigenin CR11610** | | |
| | Rep 1 | Rep 2 | Rep 3 | Rep 1 | Rep 2 | Rep 3 |
| BOTTOM | 3.8 | -0.4 | -0.9 | -0.4 | 0.5 | -2.1 |
| TOP | 94.1 | 94.1 | 103 | 106 | 99.1 | 90.8 |
| LOGIC50 | -7.23 | -7.14 | -7.22 | -7.24 | -7.20 | -7.13 |
| HILLSLOPE | ***-1.4*** | -1.0 | -0.9 | -1.0 | -1.0 | -0.8 |
| | **Methoxychlor CR11605** | | | **Ronidazole CR11611** | | |
| | Rep 1 | Rep 2 | Rep 3 | Rep 1 | Rep 2 | Rep 3 |
| BOTTOM | 1.4 | -0.3 | 1.1 | -0.1 | 0.1 | 0.5 |
| TOP | 100 | 98.5 | 100 | 103 | 103 | 101 |
| LOGIC50 | -7.14 | ***-6.97*** | -7.09 | -7.21 | -7.18 | -7.15 |
| HILLSLOPE | -1.1 | -1.0 | -1.1 | -1.0 | -1.0 | -1.0 |
| | **Aldicarb CR11606** | | | **Genistein CR11612** | | |
| | Rep 1 | Rep 2 | Rep 3 | Rep 1 | Rep 2 | Rep 3 |
| BOTTOM | 2.0 | 0.5 | -1.4 | 1.2 | 1.8 | 1.5 |
| TOP | 101 | ***113*** | 96.2 | 90 | 98.1 | 91.1 |
| LOGIC50 | -7.10 | ***-6.97*** | ***-6.94*** | -7.11 | -7.18 | -7.20 |
| HILLSLOPE | -1.1 | -1.0 | -0.9 | -1.2 | -1.2 | -1.1 |

| | p,p'-DDE CR11613 | | | Nitrofen CR11615 | | |
|---|---|---|---|---|---|---|
| | Rep 1 | Rep 2 | Rep 3 | Rep 1 | Rep 2 | Rep 3 |
| BOTTOM | 1.3 | 0.7 | 0.7 | 2.4 | 0.2 | 0.9 |
| TOP | 90.6 | 101 | 103 | 98.8 | 99.5 | 96.8 |
| LOGIC50 | -7.14 | -7.20 | -7.13 | -7.00 | -7.19 | -7.17 |
| HILLSLOPE | -1.1 | -1.0 | -1.1 | -1.2 | -1.1 | -1.0 |
| | **Alachlor CR11614** | | | **Trifluralin CR11616** | | |
| | Rep 1 | Rep 2 | Rep 3 | Rep 1 | Rep 2 | Rep 3 |
| BOTTOM | 0.1 | -3.2 | 1.7 | -0.3 | 1.3 | 0.9 |
| TOP | 106 | 104 | 108 | 107 | 98.6 | 96.8 |
| LOGIC50 | ***-6.96*** | ***-6.96*** | -7.03 | -7.02 | -7.19 | -7.17 |
| HILLSLOPE | -1.1 | -0.8 | -1.1 | -1.0 | -1.1 | -1.0 |

*Reference chemical names and codes indicate the RC run that was run concurrent with each positive control assay

Values that don't meet the performance criteria are presented in ***bold, italicized*** type.

**Table 12.3-3. Positive control assay 95% confidence intervals for model parameters**

| Chemical Code | Chemical | Technician | Run | Bottom | | TOP | | LOGIC50 | | HILLSLOPE | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *Lower* | *Upper* | *Lower* | *Upper* | *Lower* | *Upper* | *Lower* | *Upper* |
| CR11601 | Vinclozolin | 1 | 1 | -0.8 | 1.7 | 92.0 | 94.2 | -7.22 | -7.17 | -1.1 | -1.0 |
| | | | 2 | ***-7.3*** | ***5.1*** | 99.8 | 110 | ***-7.34*** | -7.12 | -1.1 | ***-0.7*** |
| | | | 3 | -2.1 | 2.5 | 99.0 | 103 | -7.24 | -7.16 | -1.2 | -0.9 |
| CR11602 | Bisphenol A | 4 | 1 | ***-7.2*** | ***8.5*** | ***88.2*** | 101 | -7.27 | ***-6.99*** | ***-1.5*** | ***-0.7*** |
| | | | 4 | -1.1 | 4.3 | 95.9 | 101 | -7.26 | -7.16 | ***-1.3*** | -1.0 |
| | | | 5 | ***-13.1*** | ***14.6*** | 94.5 | ***113*** | -7.16 | ***-6.71*** | ***-1.6*** | ***-0.6*** |
| CR11603 | Tributyltin | 2 | 1 | -1.1 | 4.3 | 95.9 | 101 | -7.26 | -7.16 | ***-1.3*** | -1.0 |
| | | | 2 | -2.8 | 3.1 | 100 | 105 | -7.23 | -7.13 | -1.2 | -0.9 |
| | | | 3 | -4.6 | 4.0 | 103 | 110 | -7.25 | -7.10 | -1.1 | ***-0.8*** |
| CR11604 | Diethylhexyl phthalate | 2 | 1 | -0.9 | ***8.5*** | ***89.6*** | 98.6 | ***-7.31*** | -7.15 | ***-1.9*** | -1.0 |
| | | | 2 | ***-5.4*** | 4.6 | 90.3 | 97.9 | -7.23 | -7.04 | -1.2 | ***-0.8*** |
| | | | 3 | ***-5.8*** | 4.0 | 98.7 | 107 | -7.30 | -7.13 | -1.1 | ***-0.7*** |
| CR11605 | Methoxychlor | 1 | 1 | ***-7.8*** | ***10.5*** | 92.9 | 107 | -7.29 | ***-6.98*** | ***-1.5*** | ***-0.6*** |
| | | | 2 | ***-7.6*** | ***7.1*** | 93.7 | 103 | -7.10 | ***-6.85*** | ***-1.2*** | ***-0.7*** |
| | | | 3 | -2.6 | 4.8 | 97.3 | 103 | -7.15 | -7.03 | ***-1.3*** | -0.9 |
| CR11606 | Aldicarb | 2 | 1 | -3.5 | ***7.6*** | 97.2 | 106 | -7.19 | -7.01 | ***-1.4*** | -0.8 |
| | | | 2 | ***-5.8*** | ***6.8*** | 109 | ***118*** | -7.07 | ***-6.88*** | ***-1.3*** | -0.8 |
| | | | 3 | ***-9.5*** | ***6.7*** | 91.0 | 101 | -7.08 | ***-6.80*** | -1.2 | ***-0.7*** |
| CR11607 | Flavone | 3 | 1 | -4.2 | ***5.6*** | 97.7 | 106 | -7.25 | -7.08 | ***-1.3*** | -0.8 |
| | | | 2 | -1.4 | 2.4 | 106 | 110 | -7.22 | -7.16 | -1.1 | -1.0 |
| | | | 3 | -1.4 | ***7.4*** | ***85.2*** | 92.8 | -7.24 | -7.07 | ***-1.6*** | -0.9 |
| CR11608 | Triadimefon | 1 | 1 | -1.1 | 2.9 | 101.0 | 104 | -7.27 | -7.20 | -1.2 | -1.0 |
| | | | 2 | -0.6 | 2.2 | 96.5 | 98.9 | -7.27 | -7.21 | -1.1 | -1.0 |
| | | | 3 | -1.4 | 2.6 | 97.2 | 101 | -7.26 | -7.19 | -1.2 | -1.0 |
| CR11609 | Imazalil | 1 | 1 | -0.4 | 2.2 | 97.0 | 99.3 | -7.25 | -7.20 | -1.1 | -1.0 |
| | | | 2 | -2.0 | 3.4 | 95.6 | 100 | ***-7.32*** | -7.21 | -1.2 | -0.9 |
| | | | 3 | -0.3 | 4.3 | 95.0 | 99.3 | -7.29 | -7.20 | ***-1.3*** | -1.0 |

**Table 12.3-3. (continued)**

| Chemical Code | Chemical | Technician | Run | Bottom | | TOP | | LOGIC50 | | HILLSLOPE | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *Lower* | *Upper* | *Lower* | *Upper* | *Lower* | *Upper* | *Lower* | *Upper* |
| CR11610 | Apigenin | 2 | 1 | -2.6 | 1.8 | 105 | 108 | -7.28 | -7.20 | -1.0 | -0.9 |
| | | | 2 | -2.5 | 3.6 | 96.6 | 102 | -7.26 | -7.15 | -1.2 | -0.9 |
| | | | 3 | ***-8.5*** | 4.3 | ***86.1*** | 95.4 | -7.26 | -7.00 | -1.0 | ***-0.6*** |
| CR11611 | Ronidazole | 4 | 1 | ***-7.5*** | ***7.3*** | 96.5 | 109 | ***-7.34*** | -7.08 | ***-1.3*** | ***-0.7*** |
| | | | 2 | -2.8 | 3.1 | 100 | 105 | -7.23 | -7.13 | -1.2 | -0.9 |
| | | | 3 | -4.8 | ***5.7*** | 97.0 | 105 | -7.24 | -7.06 | ***-1.3*** | -0.8 |
| CR11612 | Genistein | 3 | 1 | -2.2 | 4.5 | ***87.4*** | 92.7 | -7.17 | -7.05 | ***-1.2*** | -1.0 |
| | | | 2 | -0.4 | 4.0 | 96.2 | 100 | -7.21 | -7.14 | ***-1.3*** | -1.0 |
| | | | 3 | -1.9 | 4.9 | ***88.2*** | 94.0 | -7.27 | -7.14 | ***-1.3*** | -0.9 |
| CR11613 | p,p'-DDE | 3 | 1 | -1.3 | 3.8 | ***88.5*** | 92.6 | -7.18 | -7.09 | ***-1.3*** | -1.0 |
| | | | 2 | -2.1 | 3.5 | 98.3 | 103 | -7.25 | -7.15 | -1.2 | -0.9 |
| | | | 3 | -3.2 | 4.6 | 99.7 | 106 | -7.20 | -7.07 | ***-1.3*** | -0.9 |
| CR11614 | Alachlor | 3 | 1 | ***-8.1*** | ***8.4*** | 100 | ***111*** | -7.09 | ***-6.84*** | ***-1.4*** | ***-0.8*** |
| | | | 2 | ***-14.3*** | ***8.0*** | 97.4 | ***111*** | -7.14 | ***-6.77*** | -1.1 | ***-0.6*** |
| | | | 3 | -2.5 | ***5.8*** | 105 | ***111*** | -7.10 | ***-6.97*** | ***-1.3*** | -1.0 |
| CR11615 | Nitrofen | 2 | 1 | ***-15.1*** | ***19.9*** | ***86.3*** | ***111*** | -7.29 | ***-6.71*** | ***-2.2*** | ***-0.3*** |
| | | | 2 | -2.1 | 2.6 | 97.6 | 101 | -7.24 | -7.15 | -1.2 | -0.9 |
| | | | 3 | -1.5 | 3.4 | 94.8 | 98.7 | -7.22 | -7.13 | -1.1 | -0.9 |
| CR11616 | Trifluralin | 3 | 1 | ***-14.1*** | ***13.5*** | 97.6 | ***117*** | -7.24 | ***-6.80*** | ***-1.4*** | ***-0.5*** |
| | | | 2 | -1.8 | 4.4 | 95.9 | 101 | -7.24 | -7.13 | ***-1.3*** | -0.9 |
| | | | 3 | -1.5 | 3.4 | 94.8 | 98.7 | -7.22 | -7.13 | -1.1 | -0.9 |

Values in ***bold, italicized*** type are outside the performance criteria ranges

## 12.4 Reference Chemical Assay Results

**Percent of Control Values.** The assay data were entered into spreadsheets for calculation of aromatase activity (Appendix E of the original laboratory report). The aromatase activity found in each assay tube was normalized to percent of control by dividing by the average full enzyme activity for the run. The percent of control values for each reference chemical run and tube, along with the mean, SD, SEM, and CV of the percent of control across tubes within a run, are presented in Appendix H of the original laboratory report. Generally, there was little tube-to-tube variance (CV generally less than 10 percent) within a run. For some high reference chemical concentrations where the percent of control values were near zero, the CV values were higher, in part due to increased variance in the measurement of DPM at these near background levels.

The mean percent of control values across tubes for each run and the overall mean, SD, SEM, and CV across runs are presented in Table 12.4-1. Run-to-run reproducibility was good, with most CV values less than 10 percent. Again, some higher CVs were observed when percent of control was near zero.

**Table 12.4-1. Mean percent of control per run and percent of control across runs**

| Reference Chemical | Log[RC] (M) | Percent of Control | | | Overall | | | |
|---|---|---|---|---|---|---|---|---|
| | | Rep 1 | Rep 2 | Rep 3 | Mean | ±SD | SEM | CV |
| Vinclozolin (CR11601) | *-3.00* | 83.89 | NA | NA | 83.89 | NC | NC | NC |
| | -4.00 | 88.44 | 99.20 | 99.08 | 95.57 | 6.18 | 3.57 | 6.46 |
| | -5.00 | 93.78 | 104.51 | 106.61 | 101.63 | 6.88 | 3.97 | 6.77 |
| | -6.00 | 95.92 | 100.84 | 105.30 | 100.68 | 4.69 | 2.71 | 4.66 |
| | -6.30 | NA | 100.22 | 107.86 | 104.04 | 5.40 | 3.82 | 5.19 |
| | -7.00 | 95.40 | 97.64 | 101.50 | 98.18 | 3.08 | 1.78 | 3.14 |
| | -8.00 | 97.02 | 99.81 | 103.94 | 100.26 | 3.48 | 2.01 | 3.47 |
| | -9.00 | 96.08 | 99.52 | 104.03 | 99.88 | 3.99 | 2.30 | 4.00 |
| | -10.00 | 95.28 | 101.47 | 101.44 | 99.40 | 3.56 | 2.06 | 3.59 |
| Bisphenol A* (CR11602) | -3.00 | 3.23 | 4.67 | 4.59 | 4.16 | 0.81 | 0.46 | 19.35 |
| | -3.30 | NA | 19.37 | 20.85 | 20.11 | 1.05 | 0.74 | 5.20 |
| | -3.60 | NA | 42.30 | 43.50 | 42.90 | 0.85 | 0.60 | 1.98 |
| | -4.00 | 59.13 | 65.37 | 73.12 | 65.87 | 7.01 | 4.04 | 10.63 |
| | -4.30 | NA | 89.07 | 90.70 | 89.88 | 1.15 | 0.81 | 1.28 |
| | -4.60 | NA | 82.10 | 97.24 | 89.67 | 10.70 | 7.57 | 11.94 |
| | -5.00 | 87.57 | 97.80 | 104.18 | 96.51 | 8.38 | 4.84 | 8.68 |
| | -6.00 | 90.03 | 87.37 | 113.43 | 96.94 | 14.34 | 8.28 | 14.79 |
| | -7.00 | 92.17 | NA | NA | 92.17 | NC | NC | NC |
| | -8.00 | 92.30 | NA | NA | 92.30 | NC | NC | NC |
| | -9.00 | 93.27 | NA | NA | 93.27 | NC | NC | NC |
| | -10.00 | 92.93 | NA | NA | 92.93 | NC | NC | NC |

**Table 12.4-1. (continued)**

| Reference Chemical | Log[RC] (M) | Percent of Control | | | Overall | | | |
|---|---|---|---|---|---|---|---|---|
| | | Rep 1 | Rep 2 | Rep 3 | Mean | ±SD | SEM | CV |
| Tributyltin (CR11603) | -3.00 | 78.03 | 80.95 | 82.10 | 80.36 | 2.10 | 1.21 | 2.61 |
| | -4.00 | 83.25 | 81.70 | 83.89 | 82.95 | 1.12 | 0.65 | 1.36 |
| | -5.00 | 92.95 | 94.85 | 90.34 | 92.71 | 2.26 | 1.31 | 2.44 |
| | -6.00 | 98.86 | 100.75 | 99.87 | 99.83 | 0.95 | 0.55 | 0.95 |
| | -7.00 | 95.49 | 94.69 | 90.21 | 93.46 | 2.84 | 1.64 | 3.04 |
| | -8.00 | 93.89 | 94.13 | 98.74 | 95.59 | 2.73 | 1.58 | 2.86 |
| | -9.00 | 92.30 | 97.76 | 95.32 | 95.13 | 2.73 | 1.58 | 2.87 |
| | -10.00 | 93.20 | 99.24 | 92.97 | 95.14 | 3.55 | 2.05 | 3.74 |
| Diethylhexyl phthalate (CR11604) | *-3.00* | 107.10 | NA | NA | 107.10 | NC | NC | NC |
| | -4.00 | 109.21 | 100.91 | 110.52 | 106.88 | 5.21 | 3.01 | 4.88 |
| | -5.00 | 109.20 | 106.35 | 110.48 | 108.68 | 2.11 | 1.22 | 1.94 |
| | -6.00 | 97.84 | 97.03 | 102.43 | 99.10 | 2.91 | 1.68 | 2.94 |
| | -6.30 | NA | 101.01 | 103.23 | 102.12 | 1.57 | 1.11 | 1.54 |
| | -7.00 | 98.11 | 95.53 | 105.25 | 99.63 | 5.03 | 2.91 | 5.05 |
| | -8.00 | 96.65 | 93.55 | 99.16 | 96.45 | 2.81 | 1.62 | 2.91 |
| | -9.00 | 100.23 | 93.37 | 101.94 | 98.51 | 4.54 | 2.62 | 4.61 |
| | -10.00 | 95.94 | 99.72 | 102.38 | 99.35 | 3.24 | 1.87 | 3.26 |
| Methoxychlor (CR11605) | *-3.00* | 81.16 | NA | NA | 81.16 | NC | NC | NC |
| | *-4.00* | 93.68 | NA | NA | 93.68 | NC | NC | NC |
| | -5.00 | 96.26 | 109.33 | 94.01 | 99.87 | 8.27 | 4.78 | 8.29 |
| | -6.00 | 97.67 | 109.09 | 105.14 | 103.97 | 5.80 | 3.35 | 5.58 |
| | -7.00 | 91.29 | 116.65 | 103.79 | 103.91 | 12.68 | 7.32 | 12.20 |
| | -7.30 | NA | 106.99 | 94.39 | 100.69 | 8.91 | 6.30 | 8.85 |
| | -8.00 | 100.30 | 112.76 | 99.42 | 104.16 | 7.46 | 4.31 | 7.16 |
| | -8.30 | NA | 110.62 | 103.34 | 106.98 | 5.15 | 3.64 | 4.81 |
| | -9.00 | 105.41 | 117.60 | 102.25 | 108.42 | 8.10 | 4.68 | 7.47 |
| | -10.00 | 102.39 | 116.17 | 102.66 | 107.07 | 7.88 | 4.55 | 7.36 |
| Aldicarb (CR11606) | -3.00 | 86.29 | 106.30 | 84.92 | 92.50 | 11.97 | 6.91 | 12.94 |
| | -4.00 | 94.06 | 116.65 | 106.06 | 105.59 | 11.30 | 6.52 | 10.70 |
| | -5.00 | 93.84 | 113.30 | 100.16 | 102.43 | 9.93 | 5.73 | 9.69 |
| | -6.00 | 83.66 | 113.38 | 101.46 | 99.50 | 14.96 | 8.64 | 15.03 |
| | -7.00 | 93.01 | 121.81 | 95.22 | 103.35 | 16.03 | 9.25 | 15.51 |
| | -8.00 | 92.79 | 115.66 | 95.49 | 101.31 | 12.50 | 7.22 | 12.34 |
| | -9.00 | 97.68 | 114.52 | 94.93 | 102.38 | 10.60 | 6.12 | 10.36 |
| | -10.00 | 82.46 | 117.85 | 98.42 | 99.58 | 17.72 | 10.23 | 17.80 |

**Table 12.4-1. (continued)**

| Reference Chemical | Log[RC] (M) | Percent of Control | | | Overall | | | |
|---|---|---|---|---|---|---|---|---|
| | | Rep 1 | Rep 2 | Rep 3 | Mean | ±SD | SEM | CV |
| Flavone (CR11607) | *-3.00* | 1.88 | NA | NA | 1.88 | NC | NC | NC |
| | -3.60 | NA | 11.25 | 10.90 | 11.07 | 0.25 | 0.18 | 2.24 |
| | -4.00 | 24.39 | 25.24 | 26.23 | 25.28 | 0.92 | 0.53 | 3.65 |
| | -4.30 | NA | 41.60 | 40.98 | 41.29 | 0.44 | 0.31 | 1.06 |
| | -4.60 | NA | 57.88 | 57.87 | 57.87 | 0.01 | 0.00 | 0.01 |
| | -5.00 | 76.89 | 77.41 | 76.08 | 76.79 | 0.67 | 0.39 | 0.87 |
| | -5.30 | NA | 87.27 | 82.43 | 84.85 | 3.42 | 2.42 | 4.03 |
| | -6.00 | 97.72 | 98.20 | 94.18 | 96.70 | 2.20 | 1.27 | 2.27 |
| | -7.00 | 97.74 | 101.41 | 96.22 | 98.46 | 2.67 | 1.54 | 2.71 |
| | -8.00 | 98.84 | NA | NA | 98.84 | NC | NC | NC |
| | -9.00 | 98.14 | NA | NA | 98.14 | NC | NC | NC |
| | -10.00 | 100.88 | NA | NA | 100.88 | NC | NC | NC |
| Triadimefon (CR11608) | *-3.00* | 0.16 | NA | NA | 0.16 | NC | NC | NC |
| | -4.00 | 2.09 | 1.83 | 2.14 | 2.02 | 0.17 | 0.10 | 8.22 |
| | -4.30 | NA | 3.76 | 4.19 | 3.97 | 0.30 | 0.21 | 7.59 |
| | -5.00 | 16.36 | 14.85 | 16.75 | 15.99 | 1.01 | 0.58 | 6.29 |
| | -5.30 | NA | 24.98 | 27.67 | 26.33 | 1.90 | 1.34 | 7.21 |
| | -5.60 | NA | 39.30 | 42.36 | 40.83 | 2.17 | 1.53 | 5.30 |
| | -6.00 | 64.74 | 59.06 | 63.82 | 62.54 | 3.05 | 1.76 | 4.87 |
| | -7.00 | 94.33 | 90.65 | 95.73 | 93.57 | 2.63 | 1.52 | 2.81 |
| | -8.00 | 102.78 | 96.66 | 100.73 | 100.06 | 3.12 | 1.80 | 3.11 |
| | -9.00 | 101.06 | NA | NA | 101.06 | NC | NC | NC |
| | -10.00 | 99.18 | NA | NA | 99.18 | NC | NC | NC |
| Imazalil (CR11609) | *-3.00* | 0.15 | NA | NA | 0.15 | NC | NC | NC |
| | -4.00 | 0.07 | NA | NA | 0.07 | NC | NC | NC |
| | -5.00 | 0.14 | 0.26 | -0.01 | 0.13 | 0.14 | 0.08 | 103.43 |
| | -6.00 | 1.12 | 1.78 | 1.14 | 1.35 | 0.38 | 0.22 | 28.08 |
| | -7.00 | 8.95 | 14.93 | 11.54 | 11.81 | 3.00 | 1.73 | 25.38 |
| | -7.60 | NA | 40.25 | 30.28 | 35.26 | 7.06 | 4.99 | 20.01 |
| | -8.00 | 46.29 | 61.73 | 52.00 | 53.34 | 7.81 | 4.51 | 14.63 |
| | -8.60 | NA | 85.85 | 77.56 | 81.71 | 5.86 | 4.14 | 7.17 |
| | -9.00 | 90.75 | 93.30 | 84.29 | 89.45 | 4.64 | 2.68 | 5.19 |
| | -10.00 | 98.62 | 98.84 | 90.45 | 95.97 | 4.78 | 2.76 | 4.98 |

**Table 12.4-1. (continued)**

| Reference Chemical | Log[RC] (M) | Percent of Control | | | Overall | | | |
|---|---|---|---|---|---|---|---|---|
| | | Rep 1 | Rep 2 | Rep 3 | Mean | ±SD | SEM | CV |
| Apigenin (CR11610) | *-3.00* | 7.19 | NA | NA | 7.19 | NC | NC | NC |
| | *-4.00* | 16.67 | NA | NA | 16.67 | NC | NC | NC |
| | -4.30 | NA | 18.68 | 12.69 | 15.68 | 4.24 | 3.00 | 27.03 |
| | -4.60 | NA | 31.86 | 21.77 | 26.81 | 7.14 | 5.05 | 26.61 |
| | -5.00 | 51.09 | 52.45 | 38.82 | 47.45 | 7.51 | 4.33 | 15.82 |
| | -5.30 | NA | 69.55 | 54.91 | 62.23 | 10.35 | 7.32 | 16.63 |
| | -5.60 | NA | 80.97 | 66.81 | 73.89 | 10.01 | 7.08 | 13.54 |
| | -6.00 | 93.11 | 92.30 | 82.97 | 89.46 | 5.63 | 3.25 | 6.30 |
| | -7.00 | 102.32 | 97.57 | 94.99 | 98.29 | 3.72 | 2.15 | 3.78 |
| | -8.00 | 102.46 | 100.74 | 92.09 | 98.43 | 5.56 | 3.21 | 5.65 |
| | -9.00 | 99.99 | NA | NA | 99.99 | NC | NC | NC |
| | -10.00 | 100.61 | NA | NA | 100.61 | NC | NC | NC |
| Ronidazole (CR11611) | *-3.00* | 108.93 | NA | NA | 108.93 | NC | NC | NC |
| | *-4.00* | 113.17 | NA | NA | 113.17 | NC | NC | NC |
| | -5.00 | 114.44 | 102.21 | 98.25 | 104.97 | 8.44 | 4.87 | 8.04 |
| | -6.00 | 111.53 | 101.56 | 100.12 | 104.40 | 6.21 | 3.59 | 5.95 |
| | -7.00 | 107.77 | 101.48 | 100.92 | 103.39 | 3.80 | 2.20 | 3.68 |
| | -7.30 | NA | 103.13 | 81.64 | 92.38 | 15.19 | 10.74 | 16.45 |
| | -8.00 | 111.97 | 97.11 | 97.49 | 102.19 | 8.47 | 4.89 | 8.29 |
| | -8.30 | NA | 96.40 | 91.60 | 94.00 | 3.40 | 2.40 | 3.62 |
| | -9.00 | 107.88 | 102.09 | 98.56 | 102.84 | 4.71 | 2.72 | 4.58 |
| | -10.00 | 111.33 | 99.33 | 102.56 | 104.41 | 6.21 | 3.58 | 5.95 |
| Genistein (CR11612) | *-3.00* | 59.42 | NA | NA | 59.42 | NC | NC | NC |
| | *-3.30* | NA | 72.47 | 74.09 | 73.28 | 1.14 | 0.81 | 1.56 |
| | -3.60 | NA | 82.90 | 84.20 | 83.55 | 0.92 | 0.65 | 1.10 |
| | -4.00 | 86.97 | 91.82 | 85.38 | 88.05 | 3.35 | 1.94 | 3.81 |
| | -5.00 | 94.04 | 100.53 | 82.87 | 92.48 | 8.93 | 5.16 | 9.66 |
| | -6.00 | 96.72 | 97.84 | 84.77 | 93.11 | 7.24 | 4.18 | 7.78 |
| | -7.00 | 97.48 | 98.67 | 89.26 | 95.13 | 5.13 | 2.96 | 5.39 |
| | -8.00 | 98.98 | 96.51 | 88.25 | 94.58 | 5.62 | 3.24 | 5.94 |
| | -9.00 | 97.28 | 100.07 | 96.51 | 97.95 | 1.87 | 1.08 | 1.91 |
| | -10.00 | 97.33 | NA | NA | 97.33 | NC | NC | NC |

**Table 12.4-1. (continued)**

| Reference Chemical | Log[RC] (M) | Percent of Control | | | Overall | | | |
|---|---|---|---|---|---|---|---|---|
| | | Rep 1 | Rep 2 | Rep 3 | Mean | ±SD | SEM | CV |
| p,p'-DDE (CR11613) | *-3.00* | 64.90 | NA | NA | 64.90 | NC | NC | NC |
| | *-4.00* | 81.78 | NA | NA | 81.78 | NC | NC | NC |
| | -5.00 | 81.75 | 89.09 | 91.73 | 87.52 | 5.17 | 2.99 | 5.91 |
| | -6.00 | 90.86 | 97.63 | 100.42 | 96.30 | 4.91 | 2.84 | 5.10 |
| | -7.00 | 98.82 | 104.48 | 106.73 | 103.34 | 4.08 | 2.35 | 3.95 |
| | -7.30 | NA | 108.66 | 106.09 | 107.37 | 1.82 | 1.29 | 1.70 |
| | -8.00 | 99.59 | 111.49 | 96.76 | 102.61 | 7.82 | 4.51 | 7.62 |
| | -8.30 | NA | 108.74 | 101.65 | 105.19 | 5.01 | 3.54 | 4.76 |
| | -9.00 | 99.29 | 106.26 | 104.50 | 103.35 | 3.63 | 2.09 | 3.51 |
| | -10.00 | 97.96 | 107.02 | 103.77 | 102.92 | 4.59 | 2.65 | 4.46 |
| Alachlor (CR11614) | *-3.00* | 53.91 | NA | NA | 53.91 | NC | NC | NC |
| | -4.00 | 103.02 | 100.50 | 96.24 | 99.92 | 3.43 | 1.98 | 3.43 |
| | -5.00 | 104.90 | 92.89 | 102.30 | 100.03 | 6.32 | 3.65 | 6.31 |
| | -6.00 | 115.25 | 95.07 | 114.45 | 108.26 | 11.42 | 6.60 | 10.55 |
| | -6.30 | NA | 96.01 | 101.57 | 98.79 | 3.93 | 2.78 | 3.98 |
| | -7.00 | 119.26 | 99.18 | 109.18 | 109.21 | 10.04 | 5.79 | 9.19 |
| | -8.00 | 123.86 | 102.22 | 105.42 | 110.50 | 11.68 | 6.74 | 10.57 |
| | -9.00 | 99.32 | 109.93 | 101.77 | 103.67 | 5.55 | 3.21 | 5.36 |
| | -10.00 | 114.21 | 96.99 | 82.38 | 97.86 | 15.93 | 9.20 | 16.28 |
| Nitrofen (CR11615) | *-3.00* | 37.48 | NA | NA | 37.48 | NC | NC | NC |
| | *-4.00* | 50.59 | NA | NA | 50.59 | NC | NC | NC |
| | *-4.30* | NA | 41.24 | 42.20 | 41.72 | 0.67 | 0.48 | 1.62 |
| | -4.60 | NA | 44.79 | 43.47 | 44.13 | 0.93 | 0.66 | 2.11 |
| | -5.00 | 69.77 | 57.19 | 56.90 | 61.29 | 7.35 | 4.24 | 11.99 |
| | -5.30 | NA | 71.83 | 69.67 | 70.75 | 1.53 | 1.08 | 2.16 |
| | -5.60 | NA | 85.72 | 82.89 | 84.30 | 2.00 | 1.41 | 2.37 |
| | -6.00 | 107.14 | 97.04 | 89.78 | 97.99 | 8.72 | 5.03 | 8.90 |
| | -7.00 | 108.11 | 100.33 | 96.81 | 101.75 | 5.78 | 3.34 | 5.68 |
| | -8.00 | 101.55 | 100.91 | 97.92 | 100.13 | 1.94 | 1.12 | 1.94 |
| | -9.00 | 107.24 | NA | NA | 107.24 | NC | NC | NC |
| | -10.00 | 109.06 | NA | NA | 109.06 | NC | NC | NC |
| Trifluralin (CR11616) | *-3.00* | 102.30 | NA | NA | 102.30 | NC | NC | NC |
| | -4.00 | 104.84 | 100.72 | 100.88 | 102.15 | 2.34 | 1.35 | 2.29 |
| | -5.00 | 103.62 | 98.94 | 106.02 | 102.86 | 3.60 | 2.08 | 3.50 |
| | -6.00 | 103.54 | 95.69 | 104.85 | 101.36 | 4.96 | 2.86 | 4.89 |
| | -6.30 | NA | 99.54 | 100.87 | 100.20 | 0.94 | 0.66 | 0.94 |
| | -7.00 | 105.56 | 99.39 | 102.38 | 102.45 | 3.08 | 1.78 | 3.01 |
| | -8.00 | 92.98 | 99.19 | 105.97 | 99.38 | 6.50 | 3.75 | 6.54 |
| | -9.00 | 106.74 | 96.38 | 102.38 | 101.83 | 5.20 | 3.00 | 5.10 |
| | -10.00 | 89.54 | 103.85 | 102.92 | 98.77 | 8.01 | 4.62 | 8.11 |

Italicized concentrations were insoluble in the reaction mixture and data obtained are excluded from modeling.

NA Not applicable – this concentration was not assayed in this run

NC Not calculated – only one data point – no SD, SEM or CV calculated.

*Values under Rep 2 and Rep 3 are from Rep 4 and Rep 5, respectively, for Bisphenol A

**Model Fitting.** The percent of control data were fit using nonlinear regression methods in Prism 4.03 to the models described in Chapter 5. Prism outputs are presented in Appendix H of the original laboratory report. The Top, Bottom, Log$IC_{50}$, and Slope were calculated for the full (four-parameter) model while only Log $IC_{50}$ and Slope were calculated for the two-parameter model (since Top and Bottom were fixed in that reduced model). The parameters from both models are presented in Table 12.4-2. The calculated four-parameter model Top value was compared against the performance criterion that requires Top = 100 ± 10 percent, and all runs (where Top was calculable) passed this requirement except genistein (CR11612), Run 3, for which the value for Top was 189.7. There was an extremely wide 95 percent confidence interval for that (and other) parameter(s) in that run, indicating a poor curve fit for this noninhibitor.

The model fits were compared in Prism to determine if there were significant differences between them, and the results of that analysis are presented in Table 12.4-3. As expected, the more complex models fit the data better in all cases where there were significant differences in the model fits. When the model fits are not significantly different, the preferred model is the simpler two-parameter model by default. The curve fits are presented graphically in Figure 12.4-1. The plotted curves are those of the preferred model for each run.

**Table 12.4-2. Reference chemical model fit parameters**

| Chemical Name | Chemical Code | Run | Top | | Bottom | | LogIC50 | | Slope | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Eq 1 | Eq 2 | Eq 1 | Eq 2 | Eq 1 | Eq 2 | Eq 1 | Eq 2 |
| Vinclozolin | CR11601 | R1 | 95.95 | 100 | 88.04 | 0 | -4.752 | 4.929 | -1.688 | -0.1089 |
| | | R2 | | | | | | | | |
| | | R3 | | | | | | | | |
| ***Bisphenol A*** | ***CR11602*** | R1 | 92.37 | 100 | -22.92 | 0 | -3.575 | -3.912 | -0.9262 | -1.104 |
| | | R4 | 91.92 | 100 | -6.355 | 0 | -3.622 | -3.759 | -1.418 | -1.28 |
| | | R5 | 112.1 | 100 | -23.86 | 0 | -3.604 | -3.689 | -0.9766 | -1.579 |
| Tributyltin | CR11603 | R1 | 94.69 | 100 | 77.73 | 0 | -4.234 | -4.1E+07 | -1.387 | 2.46E-08 |
| | | R2 | 97.31 | 100 | 81.05 | 0 | -4.659 | -0.4193 | -2.23 | -0.2286 |
| | | R3 | 95.42 | 100 | 82.99 | 0 | -4.981 | 1.67 | -8.315 | -0.1418 |
| Diethylhexyl phthalate | CR11604 | R1 | 109.2 | 100 | 97.73 | 0 | -5.591 | -13.51 | 4.929 | 0.3997 |
| | | R2 | | | | | | | | |
| | | R3 | | | | | | | | |
| Methoxychlor | CR11605 | R1 | 103.9 | 100 | 95.07 | 0 | -7.981 | 0.5212 | -8.651 | -0.2356 |
| | | R2 | | | | | | | | |
| | | R3 | | 100 | | 0 | | -4.835 | | -7.236 |
| Aldicarb | CR11606 | R1 | | 100 | | 0 | | -549.6 | | 0.0018 |
| | | R2 | | | | | | | | |
| | | R3 | | | | | | | | |
| ***Flavone*** | ***CR11607*** | R1 | 98.94 | 100 | 14.85 | 0 | -4.665 | -4.484 | -1.342 | -1.015 |
| | | R2 | 101.8 | 100 | -3.973 | 0 | -4.438 | -4.461 | -0.9307 | -1.014 |
| | | R3 | 96.75 | 100 | -6.247 | 0 | -4.37 | -4.482 | -0.9122 | -0.9194 |
| ***Triadimefon*** | ***CR11608*** | R1 | 101.0 | 100 | -0.09823 | 0 | -5.74 | -5.728 | -0.9571 | -0.9801 |
| | | R2 | 97.55 | 100 | -0.2917 | 0 | -5.791 | -5.829 | -0.9238 | -0.8861 |
| | | R3 | 101.8 | 100 | 0.002194 | 0 | -5.757 | -5.734 | -0.9391 | -0.9691 |

**Table 12.4-2. (continued)**

| Chemical Name | Chemical Code | Run | Top | | Bottom | | LogIC50 | | Slope | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Eq 1 | Eq 2 | Eq 1 | Eq 2 | Eq 1 | Eq 2 | Eq 1 | Eq 2 |
| ***Imazalil*** | ***CR11609*** | R1 | 100.4 | 100 | 0.2596 | 0 | -8.064 | -8.056 | -0.994 | -0.9947 |
| | | R2 | 99.51 | 100 | -0.03275 | 0 | -7.776 | -7.783 | -0.9656 | -0.9556 |
| | | R3 | 91.06 | 100 | 0.2206 | 0 | -7.882 | -8.005 | -1.017 | -0.8179 |
| ***Apigenin*** | ***CR11610*** | R1 | 101.3 | 100 | 50.95 | 0 | -5.783 | -4.984 | -3.274 | -1.148 |
| | | R2 | 99.88 | 100 | 0.375 | 0 | -4.948 | -4.946 | -0.9888 | -0.9821 |
| | | R3 | 94.31 | 100 | 2.478 | 0 | -5.188 | -5.229 | -0.9968 | -0.864 |
| Ronidazole | CR11611 | R1 | | | | | | | | |
| | | R2 | | | | | | | | |
| | | R3 | | | | | | | | |
| Genistein | CR11612 | R1 | 97.81 | 100 | 75.41 | 0 | -3.958 | -0.7823 | -0.6602 | -0.2648 |
| | | R2 | 98.72 | 100 | 81.99 | 0 | -3.956 | -2.906 | -3.491 | -0.9823 |
| | | R3 | 189.7 | 100 | 84.09 | 0 | -11.52 | 6.274 | -0.3524 | -0.06876 |
| p,p'-DDE | CR11613 | R1 | 98.94 | 100 | 81.59 | 0 | -5.972 | -3.537 | -2.104 | -0.4385 |
| | | R2 | 108.4 | 100 | 87.51 | 0 | -6.049 | -4.215 | -1.014 | -1.158 |
| | | R3 | 103.0 | 100 | 91.73 | 0 | -5.933 | -4.847 | -7.743 | -6.85 |
| Alachlor | CR11614 | R1 | | | | | | | | |
| | | R2 | | | | | | | | |
| | | R3 | | | | | | | | |
| ***Nitrofen*** | ***CR11615*** | R1 | | | | | | | | |
| | | R2 | 100.9 | 100 | 39.26 | 0 | -5.265 | -4.776 | -1.496 | -0.8813 |
| | | R3 | 97.44 | 100 | 31.59 | 0 | -5.171 | -4.788 | -1.161 | -0.7552 |
| Trifluralin | CR11616 | R1 | | | | | | | | |
| | | R2 | | | | | | | | |
| | | R3 | | | | | | | | |

For runs where the models did not converge, the cells are left blank
***Bold, italicized*** type indicates the chemical was classified as an inhibitor.

**Table 12.4-3. Model curve fit comparison results**

| | **Vinclozolin CR11601** | | | ***Flavone CR11607*** | | |
|---|---|---|---|---|---|---|
| | **Rep 1** | **Rep 2** | **Rep 3** | **Rep 1** | **Rep 2** | **Rep 3** |
| Converged? | Y | DNC | DNC | Y | Y | Y |
| P value | ***0.015*** | | | 0.7977 | 0.1050 | 0.0564 |
| Preferred model | Equation 1 | | | Equation 2 | Equation 2 | Equation 2 |
| F | 5.433 | | | 0.2291 | 2.528 | 3.331 |
| | ***Bisphenol A CR11602*** | | | ***Triadimefon CR11608*** | | |
| | **Rep 1** | **Rep 1** | **Rep 1** | **Rep 1** | **Rep 2** | **Rep 3** |
| Converged? | Y | Y | Y | Y | Y | Y |
| P value | ***P<0.0001*** | 0.1182 | ***0.0003*** | 0.4240 | ***P<0.0001*** | ***0.0033*** |
| Preferred model | Equation 1 | Equation 2 | Equation 1 | Equation 2 | Equation 1 | Equation 1 |
| F | 70.71 | 2.381 | 12.28 | 0.9029 | 22.47 | 7.684 |
| | **Tributyltin CR11603** | | | ***Imazalil CR11609*** | | |
| | **Rep 1** | **Rep 2** | **Rep 3** | **Rep 1** | **Rep 2** | **Rep 3** |
| Converged? | Y | Y | Y | Y | Y | Y |
| P value | ***P<0.0001*** | ***0.0376*** | 0.1737 | 0.2510 | 0.6059 | 0.0698 |
| Preferred model | Equation 1 | Equation 1 | Equation 2 | Equation 2 | Equation 2 | Equation 2 |
| F | 23.32 | 3.883 | 1.913 | 1.518 | 0.5138 | 3.050 |
| | **Diethylhexyl phthalate CR11604** | | | ***Apigenin CR11610*** | | |
| | **Rep 1** | **Rep 2** | **Rep 3** | **Rep 1** | **Rep 2** | **Rep 3** |
| Converged? | Y | DNC | DNC | Y | Y | Y |
| P value | ***0.0004*** | | | 0.0642 | 0.9886 | ***0.0001*** |
| Preferred model | Equation 1 | | | Equation 2 | Equation 2 | Equation 1 |
| F | 12.55 | | | 3.363 | 0.01148 | 14.51 |
| | **Methoxychlor CR11605** | | | **Ronidazole CR11611** | | |
| | **Rep 1** | **Rep 2** | **Rep 3** | **Rep 1** | **Rep 2** | **Rep 3** |
| Converged? | Y | DNC | Y ( 1 model) | DNC | DNC | DNC |
| P value | 0.0879 | | No Comp | | | |
| Preferred model | Equation 2 | | Equation 2 | | | |
| F | 2.908 | | | | | |
| | **Aldicarb CR11606** | | | **Genistein CR11612** | | |
| | **Rep 1** | **Rep 2** | **Rep 3** | **Rep 1** | **Rep 2** | **Rep 3** |
| Converged? | Y ( 1 model) | DNC | DNC | Y | Y | Y |
| P value | No Comp | | | 0.2199 | 0.1320 | ***0.0172*** |
| Preferred model | Equation 2 | | | Equation 2 | Equation 2 | Equation 1 |
| F | | | | 1.658 | 2.287 | 5.212 |
| | **p,p'-DDE CR11613** | | | ***Nitrofen* CR11615** | | |

| | Rep 1 | Rep 2 | Rep 3 | Rep 1 | Rep 2 | Rep 3 |
|---|---|---|---|---|---|---|
| Converged? | Y | Y | Y | DNC | Y | Y |
| P value | 0.2151 | ***P<0.0001*** | 0.1206 | | ***0.0004*** | ***0.0002*** |
| Preferred model | Equation 2 | Equation 1 | Equation 2 | | Equation 1 | Equation 1 |
| F | 1.718 | 37.08 | 2.369 | | 12.95 | 15.17 |
| | **Alachlor CR11614** | | | **Trifluralin CR11616** | | |
| | **Rep 1** | **Rep 2** | **Rep 3** | **Rep 1** | **Rep 2** | **Rep 3** |
| Converged? | DNC | DNC | DNC | DNC | DNC | DNC |
| P value | | | | | | |
| Preferred model | | | | | | |
| F | | | | | | |

Y = yes, the models converged

DNC = neither model converged

F = the test statistic

No Comp = no comparison because only one model converged

Equation 1 = the four-parameter equation

Equation 2 = the two-parameter equation

Statistical significance level is set at P< 0.05, significant results indicated with ***bold, italicize***

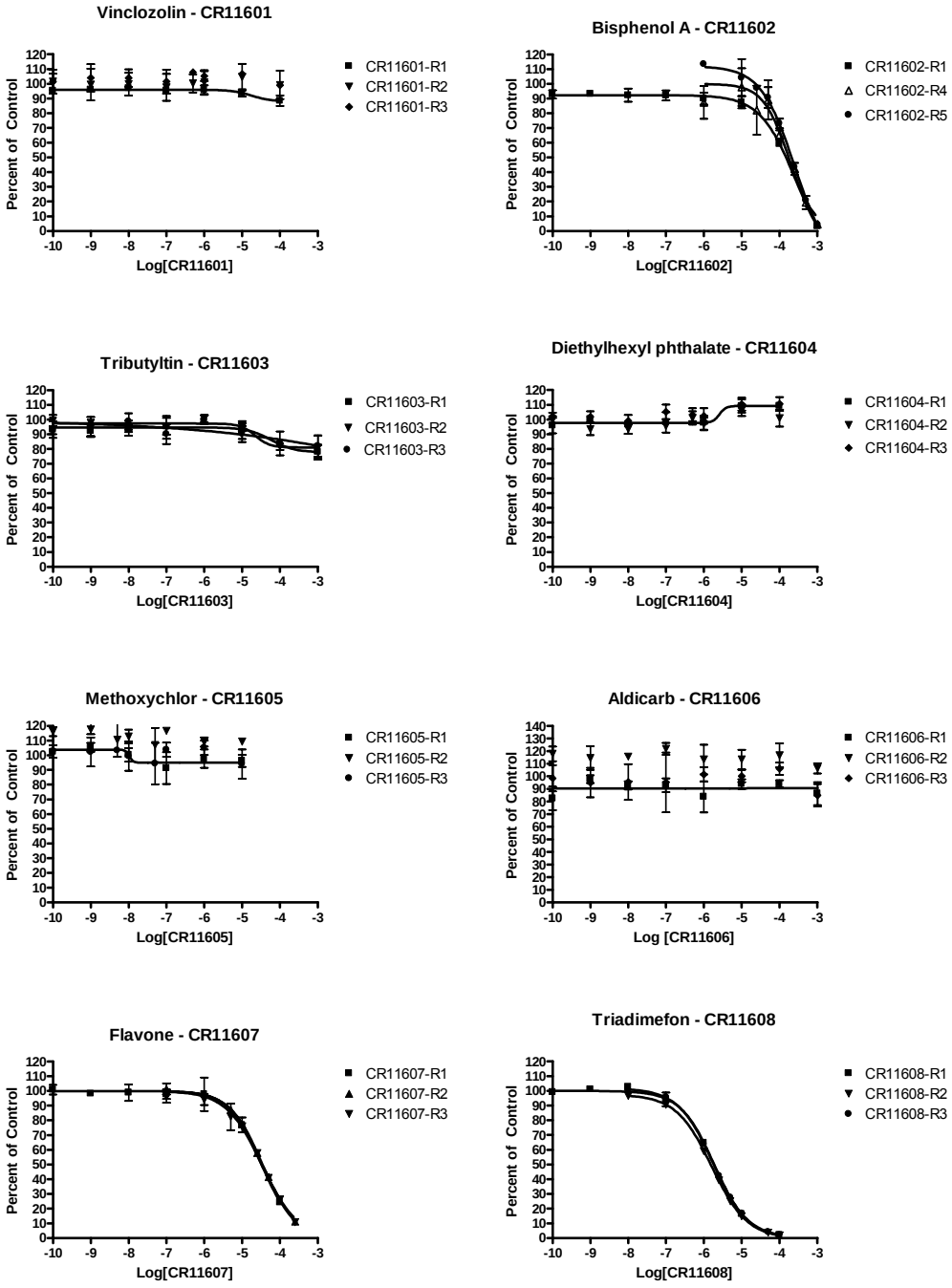


**Figure 12.4-1. Reference chemical concentration response curves**

Data are mean of 3 tubes per run with error bars denoting SEM

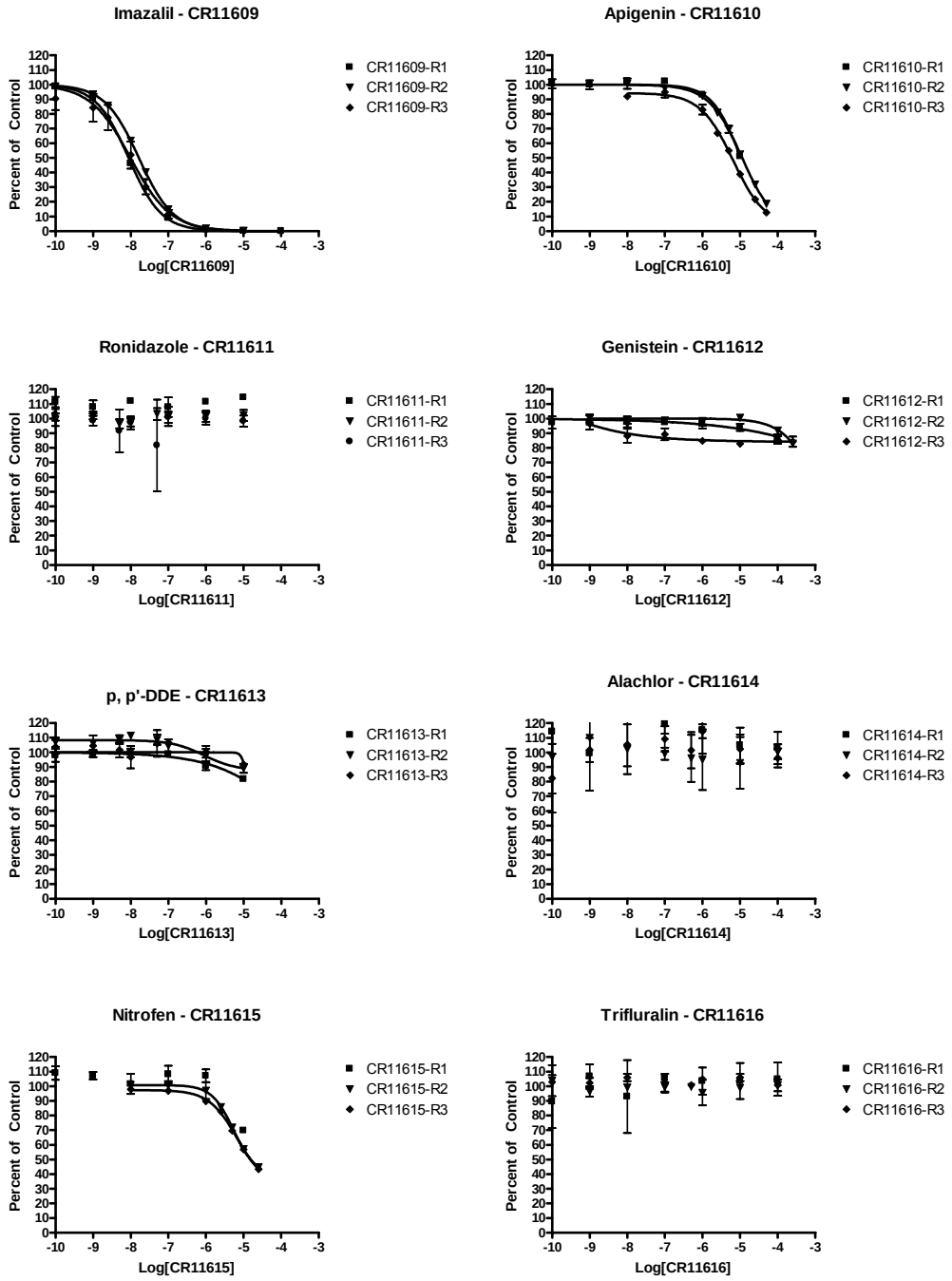


**Figure 12.4-1. Reference chemical concentration response curves (continued)**

Data are mean of 3 tubes per run with error bars denoting SEM

## 12.5 Classification of Chemicals

The classification of the reference chemicals as noninhibitors, inhibitors, or as chemicals for which the results are equivocal were assigned based on the criteria in Table 12.5-1. The

resulting classifications are presented in Table 12.5-2. Only 6 of the 16 chemicals tested had Bottom values below 75 percent, and for all of these Bottom was below 50 percent, which would lead to the classification of all six compounds as inhibitors of aromatase. These six compounds were: bisphenol A, flavone, triadimefon, imazalil, apigenin and nitrofen. The percent of control values essentially spanned the entire range 0-100 percent for five of the six inhibitors. Only nitrofen (CR11615) had a Bottom value (39 percent) that was not near 0 percent.

**Table 12.5-1. Chemical classification criteria**

| Class | Criteria |
|---|---|
| Noninhibitor | The most inhibited level lies above 75% of full activity control |
| Equivocal | The most inhibited level lies between 75% and 50% of full activity control |
| Inhibitor | The most inhibited level lies below 50% of full activity control |

**Table 12.5-2. Classification of reference chemicals**

| Chemical Name | Chemical Code | Highest Tested Soluble Concentration (M) | Classification | Results Reported in the Literature |
|---|---|---|---|---|
| Vinclozolin | CR11601 | 1.00E-04 | Noninhibitor | Noninhibitor |
| ***Bisphenol A*** | ***CR11602*** | ***1.00E-03*** | ***Inhibitor*** | ***Inhibitor*** |
| Tributyltin | CR11603 | 1.00E-03 | Noninhibitor | **Inhibitor KGN** |
| Diethylhexyl phthalate | CR11604 | 1.00E-04 | Noninhibitor | **Inhibitor KGN** |
| Methoxychlor | CR11605 | 1.00E-05 | Noninhibitor | Noninhibitor KGN |
| Aldicarb | CR11606 | 1.00E-03 | Noninhibitor | **Inhibitor KGN** |
| ***Flavone*** | ***CR11607*** | ***2.50E-04*** | ***Inhibitor*** | ***Inhibitor*** |
| ***Triadimefon*** | ***CR11608*** | ***1.00E-04*** | ***Inhibitor*** | ***Inhibitor*** |
| ***Imazalil*** | ***CR11609*** | ***1.00E-05*** | ***Inhibitor*** | ***Inhibitor*** |
| ***Apigenin*** | ***CR11610*** | ***5.00E-05*** | ***Inhibitor*** | ***Inhibitor*** |
| Ronidazole | CR11611 | 1.00E-05 | Noninhibitor | Weak inhibitor |
| Genistein | CR11612 | 5.00E-04 | Noninhibitor | Noninhibitor |
| p,p'-DDE | CR11613 | 1.00E-05 | Noninhibitor | **Inhibitor KGN** |
| Alachlor | CR11614 | !.00E-04 | Noninhibitor | Noninhibitor KGN |
| ***Nitrofen*** | ***CR11615*** | ***5.00E-05*** | ***Inhibitor*** | Noninhibitor KGN |
| Trifluralin | CR11616 | 1.00E-04 | Noninhibitor | Noninhibitor KGN |

As noted in Chapter 6, these 16 chemicals were chosen for the supplementary studies because some evidence was found for their effect on aromatase in the scientific literature. A comparison of the classification from the RTI study with the results reported in the literature shows concordance for 10 of the chemicals. However, the level of confidence in the literature result varies greatly, from a single study for 10 of the chemicals to as many as 6 studies for Imazalil (see Table 6.2-2). The data in the Morinaga (2004) KGN cell paper is of low confidence because the results were presented only in a relative graphic and no quantitative estimate was reported. In addition, it is not known how applicable the KGN cell data would be for a microsomal assay. Thus, while this comparison is interesting, the KGN data cannot be used as an indicator of the specificity or sensitivity of the microsomal aromatase assay.

### 12.6 Aromatase Inhibitors – Curve Fits

Data from the three runs of each of the chemicals classified as an inhibitor were combined and fitted to the models. The resulting concentration response curves, with 95 percent confidence intervals, are presented in Figure 12.6-1. It is clear from the graphs that there is very little run-to-run variation in the data and the curve fits. The exception occurs at the bottom of the nitrofen (CR11615) curve where the 95 percent confidence interval broadens. The curve parameters (Top, Bottom, $LogIC_{50}$, and Hillslope) calculated, using the two models, are presented in Table 12.6-1. There were no significant differences in the fitted curve parameters obtained from the two models for the chemicals flavone, triadimefon, imazalil, and apigenin. There were significant differences between the two models for bisphenol A and nitrofen. In the case of nitrofen, the values for $LogIC_{50}$ and Hillslope, obtained with Equation 1 (presented below), were markedly different from those obtained with the reduced model, and the standard errors for those parameters from Equation 1 were about 3-fold larger than those obtained with Equation 2.

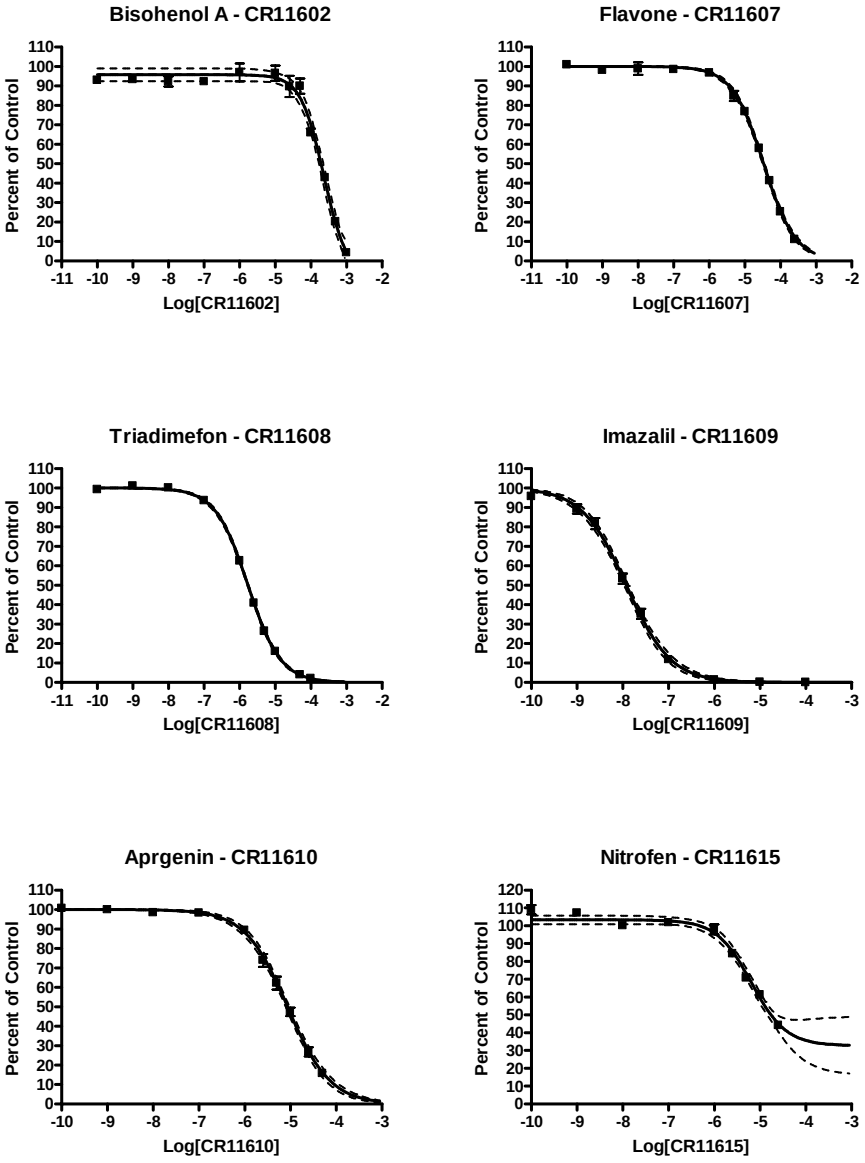


**Figure 12.6-1 Reference chemical curve fits across runs**

Symbols represent mean percent of control with error bars showing SEM
Dashed lines indicate 95% confidence intervals
Solid lines indicated the fitted curve from the preferred model.

**Table 12.6-1 Comparison of best fit estimates and their associated standard errors (SE) for the model parameters across models for aromatase inhibitors**

| Chemical | Chem Code | Model | P value | Bottom | | Top | | LogIC50 | | HillSlope | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Best Fit | SE | Best fit | SE | Best Fit | SE | Best fit | SE |
| Bisphenol A | CR11602 | Eq. 1 | 0.0091 | -8.1 | 8.4 | 95.7 | 1.66 | -3.636 | 0.075 | -1.3 | 0.2 |
| | | Eq. 2 | | 0.0 | | 100.0 | | -3.749 | 0.028 | -1.4 | 0.1 |
| Flavone | CR11607 | Eq. 1 | 0.2467 | -4.1 | 4.4 | 99.2 | 0.81 | -4.417 | 0.049 | -0.9 | 0.1 |
| | | Eq. 2 | | 0.0 | | 100.0 | | -4.473 | 0.013 | -1.0 | 0.0 |
| Triadimefon | CR11608 | Eq. 1 | 0.6846 | -0.1 | 0.7 | 100.5 | 0.54 | -5.772 | 0.013 | -0.9 | 0.0 |
| | | Eq. 2 | | 0.0 | | 100.0 | | -5.767 | 0.009 | -0.9 | 0.0 |
| Imazalil | CR11609 | Eq. 1 | 0.2283 | 0.0 | 1.2 | 96.9 | 1.82 | -7.882 | 0.034 | -1.0 | 0.1 |
| | | Eq. 2 | | 0.0 | | 100.0 | | -7.924 | 0.023 | -0.9 | 0.0 |
| Apigenin | CR11610 | Eq. 1 | 0.9262 | -1.1 | 7.6 | 99.6 | 1.24 | -5.047 | 0.086 | -0.9 | 0.1 |
| | | Eq. 2 | | 0.0 | | 100.0 | | -5.064 | 0.020 | -0.9 | 0.0 |
| Nitrofen | CR11615 | Eq. 1 | 0.0009 | 32.7 | 8.2 | 103.3 | 1.22 | -5.189 | 0.107 | -1.1 | 0.2 |
| | | Eq. 2 | | 0.0 | | 100.0 | | -4.766 | 0.034 | -0.9 | 0.1 |

The value returned as $logEC_{50}$ by Prism using the 4-parameter equation is equivalent to (Top + Bottom)/2. For data sets where the 95 percent confidence intervals of Top and Bottom encompass 100 and 0 percent, respectively, the parameter '$LogEC_{50}$' estimated by Prism is equivalent to $LogIC_{50}$ which, by definition, is the concentration that yields 50 percent inhibition. Caution should be exercised in the reporting of $IC_{50}$ estimates generated from data sets that do not span the 0 to 100 percent range for percent of control. Plots of the two- and four-parameter curve fits and their associated 95 percent confidence bands for nitrofen are presented in Figure 12.6-2. Both models fit this data set reasonably well, but the 95 percent confidence bands to broaden as the four-parameter model approaches lower percent of control values due to the poorly defined Bottom for that model. The Prism estimates for $LogEC_{50}$ are -4.776 and -5.189 for the two- and four-parameter models, respectively. As depicted graphically in Figure 12.6-2, the estimate for $LogEC_{50}$ returned by the 4-parameter model corresponds to the concentration that yields 68 percent of control activity [(Top +Bottom)/2 = (103.3+32.7)/2 = 68]. When queried for the value of X where Y=50 (defined as $LogIC_{50}$), Prism returns the estimate of -4.762 for the 4-parameter equation. Equation 3 (below) can be used in Prism to directly yield direct $LogIC_{50}$ estimates, regardless of whether the range of the inhibition spans 0 to 100 percent. The $LogIC_{50}$ value from Equation 3 (-4.762) matches that obtained as a point estimate for X at Y=50 from the 4-parameter equation. The advantage of using Equation 3 is the availability of confidence intervals from Prism for the true $LogIC_{50}$. Table 12.6-2 shows values for $LogIC_{50}$

from various models. Figure 12.6-3 shows the concentration response curve for nitrofen from Equation 3.

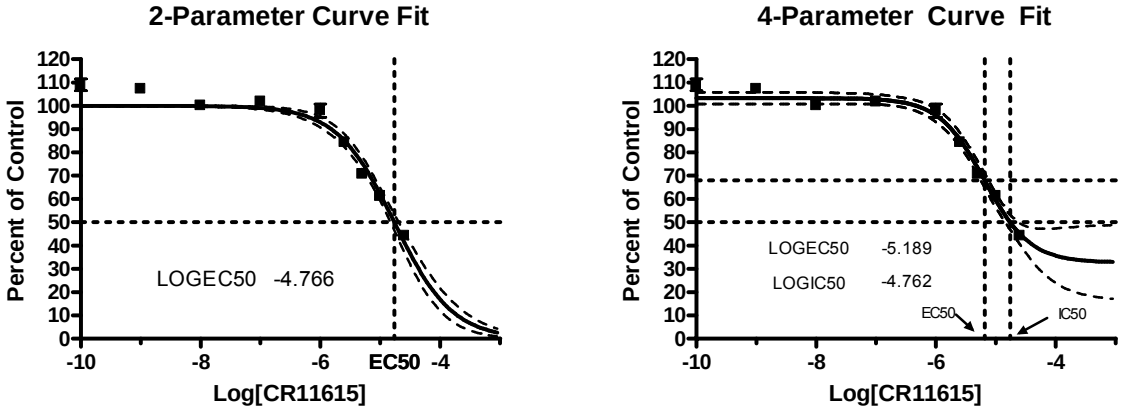


**Figure 12.6-2. Comparison of the 2- and 4-parameter model fits for nitrofen**

**Table 12.6-2. Values for $LogIC_{50}$ from various models**

| Model | $LogIC_{50}$ (from curve fit) | SE of $LogIC_{50}$ | Concentration corresponding to Y=50 ($LogIC_{50}$) |
|---|---|---|---|
| Equation 1 | -5.189 | 0.1072 | -4.762 |
| Equation 2 | -4.766 | 0.0337 | -4.766 |
| Equation 3 | -4.762 | 0.0464 | -4.762 |

**Equation 1: The standard 4-parameter model**

$$Y = Bottom + \frac{(Top-Bottom)}{1 + 10^{(Log\ EC_{50}-X)\beta}}$$

**Equation 2: The standard 2-parameter model** (Top is set at 100%; Bottom, at 0%)

$$Y = \frac{100}{1 + 10^{(Log\ EC_{50}-X)\beta}}$$

**Equation 3: The modified 4-parameter model** (Corrects for partial curves)

$$Y = Bottom + \frac{(Top-Bottom)}{1 + 10^{(Log\ EC_{50}-X)\beta + \log[(Top-Bottom)/(50-Bottom)-1]}}$$

where *Y* is the percent of control activity remaining, *X* is the $\log_{10}$ of the concentration of the chemical, *Top* and *Bottom* are the plateaus of the response curve, $\mu$ is the $\log_{10}IC_{50}$ and $\beta$ is the slope of the response curve at $\log_{10}IC_{50}$.

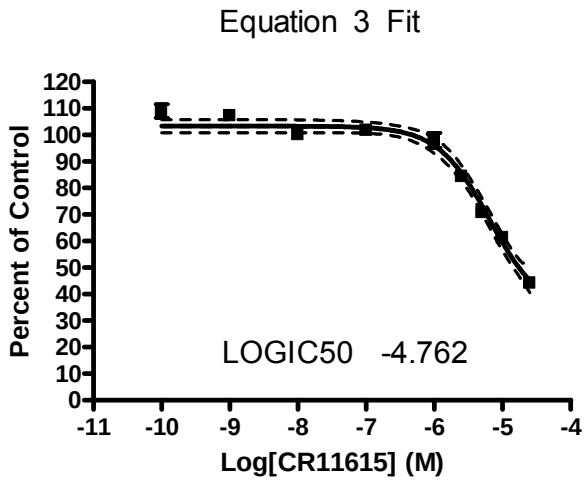


**Figure 12.6-3. Concentration response curve for nitrofen from Equation 3**

## 12.7 Application of Data Interpretation Criterion

Data interpretation criteria would be used to decide upon the proper classification of a test chemical as either an inhibitor, a noninhibitor, or as a chemical for which the data are equivocal. Chapter 11 discussed several approaches for the interpretation of data. RTI used the one based on the upper and lower 95 percent confidence bounds of the estimated response curve, but with slightly different cut-points.

According to RTI, if both confidence bands for the estimated response curve extend below the 50 percent line on the graph, then the chemical can be classified as an inhibitor with only a small chance of a classification error. If both confidence bands for the estimated response curve stay above the 75 percent line on the graph, then the chemical can be classified as a noninhibitor with only a small chance of a classification error. More analyses would need to be performed to determine the classification rules if at least one of the confidence bands remains between the 50 percent and 75 percent lines for the highest concentrations of the chemical.

In the present data set, there are six compounds that are clearly inhibitors and 10 that are clearly noninhibitors. In no case was the most inhibited level between 75 percent and 50 percent of control which would lead to the classification of equivocal.

Five of the six inhibitors exhibited maximal inhibition of less than 20 percent of control; the other inhibitor (CR11615) had a maximal inhibition of about 32 percent of control. These are all well within the 'inhibitor' classification range. The 95 percent confidence bands on the curve fits (Figure 5 and Appendix H of the original laboratory report) were examined to determine if

both confidence bands extended below 50 percent of control at the highest concentration. In all cases, the confidence bands were well below 50 percent of control at the highest concentration.

Generally, there was no concentration-dependent response for the noninhibitors, and Prism was often unable to fit the model to the data. Under these circumstances, there can be no examination of the 95 percent confidence bands, and no further analysis is necessary to classify these compounds as noninhibitors. In some cases, Prism returned a fitted curve, but the 95 percent confidence intervals were very large and so the curve fits were meaningless.

In order to mimic data one might obtain for equivocal inhibitors and to generate a test set for evaluation of the 95 percent confidence interval bounds as an interpretation criterion, the data from the six inhibitors was limited to those concentrations where activity was > 40 to 60 percent of control and Prism was used to fit the four-parameter model. The resulting plots are presented in Figure 12.7-1 and include the 95 percent confidence intervals. The data sets for which there are wide variances in the curve fit parameters are obvious by their wide confidence intervals, and all of these would necessarily be classified as equivocal. There were not enough data in some of the limited data sets to fit a curve. The other data sets show fairly narrow confidence intervals, and in all cases where the lowest mean percent of control activity was <50 percent, both confidence interval bounds also fell below 50 percent allowing the classification of inhibitor to be assigned. Also, in each case when the lowest mean percent of control activity was between 75 percent and 50 percent of control, both confidence interval bounds fell completely within that range and the chemical would be classified as an equivocal inhibitor.

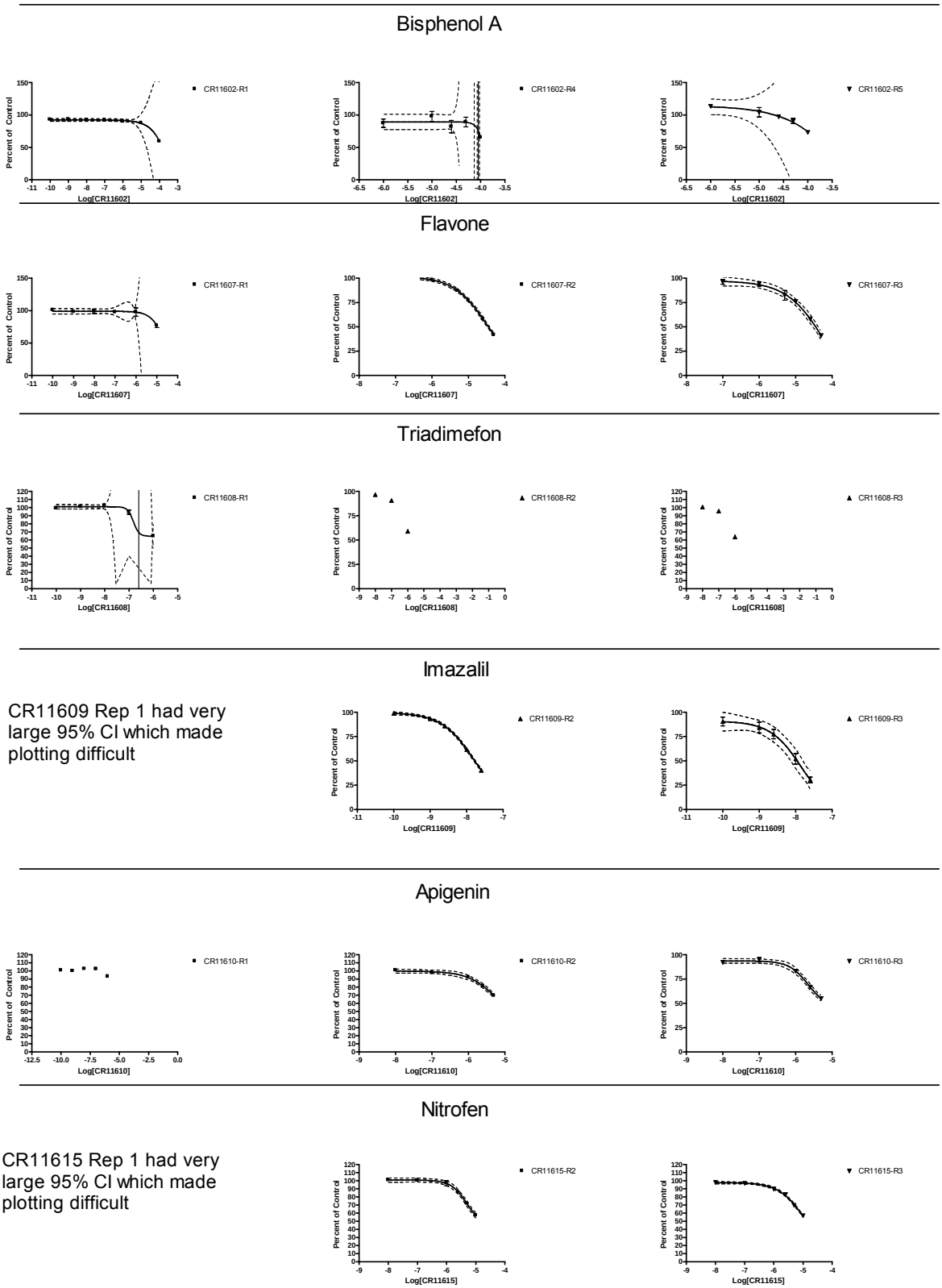


**Figure 12.7-1. Concentration response curves from data sets with percent of control limited to >40-60%**

## 12.8 Performance Criteria for Test Chemicals

These studies with the16 test chemicals were also used in an attempt to craft some performance criteria for test chemicals, specifically to identify poor quality runs of the test chemical so that the run could be repeated.

In order to assess whether an individual run of the assay is acceptable, coefficients of variation (CVs) for the percent of control values at each concentration of the test chemicals and the mean CV across all concentrations were examined. The CVs obtained for each reference chemical run in the supplementary study are presented in Table 12.8-1. It is clear that within run CVs at each concentration are often less than 10 percent, but that sometimes they range to 15 percent or even higher. In cases where the CVs are higher than 15 percent, the data should be examined for outliers that might indicate experimental error. In addition, it is important to note that when the DPM data approach background values (as when percent of control is less than approximately 5 percent) the inherent uncertainty (measurement error) in the data rises to nearly 15 percent and, therefore, CVs calculated for data at very low percent of control values are primarily comprised of measurement error. There are enough CVs in the 10 to 15 percent range to warrant setting the standard for CV at each concentration to be no tighter than ≤15 percent. The effect of the higher individual concentration CVs on the curve fit can be assessed by examining the 95 percent confidence bands of the fitted curves. Only two runs in the data set (CR11602- Rep 4 and CR11609 – Rep 3) had both individual CVs >15 percent and produced fitted curves in Prism. For both of these, the 95 percent confidence bands are narrow across the entire curve (Figure 12.8-1), indicating that the fitted curves were well defined even when there were some instances of high individual CVs in the data set. Three other runs (CR11611- Rep 3, CR11614-Rep 2 and CR11616- Rep 1) had individual CVs above 15 percent, but these chemicals were all noninhibitors.

Another approach would be to look at the mean CV across concentrations within a run. As can be seen in the data presented in Table 12.8-1, those mean CVs were ≤15 percent for 47 of the 48 assays and were ≤10 percent in 41 of the 48 assays. Of the seven runs that had CV means greater than 10 percent, only two (CR11602, Rep 4 and CR11609, Rep 3) were from chemicals for which Prism could fit the model; the others were from chemicals that were clearly noninhibitors. As discussed above, the 95 percent confidence bands on these curve fits are narrow, indicating that well-defined curves were obtained from the data.

**Table 12.8-1. Coefficients of Variance (%CV) for each Reference Chemical Run and Concentration (Recombinant Aromatase Assay)**

| Chemical | Log[RC]* | Rep 1 | Rep 2 | Rep 3 | Chemical | Log[RC]* | Rep 1 | Rep 2 | Rep 3 |
|---|---|---|---|---|---|---|---|---|---|
| CR11601 | -4 | 4.1 | 9.7 | 1.5 | CR11609 | -7 | 0.2 | 2.4 | ***24.8*** |
| Vinclozolin | -5 | 2.7 | 8.6 | 1.3 | Imizalil | -7.6 | | 1.9 | ***17.2*** |
| | -6 | 3.1 | 8.2 | 3.2 | | -8 | 0.1 | 1.1 | ***17.8*** |
| | -6.3 | NA | 6.1 | 1.2 | | -8.6 | | 1.3 | 11.0 |
| | -7 | 0.7 | 9.3 | 8.0 | | -9 | 0.3 | 2.3 | 11.3 |
| | -8 | 1.4 | 7.7 | 5.7 | | -10 | 1.0 | 1.1 | 8.7 |
| | -9 | 1.3 | 10.8 | 9.0 | | | | | |
| | -10 | 1.0 | 8.1 | 5.4 | | | | | |
| | **Mean** | **2.0** | **8.6** | **4.4** | | **Mean** | **0.4** | **1.7** | **15.1** |
| | SD | 1.3 | 1.4 | 3.1 | | SD | 0.4 | 0.6 | 6.0 |
| CR11602† | -3.3 | NA | ***23.3*** | 2.5 | CR11610 | -4.3 | NA | 7.7 | 1.8 |
| Bisphenol A | -3.6 | NA | 9.3 | 2.1 | Apigenin | -4.6 | NA | 2.7 | 5.7 |
| | -4 | 1.3 | 4.7 | 4.5 | | -5 | 0.8 | 0.5 | 4.7 |
| | -4.3 | NA | 14.9 | 7.7 | | -5.3 | NA | 3.4 | 2.3 |
| | -4.6 | NA | ***20.3*** | 1.6 | | -5.6 | NA | 2.9 | 0.8 |
| | -5 | 4.8 | 13.2 | 12.2 | | -6 | 1.4 | 1.6 | 4.1 |
| | -6 | 4.6 | 12.7 | 1.3 | | -7 | 1.0 | 0.1 | 3.8 |
| | -7 | 3.7 | NA | NA | | -8 | 1.6 | 3.5 | 1.2 |
| | -8 | 4.9 | NA | NA | | -9 | 3.0 | NA | NA |
| | -9 | 1.3 | NA | NA | | -10 | 3.2 | NA | NA |
| | -10 | 3.0 | NA | NA | | | | | |
| | **Mean** | **3.4** | **14.0** | **4.6** | | **Mean** | **2.0** | **2.8** | **3.1** |
| | SD | 1.6 | 6.3 | 4.0 | | SD | 1.0 | 2.3 | 1.8 |

**Table 12.8-1. (continued)**

| Chemical | Log[RC] | Rep 1 | Rep 2 | Rep 3 | Chemical | Log[RC] | Rep 1 | Rep 2 | Rep 3 |
|---|---|---|---|---|---|---|---|---|---|
| CR11603 | -3 | 5.8 | 10.0 | 8.8 | CR11611 | -5 | 1.7 | 3.7 | 3.9 |
| Tributyltin | -4 | 2.7 | 2.9 | 9.6 | Ronidazole | -6 | 1.1 | 3.8 | 4.1 |
| | -5 | 6.6 | 2.9 | 6.0 | | -7 | 6.2 | 6.5 | 3.8 |
| | -6 | 1.8 | 2.5 | 3.0 | | -7.3 | NA | 3.9 | ***38.3*** |
| | -7 | 5.9 | 8.2 | 7.5 | | -8 | 1.7 | 4.5 | 2.8 |
| | -8 | 2.6 | 5.4 | 5.7 | | -8.3 | NA | 3.2 | ***16.0*** |
| | -9 | 4.5 | 0.8 | 6.8 | | -9 | 4.3 | 2.5 | 3.5 |
| | -10 | 6.1 | 4.0 | 3.0 | | -10 | 3.2 | 4.4 | 3.9 |
| | **Mean** | **4.5** | **4.6** | **6.3** | | **Mean** | **3.0** | **4.1** | **9.5** |
| | SD | 1.9 | 3.1 | 2.4 | | SD | 2.0 | 1.2 | 12.4 |
| CR11604 | -4 | 1.3 | 5.6 | 4.3 | CR11612 | -3.3 | NA | 5.8 | 1.1 |
| Diethyl- | -5 | 4.1 | 3.8 | 3.9 | Genistein | -3.6 | NA | 2.3 | 4.1 |
| phthalate | -6 | 4.8 | 4.5 | 5.1 | | -4 | 2.8 | 2.3 | 3.1 |
| | -6.3 | | 4.5 | 4.3 | | -5 | 2.5 | 2.0 | 1.5 |
| | -7 | 7.1 | 2.4 | 4.6 | | -6 | 3.5 | 2.8 | 1.4 |
| | -8 | 6.7 | 3.4 | 0.7 | | -7 | 1.8 | 2.0 | 4.6 |
| | -9 | 1.6 | 4.4 | 3.6 | | -8 | 2.5 | 2.5 | 5.4 |
| | -10 | 5.5 | 2.4 | 2.3 | | -9 | 5.0 | 2.7 | 1.6 |
| | | | | | | -10 | 4.3 | NA | NA |
| | **Mean** | **4.4** | **3.9** | **3.6** | | **Mean** | **3.2** | **2.8** | **2.9** |
| | SD | 2.3 | 1.1 | 1.4 | | SD | 1.1 | 1.2 | 1.7 |
| CR11605 | -5 | 4.4 | 1.3 | 10.6 | CR11613 | -5 | 1.2 | 3.6 | 0.3 |
| Methoxychlor | -6 | 6.4 | 2.8 | 4.7 | p,p'-DDE | -6 | 3.5 | 5.0 | 4.0 |
| | -7 | 11.9 | 1.6 | 4.7 | | -7 | 1.1 | 4.2 | 1.6 |
| | -7.3 | NA | 10.9 | 14.9 | | -7.3 | NA | 3.1 | 8.6 |
| | -8 | 4.7 | 4.2 | 10.0 | | -8 | 3.0 | 1.7 | 8.0 |
| | -8.3 | NA | 10.4 | 0.7 | | -8.3 | NA | 2.9 | 5.0 |
| | -9 | 2.9 | 2.7 | 9.6 | | -9 | 2.5 | 5.1 | 3.6 |
| | -10 | 1.1 | 2.7 | 4.2 | | -10 | 4.5 | 2.9 | 4.6 |
| | **Mean** | **5.2** | **4.6** | **7.4** | | **Mean** | **2.6** | **3.5** | **4.5** |
| | SD | 3.7 | 3.9 | 4.6 | | SD | 1.3 | 1.1 | 2.8 |
| CR11606 | -3 | 10.6 | 3.9 | 10.5 | CR11614 | -4 | 10.7 | 5.0 | 6.7 |
| Aldicarb | -4 | 3.0 | 8.3 | 4.7 | Alachlor | -5 | 11.6 | ***19.0*** | 9.8 |
| | -5 | 4.0 | 6.8 | 4.8 | | -6 | 14.0 | ***21.8*** | 4.1 |
| | -6 | 14.7 | 10.4 | 5.8 | | -6.3 | NA | ***16.7*** | 12.2 |
| | -7 | 5.9 | 3.9 | ***24.9*** | | -7 | 5.3 | 4.2 | 7.9 |
| | -8 | 3.5 | 2.1 | 14.8 | | -8 | 8.0 | ***16.7*** | 14.2 |
| | -9 | 3.2 | 8.3 | 12.4 | | -9 | ***25.6*** | 1.4 | 8.2 |
| | -10 | 11.3 | 4.9 | 10.5 | | -10 | 13.3 | ***25.9*** | ***28.6*** |
| | **Mean** | **7.0** | **6.1** | **11.1** | | **Mean** | **12.6** | **13.8** | **11.5** |
| | SD | 4.5 | 2.8 | 6.7 | | SD | 6.4 | 9.1 | 7.6 |

**Table 12.8-1. (continued)**

| Chemical | Log[RC] | Rep 1 | Rep 2 | Rep 3 | Chemical | Log[RC] | Rep 1 | Rep 2 | Rep 3 |
|---|---|---|---|---|---|---|---|---|---|
| CR11607 | -3.6 | NA | 2.2 | 1.7 | CR11615 | -4.3 | NA | 4.3 | 4.0 |
| Flavone | -4 | 4.9 | 2.2 | 2.9 | Nitrofen | -4.6 | NA | 0.9 | 0.8 |
| | -4.3 | NA | 3.4 | 0.9 | | -5 | 2.8 | 3.0 | 2.0 |
| | -4.6 | NA | 2.8 | 1.7 | | -5.3 | NA | 3.2 | 1.7 |
| | -5 | 6.6 | 1.3 | 1.9 | | -5.6 | NA | 2.3 | 1.5 |
| | -5.3 | NA | 2.3 | 10.9 | | -6 | 4.1 | 5.9 | 0.7 |
| | -6 | 11.7 | 1.3 | 4.1 | | -7 | 5.5 | 3.3 | 1.7 |
| | -7 | 3.3 | 3.8 | 4.5 | | -8 | 6.7 | 2.0 | 0.4 |
| | -8 | 5.6 | NA | NA | | -9 | 2.4 | NA | NA |
| | -9 | 2.2 | NA | NA | | -10 | 4.2 | NA | NA |
| | -10 | 3.2 | NA | NA | | | | | |
| | **Mean** | **5.3** | **2.4** | **3.6** | | **Mean** | **4.3** | **3.1** | **1.6** |
| | SD | 3.2 | 0.9 | 3.2 | | SD | 1.6 | 1.5 | 1.1 |
| CR11608 | -5 | 2.7 | 4.2 | 1.4 | CR11616 | -4 | 10.9 | 4.0 | 1.1 |
| Triadimefon | -5.3 | NA | 0.4 | 0.6 | Trifluralin | -5 | 11.9 | 1.8 | 2.3 |
| | -5.6 | NA | 3.9 | 0.4 | | -6 | 9.0 | 9.1 | 0.8 |
| | -6 | 1.6 | 1.8 | 0.6 | | -6.3 | NA | 1.5 | 1.3 |
| | -7 | 4.9 | 0.3 | 2.4 | | -7 | 2.1 | 3.5 | 5.8 |
| | -8 | 2.0 | 0.5 | 1.4 | | -8 | ***26.7*** | 2.2 | 2.3 |
| | -9 | 1.6 | NA | NA | | -9 | 7.8 | 3.7 | 3.3 |
| | -10 | 1.9 | NA | NA | | -10 | ***20.2*** | 10.2 | 1.6 |
| | **Mean** | **2.5** | **1.8** | **1.1** | | **Mean** | **12.7** | **4.5** | **2.3** |
| | SD | 1.3 | 1.8 | 0.8 | | SD | 8.2 | 3.3 | 1.6 |

* Concentrations showing insolubility were excluded.

†CR11602 runs reported are 1, 4 and 5.

NA Not applicable – this concentration was not assayed in this run

***Bold, italicized*** type denotes CVs > 15%

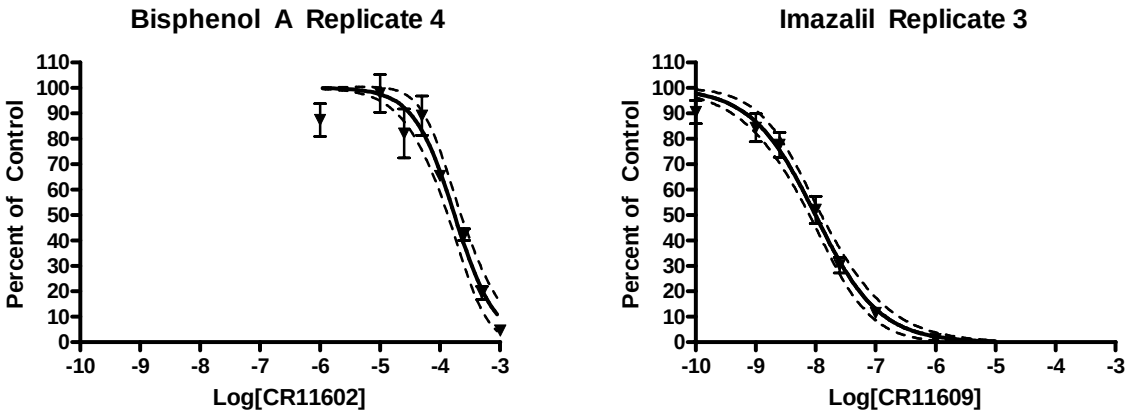


**Figure 12.8-1 Concentration response curves for runs with CV%>15%**

## 12.9 Discussion

The response of the aromatase activity in recombinant microsomes to the presence of 16 chemicals was determined in this task order. The assay conditions used were the same as those used in the interlaboratory validation for the assay. Each chemical was assayed in triplicate tubes for each of 8 concentrations in three runs of the aromatase assay. Controls for the assay included both full enzyme activity (no test inhibitor present) and background activity (no NADPH present) controls. In addition, each assay included duplicate tubes for each of 8 concentrations of the known aromatase inhibitor, 4-OH ASDN, which served as a positive control.

Each technician (4 total) who participated in the task order was required to obtain satisfactory results in the assay of the positive control chemical, 4-OH ASDN, before proceeding to the reference chemical testing. All technicians, each of whom had varying degrees of prior experience with the assay, successfully completed the positive control assay on the first try.

Full activity and background controls were run with each assay. The mean (± SD) full aromatase activity for this batch of recombinant microsomes was 0.313 ± 0.085 nmol/mg/min. In only 1 (of 50 total conducted) assay was the full activity <0.1 nmol/mg/min, and this assay was excluded from analysis. The mean (± SD) uncorrected background activity was 0.002 ± 0.001 nmol/mg/min, which corresponded to 0.7 ± 0.3 percent of control. There were five assays where the uncorrected background activity was more than 1 percent of control; the highest was at 1.8 percent of control. In each of these cases, there seemed to be a lower than normal full aromatase activity rather than an elevated background activity. Since background activity was in a normal range and all data is corrected for background before calculating percent of control, assays with these slightly elevated uncorrected backgrounds as percent of control values were not excluded from the data sets.

Results from the positive control assays that were run in conjunction with the reference chemical runs indicated no significant difference in percent of control values over all concentrations between technicians. There were some statistically significant differences in percent of control values from run-to-run within a technician. Proposed performance criteria for the four model fit parameters (Top, Bottom, $LogIC_{50}$, and HillSlope) for the positive control assay were met in 39 of 48 assays, and there was some indication that failures in 4 of the assays were due to a solution preparation problem. Most of the failures involved only one of the four parameters, and the values were usually just outside the criteria ranges. In fact, the 95 percent confidence interval bands for each parameter always intersected the performance criteria ranges. Data and curve fits for the assays which had performance criteria failures were examined and did not indicate any serious flaw in the functioning of the aromatase assay. If a laboratory obtains 3 of the 4 criteria, and has reasonable justification for why the $4^{th}$ criteria was not met, it may be possible to proceed using that data set. If one or more of the criteria lies unreasonably outside of the criteria ranges, then that run will likely need to be repeated.

It may be advisable to allow acceptance of data sets where only one of the four parameters fails the performance criteria, especially in cases where the 95 percent confidence interval band for the parameter in question includes values within the performance criteria ranges.

Reference chemicals were formulated by the Chemical Repository and were coded (blind) prior to distribution. All runs of a given reference chemical were conducted by the same technician.

Percent of control data from each run for each reference chemical were fitted to both a four- and two-parameter logistic equation to calculate estimates for Top, Bottom, Log $IC_{50}$, and HillSlope. Top and Bottom were fixed at 100 and 0 percent, respectively, for the two-parameter equation.

All but 1 of the 48 assays of reference chemicals met the performance criterion that required that Top = 100 ± 10 percent. The one failure was associated with a chemical that was not an aromatase inhibitor for which the model fit was extremely poor with huge 95 percent confidence intervals for all parameters. In fact, the data set for that run contained no percent of control values higher than 100 percent. The data indicate that requiring Top = 100 ± 10 percent is an acceptable performance criterion for the assay of test chemicals.

The two model curve fits were compared in Prism to determine if they were statistically different. In about half of the cases where Prism was able to fit both models, there were significant differences in the model fits. There was no discernible pattern in the data that would allow prediction of the best model for a particular data set. When the data nearly span the range 0-100 percent of control, either model can produce good parameter estimates. The increased flexibility of the 4-parameter model usually leads to better curve fits when the inhibited range does not span the full range of 0 to 100 percent. However, care must be taken in interpreting Prism results from the 4-parameter equation in cases where the 95 percent confidence intervals of Top and Bottom do not encompass 100 and 0 percent, respectively. Prism estimates '$LogEC_{50}$' as the concentration corresponding to the median of Top and Bottom, and this is not equivalent to the concentration corresponding to 50 percent inhibition ($LogIC_{50}$), when Top and Bottom are not encompassed by 100 and 0 percent, respectively. Equation 3, a modification to the 4-parameter model described in the Results section, can be used to accurately estimate $LogIC_{50}$ in those cases.

In order to develop a second performance criterion for the test chemical assays, within run coefficients of variation (CV) for the percent of control values at each concentration were examined. Often the CVs were less than 10 percent, but in some cases they ranged to 15 percent or higher. High (>15 percent) CVs are most common at low percent of control activity because of the higher measurement variance in the LSS data at these near background levels. The mean CVs across all concentrations within a run were <15 percent in 47 of the 48 assays conducted and were ≤10 percent in 41 of the 48 assays. Of the seven with CV>10 percent, only two assays involved chemicals that inhibited aromatase. The 95 percent confidence interval bands on the curve fits for those two runs were narrow, indicating a good fit for the model. This suggests that data sets should not be rejected automatically even when CVs range up to about 15 percent, but rather that higher CVs should prompt further review of the data, including review of percent of control values for outliers that might indicate experimental error and review of the 95 percent confidence intervals for the fitted curve and parameter estimates.

Only 6 of the 16 test chemicals inhibited aromatase activity >50 percent and these were all classified as inhibitors. These six compounds were: bisphenol A, flavone, triadimefon, imazalil, apigenin, and nitrofen. None of the test chemicals had a maximal inhibition in the range 75 to 50 percent of control, so none of them was classified as a chemical for which the data were equivocal. Ten of the test chemicals were classified as noninhibitors since they did not inhibit aromatase activity more than 25 percent at any tested concentration. The absence of assay data with the highest degree of inhibition near the classification boundaries of 75 percent or 50 percent makes evaluation of any data interpretation criterion difficult. In order to generate data that might be useful for evaluating data interpretation at the classification boundaries, the data sets from the chemicals classified as inhibitors were limited so that the maximal inhibition was ca. 40 to 60 percent of control. For some runs, this resulted in there not being enough data to fit a curve. When the curve fit was successful, the 95 percent confidence interval bands were reviewed in order to assess their utility in data evaluation to 'classify' the chemicals. The 95 percent confidence interval bounds were narrow and in all cases where the lowest mean percent of control activity was <50 percent, both confidence interval bounds also fell below 50 percent which would allow the classification of inhibitor to be assigned. Also, in each case when the lowest mean percent of control activity was between 75 percent and 50 percent of control, both confidence interval bounds fell completely within that range and the chemical could be classified as an equivocal inhibitor.

Assessment of any aromatase assay data set for test chemicals should begin with a review of the tube-to-tube variance in percent of control. Variances greater than 10 to 15 percent should be reviewed for any contribution from experimental error and outliers excluded as appropriate. Concentrations that yield percent of control values <10 percent can be expected to have higher CVs between tubes due to limitations of LSS data near background levels. Except in cases where the test chemical was not soluble (which should always be excluded from analysis), data from these concentrations should be allowed to remain in the data set. The data sets should be examined to determine the mean maximum inhibition. Chemicals for which the mean maximum inhibition is >80 percent of control will likely lead to model fit failures or meaningless curve fits and may be classified as non-inhibitors without further analysis. The data show that the 4-parameter model will yield good parameter estimates when the inhibited range spans approximately 100 to 0 percent of control; in cases where the full range of inhibition is less than 0 to 100 percent, an alternative model (Equation 3) is preferred. The fitted curve, 95 percent confidence bounds for the curve and estimated parameters Top and $LogIC_{50}$ should be examined. Overall review of the data from the test chemicals that were classified as inhibitors indicates that it is reasonable to keep Top = 100 ± 10 percent as a performance criterion. Bottom and Hillslope are not as useful in evaluating assay performance because, especially in the case of partial inhibition curves where a bottom plateau is not defined by the data, there can be high variances in these two parameters, even when other parameters (Top, $LogIC_{50}$) have narrow variances. An important indicator of a good curve fit is whether the 95 percent confidence interval bounds conform to the shape of the fitted curve along its entire length. If the 95 percent confidence interval bounds diverge from the fitted curve, further examination of the data is warranted. In that case, it might be necessary to choose a different model (such as Equation 3 instead of the 4-parameter equation) or to reject the data set. As discussed above, the 95 percent confidence

interval bounds of the most inhibited point can be used as a data interpretation criterion to distinguish between the three classifications or to determine that the data is equivocal.

# 13.0 General Conclusions and Compliance with Validation Criteria

## 13.1 General Conclusions

The extensive use of the human placental microsomal aromatase assay in academic labs and pharmaceutical companies as a method to quickly identify potential steroidal and nonsteroidal aromatase inhibitors was the basis of the EDSTAC recommendations for its inclusion in the EDSP Tier I screening battery. However, since the assay had never been standardized, EPA conducted a series of studies to optimize and standardize the protocol for the assay. Early in the process, EPA showed that the substitution of non-human placenta as a source of enzyme in this assay would not be feasible for use as an in vitro screen. In addition, due to the fairly recent availability of recombinant aromatase and its potential ease of use as a source of enzyme for the assay, EPA selected both the human placental microsomal assay and human recombinant aromatase assay for standardization and validation. EPA determined the optimal assay conditions for both versions of the assay and standardized the protocols, demonstrating that both the human placental and the human recombinant assays are reproducible and reliable across chemicals and testing laboratories

EPA successfully established the relevance and reliability of both versions of this assay and demonstrated their equivalence by testing 10 coded chemicals in four laboratories. The laboratories produced highly reproducible results and correctly identified the eight inhibitors and two non-inhibitors in both assays (Tables 13.1-5 and 13.1-6). For the 10 reference chemicals in the interlaboratory studies, the recombinant assay showed slightly greater variability and, therefore, broader 95 percent confidence intervals of the mean log $IC_{50}$s than the placental assay. In all but one case, the 95 percent confidence bands of the log $IC_{50}$s for the same chemical overlapped between labs for the placental assay, and in five cases the 95 percent confidence intervals of the log $IC_{50}$s of the placental assay lay within the 95 percent confidence intervals of the recombinant assay (Table 13.1-1).

**Table 13.1-1. A Comparison of the 95% Confidence Limits of Log $IC_{50}$s**

| | Placental | | Recombinant | | Relative Position |
|---|---|---|---|---|---|
| **Chemical** | Lower Limit | Upper Limit | Lower Limit | Upper Limit | |
| Aminoglutethimide | -5.422 | -5.359 | -5.411 | -5.239 | Overlap |
| Ketoconazole | -5.202 | -5.161 | -5.428 | -4.943 | Within |
| Prochloraz | -7.736 | -7.566 | -7.772 | -7.353 | Within |
| Nonylphenol | -4.596 | -4.570 | -4.798 | -4.665 | Outside |
| Fenarimol | -5.235 | -5.153 | -5.334 | -5.150 | Within |
| Econazole | -8.953 | -8.682 | -8.768 | -8.561 | Overlap |
| Chrysin | -5.763 | -5.490 | -5.773 | -5.458 | Within |
| Dicofol | -4.549 | -4.318 | -5.081 | -4.272 | Within |

The behavior of nonylphenol seemed to be anomalous, appearing as if it were a non-binder when tested at lower concentrations ( 0.001-1 μM) and then giving an unusually steep concentration-

inhibition response curve when subsequently tested at higher concentrations ( 10-100μM) in the interlaboratory studies. It was also reported to be negative in one study in the literature. To resolve this issue, the Ki of nonylphenol was determined, proving that indeed it was a competitive inhibitor. This and the partial inhibition curve obtained by one laboratory for dicofol in the interlaboratory studies underscore the importance of testing at higher concentration levels to avoid false negative or equivocal results. EPA is therefore requiring that, if a chemical is insoluble at $10^{-3}$ M, that the half-log concentration ($10^{-3.3}$ M) be tried, and if that fails, $10^{-4}$ M. However, EPA was encouraged that all of the labs that tested nonylphenol obtained the same steep and abnormally-shaped curves, using both the placental and recombinant aromatase. This clearly demonstrates the reproducibility of the aromatase assays.

To obtain additional data on a wider variety of chemicals, 16 additional chemicals were subsequently tested in the lead laboratory. This group of chemicals covered a broader range of chemical structures, and studies documenting the effects on aromatase activity had been previously reported in the literature. For this study, EPA modified the protocol to include a full eight-concentration curve for the positive control , 4-OH-ASDN, since earlier studies using a single concentration of the positive control, 4-OH ASDN, which showed a 20 percent variation in the interlaboratory studies, was not an adequate indicator of laboratory performance

In addition to demonstrating applicability of the assay to a wider range of chemicals, the testing of the 16 chemicals provided an opportunity to apply proposed performance criteria for the four model fit parameters (Top, Bottom, $LogIC_{50}$, and HillSlope). These four parameters for the positive control assay were met in 39 of 48 assays. Most of the failures involved only one of the four parameters, and the values were usually just outside the criteria ranges. In fact, the 95 percent confidence interval bands for each parameter always intersected the performance criteria ranges. Data and curve fits for the assays that had performance criteria failures were examined and did not indicate any serious flaw in the functioning of the aromatase assay. EPA adjusted only the criterion for the bottom parameter, raising it from 5% to 6% based on a tolerance interval of 80% with 95% confidence.

EPA evaluated the use of the two-parameter model, in which the top and bottom are set at 110% and 0% respectively, and the four-parameter model, in which the top and bottom are assigned values by the model as part of the curve fitting process, and concluded that in about half of the cases where Prism was able to fit both models, there were significant differences in the model fits. When the data nearly span the range 0 to 100 percent of control, either model can produce good parameter estimates. EPA has concluded that the increased flexibility of the four-parameter model usually leads to better curve fits when the inhibited range does not span the full range of 0 to 100 percent and is generally requiring its use; however, care must be taken in cases where the 95 percent confidence intervals of Top and Bottom do not encompass 100 and 0 percent, respectively, as in these cases the estimates of $LogEC_{50}$, the concentration corresponding to the median of Top and Bottom, is not equivalent to the concentration corresponding to 50 percent inhibition ($LogIC_{50}$). A modified version of the modeling equation, discussed in Chapter 5, is necessary in these cases.

Recognizing that good performance with the positive control will generally, but not always, ensure good performance with the test chemical, EPA developed performance criterion for the positive controls (Tables 13.1-2 and 13.1-3). The Agency will allow some leeway (with reasonable justification) in their application to the positive control in view of these results.

**Table 13.1-2. Full Activity and Background Control Criteria***

| | Parameter | Value |
|---|---|---|
| **Full Activity Control** | **Minimum Activity** | **0.100 nmol/mg/min** |
| **Background Control** | **Maximum Activity** | **1% of full activity control** |

*The full activity control for the placental assay is 0.03 nmol/mg/min

**Table 13.1-3 Performance Criteria for the Positive Control**

| | Parameter | Lower limit | Upper Limit |
|---|---|---|---|
| **Positive Control** | **Slope** | **-1.2** | **-0.8** |
| | **Top (%)** | **90** | **110** |
| | **Bottom (%)** | **-5** | **+6** |
| | **Log $IC_{50}$** | **-7.3** | **-7.0** |

EPA did not adopt criteria for test chemicals but concluded that within-run mean coefficients of variation (CVs) for the percent of control values should be less than 15 percent and that higher CVs should prompt further review of the data, including review of percent of control values for outliers that might indicate experimental error and a review of the 95 percent confidence intervals for the fitted curve and parameter estimates.

EPA considered three approaches for interpreting data. The first focused on the calculation of an $IC_{50}$. EPA concluded that extrapolation by the model may result in too many false positives and the focus turned to whether or not the regression curve itself was supported by data below 50%. This lead EPA to an approach in which the inhibition curve generated by the non-linear regression model is compared with specified criteria (i.e. less than 50% for a positive and greater than 75% for a negative). The third approach EPA considered involved comparison of the 95 percent confidence limits with specified criteria. Although there is considerable appeal to the use of the confidence limits, EPA concluded that the latter two different approaches performed comparably on the data sets generated in the validation program and decided to adopt the second option, simple criteria involving the best fit curve, as the most practical approach. Without firm data acceptance criteria for test chemicals, EPA was concerned that the confidence interval approach would lead to wide confidence intervals and a disproportionate number of chemicals being classified as equivocal.

**Table 13.1-4. Adopted Data Interpretation Criteria**

| Criteria | | Classification |
|---|---|---|
| Data fit 4-parameter nonlinear regression model | Curve crosses 50% | Inhibitor |
| | Lower portion of curve is between 50% and 75% activity | Equivocal |
| Data do not fit the model | Data points at highest concentration range below 75% | |
| | Data points at the highest concentration are above 75% | Non-inhibitor |

**Table 13.1-5. Placental Aromatase Assay: Classification Based on 3 Runs for Each Chemical**

| Chemical | RTI | Battelle | In Vitro | WIL |
|---|---|---|---|---|
| **Aminoglutethimide** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Ketoconazole** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Prochloraz** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **4-Nonylphenol** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Dibenz[a,h]anthracene** | Non-Inhibitor | Non-Inhibitor | Non-Inhibitor | Non-Inhibitor |
| **Fenarimol** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Econazole** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Chrysin** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Dicofol** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Atrazine** | Non-Inhibitor | Non-inhibitor | Non-inhibitor | Non-inhibitor |

**Table 13.1-6. Recombinant Aromatase Assay: Classification Based on 3 Runs for Each Chemical**

| Chemical | RTI | Battelle | In Vitro | WIL |
|---|---|---|---|---|
| **Aminoglutethimide** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Ketoconazole** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Prochloraz** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **4-Nonylphenol** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Dibenz[a,h]anthracene** | Non-Inhibitor | Non-Inhibitor | Non-Inhibitor | Non-Inhibitor |
| **Fenarimol** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Econazole** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Chrysin** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Dicofol** | Inhibitor | Inhibitor | Inhibitor | Inhibitor |
| **Atrazine** | Non-inhibitor | Non-inhibitor | Non-inhibitor | Non-inhibitor |

## FINAL CONCLUSIONS

EPA found that both the human placental and recombinant aromatase assays provide reproducible results consistently across labs for all of the chemicals tested. The data were also

well matched to similar assays in the literature. When deviations did occur from historical values, there was usually a difference in test systems (e.g. a non-human source of enzyme or a recombinant cell line was used). Furthermore, the consistency across labs with observation of an unusual inhibition curve for nonylphenol indicated that this is a true result and emphasize that both assays provide reproducible and reliable data across labs.

## 13.2 Compliance with Validation Criteria

Table 13.2 lists the nine criteria for assay validation as outlined by ICCVAM (NIEHS, 1997), and whether or not each criterion has been met, along with discussion and explanation.

### Table 13.2 Status of validation criteriaa

| Principles | Criteria met/ not met: explanation and justification |
|---|---|
| **a) The rationale for the test method should be available.** This should include a clear statement of the scientific basis, regulatory purpose and need for the test. | The scientific basis and rationale for the test method and its role in the EDSP is described in Chapter 3.0 |
| **b) The relationship between the test method's endpoint(s) and the (biological) phenomenon of interest should be described.** This should include a reference to scientific relevance of the effect(s) measured by the test method in terms of their mechanistic (biological) or empirical (correlative) relationship to the specific type of effect/toxicity of interest. Although the relationship may be mechanistic or correlative, test methods with biological relevance to the effect/toxicity being evaluated are preferred. | Interference with steroid hormone synthesis has been demonstrated to be one way in which chemicals can interfere with the endocrine system. The microsomal aromatase assays detect inhibition of aromatase, the enzyme responsible for the conversion of androgens to estrogens, thereby identifying chemicals that affect this stage of steroid hormone synthesis. |
| **c) A detailed protocol for the test method should be available.** The protocol should be sufficiently detailed and should include, *e.g.,* a description of the materials needed, such as specific cell types that could be used for the test (if applicable), a description of what is measured and how it is measured, a description of how data will be analyzed, decision criteria for evaluation of data and what are the criteria for acceptable test performance. | A detailed protocol, containing all of the required elements, may be found in Appendix A. In addition, the test procedure is described in Chapter 4.0. The protocol specifies what is measured, how it is measured, how data are to be interpreted, and performance criteria. |
| **d) The intra-, and inter-laboratory reproducibility of the test method should be demonstrated.** Data should be available revealing the level of reproducibility and variability within and among laboratories over time. The degree to which biological variability affects the test method reproducibility should be addressed. | The intra and interlaboratory variability were determined using 10 reference chemicals in four laboratories (Chapter 9). In addition, a total of 16 additional chemicals were also tested in the lead laboratory as discussed in Chapter 12. |

| Principles | Criteria met/ not met: explanation and justification |
|---|---|
| **e) Demonstration of the test method's performance should be based on the testing of reference chemicals representative of the types of substances for which the test method will be used.** A sufficient number of reference chemicals should be tested under code to exclude bias. | A total of 35 chemicals representing a variety of chemical classes were tested, most of these under code (Chapters 7.0, 9.0, 12.0). The selection of these chemicals, based on literature test results and a consideration of structural variety, is described in Chapter 6. |
| **f) The performance of the test method should have been evaluated in relation to relevant information from the existing relevant toxicity testing data.** | Data from a variety of literature sources—including recombinant assays, placental assays, and cell based assays --were compiled for the assessment of the performance of the assay. See Chapter 6 for chemical selection and Chapters 9, 10 and 12 for an assessment of the performance of the assay. |
| **g) The limitations of the assay should be described.** | The limitations of the assay are as follows: The assay cannot detect chemicals that induce aromatase and thereby increase aromatase activity; denaturation of the receptor may give false positive results; it cannot test poorly soluble chemicals; and the assay has limited to no ability to metabolize xenobiotics. (Chapter 3.4) |
| **h) Ideally, all data supporting the validity of a test method should have been obtained in accordance with the principles of GLP.** Aspects of data collection not performed according to GLP should be clearly identified and their potential impact on the validation status of the test method should be indicated. | Some laboratories operated under GLP conditions, others did not. All data were audited by the Contractor's Quality Assurance Unit. Final laboratory reports were issued only in draft because of the expiration of the contract performance period. EPA does not believe this affected the quality of the data or compromised the validation effort. |
| **i) All data supporting the assessment of the validity of the test method should be available for expert review.** The detailed test method protocol should be readily available and in the public domain. The data supporting the validity of the test method should be organized and easily accessible to allow for independent review(s), as appropriate. The test method description should be sufficiently detailed to permit an independent laboratory to follow the procedures and generate equivalent data. Benchmarks should be available by which an independent laboratory can itself assess its proper adherence to the protocol. | This Integrated Summary Report is intended to be the primary vehicle for the peer review, but all underlying reports and raw data are available to the peer review panel. Performance criteria have been developed to provide feedback on laboratory performance. |

# 14.0 References

## 14.1 Reports Supporting the Validation of the Aromatase Assay

*[Editor's note: The documents supporting the validation of aromatase track the various stages of the validation process and are referred to numerically in the text, for example, (Reference 1). In addition to the validation-related reports listed below, for each of the multiple laboratory studies (studies 7 through 12), separate laboratory reports exist for the work performed at RTI, Battelle, WIL, and In Vitro.]*


1. Final Detailed Review Paper on Aromatase--Report of EPA Contract No. 68-W-01-023. Work Assignments 2-7 and 5-5, Task 2. Battelle, March 30, 2005.

2. Prevalidation of the Aromatase Assay Using Human, Bovine and Porcine Placental Microsomes –Report of EPA Contract No. 68-W-01-023 Work Assignment 2-24. Battelle, June 30, 2005.

3. Microsomal Aromatase Prevalidation Supplementary Study: Determine Day to Day and Technician Variability–Report of EPA Contract No. 68-W-01-023. Work Assignment 4-10, Task 3. Battelle, January 31, 2006.

4. Microsomal Aromatase Prevalidation Supplementary Study: Establish Inhibition Curves and IC50s for Two Reference Chemicals—Report of EPA Contract No. 68-W-01-023. Work Assignment 4-10, Task 4. Battelle, January 31, 2006.

5. Microsomal Aromatase Prevalidation Supplementary Study: Compare Estrone and Tritiated Water Measurement Methods—Report of EPA Contract No. 68-W-01-023. Work Assignment 4-10, Task 5. Battelle, January 31, 2006.

6. Microsomal Aromatase Prevalidation Supplementary Study: Summation of Findings and Revised Aromatase Protocol—Report of EPA Contract No. 68-W-01-023. Work Assignment 4-10, Task 6. Battelle, January 31, 2006

7. Placental Aromatase Assay Validation: Positive Control Study—Report of EPA Contract No. 68-W-01-023. Work Assignment 4-16, Task 4. Battelle, November 18, 2005.

8. Placental Aromatase Assay Validation: Multiple Chemicals Studies with Centrally Prepared Microsomes—Report of EPA Contract No. 68-W-01-023. Work Assignment 4-16, Task 5. Battelle, February 2006.

9. Placental Aromatase Assay Validation: Prepare Microsomes in Two Participating Laboratories—Report of EPA Contract No. 68-W-01-023. Work Assignment 4-16, Task 6. Battelle, March 2006.

10. Placental Aromatase Assay Validation: Multiple Chemical Studies with Microsomes Prepared in Participating Laboratories—Report of EPA Contract No. 68-W-01-023. Work Assignment 4-16, Task 7. Battelle, June 2006.

10-A. Placental Aromatase Validation Study: Conduct Multiple Chemical Studies with Microsomes Prepared in Participating Laboratories, Audited Draft Task Report prepared for U.S. EPA Contract 68-W-01-023, WA 4-16, Task 7, by RTI International, Research Triangle Park, NC, March 24, 2006.

10-B. Aromatase Assay Validation: Conduct Multiple Chemical Studies with [Placental] Microsomes Prepared in Participating Laboratories, Draft Report prepared for U.S. EPA Contract 68-W-01-023, WA 4-16, Task 7, by Battelle, Columbus, OH, March 24, 2006.

10-C. Placental Aromatase Validation Study: Evaluation of the Potential of Reference Chemicals to Inhibit Aromatase Activity in Human Placental Microsomes, Draft Final Task Report prepared for U.S. EPA Contract 68-W-01-023, WA 4-16, Task 7, by In Vitro Technologies, Inc., Baltimore, MD, March 25, 2006.

10-D. Validation of the Placental Aromatase Assay for Endocrine Disruptor Screening Using Prepared Microsomes, Audited Task Report prepared for U.S. EPA Contract 68-W-01-023, WA 4-16, Task 7, WIL-431009, by WIL Laboratories, LLC, Ashland, OH, January 18, 2006.

11. Human Recombinant Microsomal Aromatase Assay Validation Study: Positive Control Study—Report of EPA Contract No. 68-W-01-023. Work Assignment 4-17, Task 3. Battelle, January 2006.

12. Recombinant Aromatase Validation Study: Conduct Multiple Chemical Studies with Recombinant Microsomes, Draft Final Report of EPA Contract No. 68-W-01-023. Work Assignment 4-17, Task 4, by Battelle, Columbus, OH, June 2006.

12-A. Recombinant Aromatase Validation Study: Conduct Multiple Chemical Studies with Microsomes, Draft Task Report prepared for U.S. EPA Contract 68-W-01-023, WA 4-17, Task 4, by RTI International, Research Triangle Park, NC, March 24, 2006.

12-B. Aromatase Assay Validation: Conduct Multiple Chemical Studies with Recombinant Microsomes, Draft Report prepared for U.S. EPA Contract 68-W-01-023, WA 4-17, Task 4, by Battelle, Columbus, OH, June 14, 2006.

12-C. Recombinant Aromatase Validation Study: Evaluation of the Potential of Reference Chemicals to Inhibit Activity of Recombinant Aromatase, Draft Final Task Report prepared for U.S. EPA Contract 68-W-01-023, WA 4-17, Task 4, by In Vitro Technologies, Inc., Baltimore, MD, June 15, 2006.

12-D. Validation of the Aromatase Assay for Endocrine Disruptor Screening Using Recombinant Microsomes, Audited Task Report prepared for U.S. EPA Contract 68-W-01-023, WA 4-17, Task 4, WIL-431011, by WIL Laboratories, LLC, Ashland, OH, January 18, 2006.

13. Characterization of the Inhibition of Aromatase Activity by Nonylphenol—Report of EPA Contract No. EP-W-06-026. Task Order 3. RTI International, March 2007.

14. Supplementary Testing of 16 Chemicals in the Recombinant Aromatase Assay—Report of EPA Contract No. EP-W—6-026. Task Order 9. RTI International, June 28, 2007.

## 14.2 Other References Cited in this Report

*[Editor's note: For convenience in tracking the two kinds of sources cited in this report, the other references listed below are referred to in the text using the parenthetical author-date method, for example, (Ayub and Levell 1988).*

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Banting, L., Smith, H. J., James, M., Jones, G., Nazareth, W., Nicholls, P. J., Hewlins, M. J., and Rowlands, M. G. Structure-activity relationships for non-steroidal inhibitors of aromatase. J. Enzyme Inhib., 2: 215-229, 1988.

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**APPENDIX A**

# Aromatase Assay Protocol

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Study Code: To be determined (TBD)

TITLE: Assay to Determine the Effect of Test Chemicals on the Aromatase Activity of Human Placental Microsomes

TESTING FACILITY: TBD

PROPOSED EXPERIMENTAL START DATE: TBD
PROPOSED EXPERIMENTAL END DATE: TBD

AMENDMENTS:

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

**Approved By:**

| | |
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| Study Director | Date |
| Laboratory Director | Date |

**Reviewed By:**

| | |
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| Quality Assurance Specialist | Date |

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# 1.0 OBJECTIVE

The objective of this protocol is to describe procedures for conduct of the aromatase assay as a Tier 1 screen using either human placental or recombinant microsomes.

# 2.0 MATERIALS RECEIPT AND/OR PREPARATION

A sufficient supply of chemical reagents, radiolabeled and non-radiolabeled androstenedione, and microsomal preparation from the human placenta will be obtained prior to initiation of the first set of experiments to ensure that sufficient quantities are available to conduct the studies. [Note: Use caution when working with human tissue, follow institutional safety regulations for working with human tissue].

## 2.1 Substrate

### 2.1.1 Substrate Name/Supplier

The substrate for the aromatase assay is androstenedione (ASDN). Non-radiolabeled and radiolabeled androstenedione ([1β-$^3$H]-androstenedione, [$^3$H]ASDN) will be used. The non-radiolabeled ASDN should be ≥ 98% pure. The radiolabeled ASDN should be ≥ 95% radiochemically pure and is usually supplied at a specific activity of 20-30 μCi/mmol. The [$^3$H]ASDN must have a specific activity of no less than 500 μCi/mmol. All applicable information regarding supplier, lot numbers and reported/measured purity for the substrate will be included in study reports.

### 2.1.2 Radiochemical Purity

The radiochemical purity of the [$^3$H]ASDN will be determined using high performance liquid chromatography (HPLC) and liquid scintillation counting.

The HPLC method uses a 4.6 x 250 mm column with a mobile phase of 55:15:30 (v:v:v) distilled, deionized water: tetrahydrofuran: methanol and a flow rate of 1 mL/min. The eluant will be monitored by UV absorbance at 240 nm and by a flow-through radiochemical detector. Eluant fractions will be collected manually into vials and assayed for radiochemical content by liquid scintillation spectrometry (LSS). A reference standard of nonradiolabeled ASDN will be analyzed by the same method and coelution of the nonradiolabeled and radiolabeled ASDN will be confirmed.

The radiochemical purity of the [$^3$H]ASDN shall be greater than approximately 95 percent. If the radiochemical purity is less than 95 percent, then a new batch of radiochemical shall be obtained.

### 2.1.3 Preparation of Substrate Solution for use in Aromatase Assay

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

The following example illustrates the preparation of a substrate solution using a stock of [$^3$H]ASDN with a specific activity of 25.3 Ci/mmol and a concentration of 1 mCi/mL.

- Prepare a 1:100 dilution of the radiolabeled stock in buffer.
- Prepare a 1 mg/mL solution of ASDN in ethanol and then prepare dilutions in buffer to a final concentration of 1 µg/mL.
- Combine 4.5 mL of the 1 µg/mL solution of ASDN, 800 µL of the [$^3$H]ASDN dilution and 2.7 mL buffer to make 8 mL of substrate solution (enough for 80 tubes).
- Record the weight of each component added to the substrate solution. After mixing the solution well, create 20 µL aliquots and combine with scintillation cocktail for radiochemical content analysis.
- Add 100 µL of the substrate solution to each 2 mL assay volume to yield a final [$^3$H]ASDN concentration of 100 nM with 0.1 µCi/tube.

## 2.2 Test Chemicals

The information listed below should be provided for each test chemical.

CAS No:
Molecular Formula/Weight:
Supplier/source:
Lot No.
Purity:
Storage Conditions:
Solubility:

Test chemical stock solutions will be prepared and analyzed and the method of analysis stated. Test chemicals will be formulated in buffer, absolute ethanol or dimethylsulfoxide (DMSO). The total volume of test chemical formulation used in each assay should be no more than 1 percent of the total assay volume (i.e., 20 $\mu$L in a 2 mL assay) in order to minimize the potential of the solvent to inhibit the enzyme. Fresh dilutions of the stock solution will be prepared in the same solvent as the stock solution on the day of use such that the target concentration of test chemical can be achieved by the addition of 20 $\mu$L of the dilution to a 2 mL assay volume. Information on storage conditions for test chemical stock solutions will be reported.

## 2.3 Positive Control

The known aromatase inhibitor, 4-hydroxyandrostendione (4-OH ASDN), is used as the positive control. Table 1 contains identity and property information for 4-OH ASDN.

**Table 1. Positive Control Substance**

| Test Substance | CAS Number | Molecular Formula | Molecular Weight (g/mol) |
|---|---|---|---|
| 4-OH ASDN | 566-48-3 | $C_{19}H_{26}O_3$ | 302.4 |

The positive control stock solution will be prepared and analyzed and the method of analysis stated. The 4-OH ASDN will be formulated in buffer, absolute ethanol or DMSO. The total volume of control substance formulation used in each assay should be no more than 1 percent of the total assay volume (i.e., 20 $\mu$L in a 2 mL assay) in order to minimize the potential of the solvent to inhibit the enzyme. Fresh dilutions of the stock solution will be prepared in the same solvent as the stock solution on the day of use. Dilutions will be prepared such that the target concentrations of control substance (Table 4) can be achieved by the addition of 20 $\mu$L of the dilution to a 2 mL assay volume. Information on storage conditions for the control substance stock solutions will be reported.

## 2.4 Microsomes

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

### 2.4.1 Human Placental Microsomes

Human placental microsomes will be prepared. These samples should be treated as potentially infectious, and appropriate precautions must be employed. The microsomes must be stored at -70 to -80°C. The approximate protein content of the microsomes shall be 14 mg/mL.

**2.4.1.1 Source of the Placentas.** Human placenta will be obtained from a local hospital. The exact source of placentas must be documented in the study records. Human placentas are to be from non-smoking, 21-40 year old mothers with full term deliveries. Within 30 minutes of the delivery of the placenta by the mother, it will be placed in a tissue bag, sealed, and packed in wet ice in an insulated shipping container. Placenta tissue bags will be labeled with date and time of delivery. Laboratory personnel will be on-call and will be responsible for transporting

placentas to their laboratory for processing into microsomes, as described below. Efforts will be made to minimize the time from delivery to the initiation of microsome preparation.

**2.4.1.2 Placental Microsome Preparation.** While keeping the placenta chilled on ice, the membrane and fibrous material are dissected, removed and discarded, and the tissue is then homogenized in portions using a Potter Elvejhem homogenizer (or similar) in a buffer (2:1, w:v) containing 0.25 M sucrose, 0.05 M sodium phosphate (pH 7.0), and 0.04 M nicotinamide. Next, the microsomal preparation is isolated by differential centrifugation procedures:

- Homogenize the tissue and centrifuge at a setting of 10,000g for 30 minutes in an IEC B-22M centrifuge (or similar);
- Isolate the supernatant and centrifuge at a setting of 35,000 rpm (which is equivalent to approximately 100,000g) in a Beckman L5-50B Ultracentrifuge (or similar) for one hour to obtain the crude microsomal pellet.
- Remove the supernatant and resuspend the microsomal pellet in 0.1 M sodium phosphate buffer (pH 7.4),
- Recentrifuge at a setting of 35,000 rpm (ca. 100,000g, Beckman L5-50B) for one hour to wash the microsomes.
- Repeat the washing procedure one additional time.
- Resuspend the twice-washed microsomal pellet in 0.1 M sodium phosphate buffer (pH 7.4) containing 0.25 M sucrose, 20 percent glycerol, and 0.05 mM dithiothreitol.
- Aliquot the suspension into vials (labeled with the notebook page reference for the preparation of the microsomes) that are convenient for conducting the aromatase assay [The Cytochrome P450 spectrum assay uses 2 mg/mL and the aromatase assay uses approximately 0.1 mg/mL of microsomal protein.]
- Flash freeze the aliquot vials in liquid nitrogen and store at approximately -70 C until removed for use. Under these storage conditions, the microsomal suspension retains aromatase activity for more than one year.

**2.4.1.3 Use of the Placental Microsomes**

On the day of use, microsomes are thawed quickly in a 37 ± 1° C water bath and then are immediately transferred to an ice bath.

- Rehomogenize the microsomes using a Potter-Elvejhem (or similar) homogenizer (about 5-10 passes) prior to use.
- Dilute the microsomes in buffer (serial dilutions may be necessary) to an approximate protein concentration of 0.025 mg/mL. The addition of 1 mL of that microsome dilution will result in a final approximate protein concentration of 0.0125 mg/mL in the assay tubes.
- Keep all microsome samples on ice until they are placed in the water bath just prior to their addition to the aromatase assay. It is recommended that microsomes not be left on ice for longer than approximately 2 h before proceeding with the assay or microsomal enzyme activity may be decreased.

Excess undiluted stock microsomes may be flash frozen in liquid nitrogen and returned to -70 to -80 °C storage for future use. It is strongly recommended that stock microsomes to be refrozen be divided into aliquots appropriate for use prior to refreezing in order to minimize the number of freeze/thaw cycles.

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

### 2.4.2 Human Recombinant Microsomes

**2.4.2.1 Source of the Human Recombinant Microsomes.** Human recombinant microsomes are available from Gentest™ (Woburn, MA; www.gentest.com). The product name is Human CYP19 (Aromatase) Supersomes™ and the catalog number is 456260. The Supersomes™ package size is 0.5 nmoles cytochrome P450 in 0.5 mL. The cytochrome P450 content is 1000 pmol/mL. The representative total protein concentration is 4.0 mg/mL in 100 mM potassium phosphate (pH7.4). The representative aromatase activity is 1200 pmol product/mg-protein/min, 5 pmol product/pmol P450/min. (Supplier-provided values for protein concentration, cytochrome c reductase activity, and aromatase activity will be found on the data sheet accompanying each shipment and will be included in the report.) The microsomes should be stored at -70° C.

**2.4.2.2 Human Recombinant Microsome Preparation.** Preparation of the human recombinant microsomes will involve thawing the microsomes rapidly in a 37 ± 1° C water bath and placing them in an ice bath and aliquoting them into individual vials based on the protein content of the batch. This minimizes the freeze-thaw cycles. The assay uses approximately 0.004 mg/ml (final concentration) of microsomal protein. After aliquoting the microsomes into individual vials, the vials that are not planned for immediate use will be flash frozen in liquid nitrogen and then returned to the -70° C freezer for storage.

## 2.5 Other Assay Components

### 2.5.1 Buffer

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

### 2.5.2 Propylene Glycol

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

### 2.5.3 NADPH

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

## 3.0 PRELIMINARY EXPERIMENTS TO DETERMINE SUITABILITY OF MICROSOME PREPARATIONS

### 3.1 Protein Assay

The protein concentration of the microsome preparation will be determined on each day of use of the microsomes in the aromatase assay. A 6-point standard curve will be prepared using bovine serum albumin (BSA), ranging from 0.13 to 1.5 mg protein/mL. Protein will be determined by using a standard protein assay kit such as the DC Protein Assay kit purchased from Bio-Rad (Hercules, CA) and the manufacturer's directions followed.

For the BioRad kit, a 25 µL aliquot of unknown or standard is combined with 125 µL of the BioRad DC Protein Kit Reagent A and mixed. Next, 1 mL of BioRad DC Protein Kit Reagent B is added to each standard or unknown and the samples vortexed. The samples are 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) is transferred to disposable polystyrene cuvettes and the absorbance (@ 750 nm) measured using a spectrophotometer. The protein concentration of the microsomal sample is determined by extrapolation of the absorbance value using the curve developed using the protein standards.

### 3.2 Cytochrome P450 (CYP19) Aromatase Activity

Aromatase activity of the microsome preparations will be determined prior to their use in the assay to determine that they have sufficient activity. Each preparation will be run in triplicate tubes in the aromatase assay using the optimized conditions presented in Table 2 and the method in Section 4.0. Placental aromatase activity must be at least 0.03 nmol/mg-protein/min. The minimum acceptable aromatase activity in human recombinant microsomes is 0.1 nmol/mg-protein/min. If the aromatase activity for the microsomal preparation is below the minimum level, it cannot be used in the assay.

## 4.0 AROMATASE ASSAY METHOD

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

- Label each test tube by applying a label or writing directly on the test tube.
- Adjust the volume of buffer used so the total incubation volume will be 2 mL.

- Combine the following in the test tubes in a total volume of 1 mL: Propylene glycol (100 µL), [$^3$H]ASDN, NADPH, and buffer (0.1 M sodium phosphate buffer, pH 7.4).
- Add the test chemical solution (or vehicle) to the mixture of propylene glycol, substrate, NADPH and buffer in a volume not to exceed 20 µL prior to preincubation of that mixture. The final concentrations for the assay components are presented in Table 3.

- Place the tubes and the microsomal suspension at 37 ± 1°C in the water bath for five minutes prior to initiation of the assay by the addition of 1 mL of the diluted microsomal suspension.
- The total assay volume will be 2 mL, and the tubes will be incubated for 15 min.
- Stop the incubations by the addition of methylene chloride (2 mL);
- Vortex-mix the tubes for ca. 5 s and placed on ice.
- Vortex-mixed the tubes an additional 20-25 s.
- Centrifuged the tubes using a Beckman GS-6R centrifuge with GH-3.8 rotor for 10 minutes at a setting of 1000 rpm.
- Remove the methylene chloride layer and discard;
- Extract the aqueous layers again with methylene chloride (2 mL) and discard the methylene chloride layer.
- Perform this extraction procedure one additional time, discarding the methylene chloride layer.
- Transfer and separate the aqueous layers into two 20-mL liquid scintillation counting vials as duplicate aliquots (0.5 mL).
- Add liquid scintillation cocktail (Ultima Gold, Packard, 10 mL) to each counting vial and shake to mix the solution. The radiochemical content of each aliquot will be determined as described below.

**Table 2. Optimized Aromatase Assay Conditions**

| Assay factor (units) | Human Placental | 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 |

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

Results will be presented as the amount of estrone formed and activity (velocity) of the enzyme reaction. The amount of estrone formed is determined by dividing the total amount of $^3H_2O$ formed by the specific activity of the [$^3$H] ASDN substrate (expressed in dpm/nmol). The activity of the enzyme reaction is expressed in nmol/ mg protein/min and is calculated by dividing the amount of estrone formed by the product of mg microsomal protein used times the incubation time, i.e., 15 minutes.

## 5.0 POSITIVE CONTROL ASSAY

A run is defined as an independent experiment. Each run will contain replicate tubes for full activity control, background activity control, and positive control.

Prior to conducting the assay for the first time, each technician should conduct at least one single run of the positive control experiment as outlined in Table 4. These data should be assessed against the following criteria:

1. The minimum level of mean aromatase activity in the full activity control samples shall be 0.100 nmol/mg/ protein/min.
2. The mean background control activity shall be ≤ 1% of the full activity control.
3. The concentration response curve generated for the 4-OH ASDN should meet the conditions listed in Table 3.

**Table 3: Performance Criteria for the Positive Control**

| | Parameter | Lower limit | Upper Limit |
|---|---|---|---|
| **Positive Control** | **Slope** | **-1.2** | **-0.8** |
| | **Top (%)** | **90** | **110** |
| | **Bottom (%)** | **-5** | **+6** |
| | **Log $IC_{50}$** | **-7.3** | **-7.0** |

**Table 4: Positive Control Study Design**

| Sample Type | Repetitions (tubes) | Description | 4-OH ASDN Conc. (M) |
|---|---|---|---|
| Full Activity Control | 4 | All test components. No inhibitor | N/A |
| Background Activity Control | 4 | Same as full activity control, but no NADPH | N/A |
| 4-OH ASDN Conc 1 | 3 | Complete assay with 4-OH ASDN (positive control) added | $1X10^{-5}$ |
| 4-OH ASDN Conc 2 | 3 | Same | $1X10^{-6}$ |
| 4-OH ASDN Conc 3 | 3 | Same | $1X10^{-6.5}$ |
| 4-OH ASDN Conc 4 | 3 | Same | $1X10^{-7}$ |
| 4-OH ASDN Conc 5 | 3 | Same | $1X10^{-7.5}$ |
| 4-OH ASDN Conc 6 | 3 | Same | $1X10^{-8}$ |
| 4-OH ASDN Conc 7 | 3 | Same | $1X10^{-9}$ |
| 4-OH ASDN Conc 8 | 3 | Same | $1X10^{-10}$ |

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

# 6.0 DETERMINATION OF THE RESPONSE OF AROMATASE ACTIVITY TO TEST CHEMICALS

A run is an independent experiment. Each run will contain replicate tubes for full activity control, background activity control, positive control, and test chemical as shown in Table 5.

Each run will test the response of aromatase activity to the presence of eight concentrations of a test chemical run in triplicate (i.e., there are three tubes of each test chemical concentration per run of the assay). A chemical shall be tested in three independent runs. Each run for a given test chemical must be conducted entirely independently of the other runs for that test chemical. There will be three (triplicate) repetitions for each concentration of a test chemical. A single run of a given test chemical is described in Table 6.

Three types of control samples will be included for each run. These include:

- full enzyme (aromatase) activity controls (substrate, NADPH, propylene glycol, buffer, vehicle [used for preparation of test substance solutions] and microsomes)
- background activity controls (all components that are in the full aromatase activity controls, except NADPH)
- positive controls (4-OH ASDN run at eight concentrations in the same manner as test chemicals)

Four test tubes of the full enzyme activity control and background activity controls are included with each run. The full enzyme and background activity controls sets will be split so that two tubes (of each control type) are run at the beginning and two at the end of each run. The positive control will be tested at eight concentrations in each run as indicated in Table 5. All controls are treated the same as the other samples.

The aromatase assay will be conducted as described in Section 4.0.

After completion of the first run, the data will be reviewed and, if necessary, the concentration of test chemical used in the second and third runs can be adjusted. The decision should be based on the results from the first run with the following guidelines in mind:

- If insolubility (cloudiness or a precipitate) is observed at the highest concentration ($10^{-3}$ M), then set the highest concentration for the second and third runs at the highest concentration that appeared to be soluble using mid-log concentrations; i.e., try $10^{-3..3}$ M if the test chemical is insoluble at $10^{-3}$ M. It is important to define the lower portion of the curve. Do not use a concentration lower than $10^{-5}$ M for the highest concentration tested.
- If the highest concentration to be tested is lowered to $10^{-4}$ or $10^{-5}$ M, then add mid-log concentration(s) near the lower end of the curve (higher concentrations) and around the estimated $IC_{50}$ based on the results of the first run in order to keep eight concentrations in the test set.
- The lowest concentration to be tested is $10^{-10}$ M.

**Table 5. Test Chemical Study Design**

| Sample Type | Repetitions (tubes) | Description | Reference or Chemical Conc (M) |
|---|---|---|---|
| Full Activity Control | 4 | Complete assay[a] with inhibitor vehicle control, no inhibitor | N/A |
| Background Activity Control | 4 | Same as full activity control, but no NADPH | N/A |
| Positive Control Conc 1 | 2 | Complete assay with 4-OH ASDN added | $1X10^{-5}$ |
| Positive Control Conc 2 | 2 | Same | $1X10^{-6}$ |
| Positive Control Conc 3 | 2 | Same | $1X10^{-6.5}$ |
| Positive Control Conc 4 | 2 | Same | $1X10^{-7}$ |
| Positive Control Conc 5 | 2 | Same | $1X10^{-7.5}$ |
| Positive Control Conc 6 | 2 | Same | $1X10^{-8}$ |
| Positive Control Conc 7 | 2 | Same | $1X10^{-9}$ |
| Positive Control Conc 8 | 2 | Same | $1X10^{-10}$ |
| Test Chem. Conc 1 | 3 | Complete assay with test chemical added | $1X10^{-3}$ |
| Test Chem. Conc 2 | 3 | Same | $1X10^{-4}$ |
| Test Chem. Conc 3 | 3 | Same | $1X10^{-5}$ |
| Test Chem. Conc 4 | 3 | Same | $1X10^{-6}$ |
| Test Chem. Conc 5 | 3 | Same | $1X10^{-7}$ |
| Test Chem. Conc 6 | 3 | Same | $1X10^{-8}$ |
| Test Chem. Conc 7 | 3 | Same | $1X10^{-9}$ |
| Test Chem. Conc 8 | 3 | Same | $1X10^{-10}$ |

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

# 7.0 DATA ANALYSIS

## 7.1 Aromatase Activity and Percent of Control Calculations

Relevant data are entered into the assay spreadsheet for calculation of aromatase activity and percent control. The spreadsheet (Appendix 1) calculates DPM/mL for each aliquot of extracted aqueous incubation mixture and average DPM/mL and total DPM for each aqueous portion (after extraction). The volume (mL) of substrate solution added to the incubation multiplied by the substrate solution's specific activity (DPM/mL), yields the total DPM present in the assay tube at initiation. The total DPM remaining in the aqueous portion after extraction divided by the total DPM present in the assay tube at initiation times 100 yields the percent of the substrate that was converted to product. The total DPM remaining in the aqueous portion after extraction is corrected for background by subtracting the average DPM present in the aqueous portion of the negative control tubes. This corrected DPM is then converted to nmol product formed by dividing by the substrate specific activity (DPM/nmol). The activity of the enzyme reaction is expressed in nmol (mg product)$^{-1}$ min$^{-1}$ and is calculated by dividing the amount of estrone formed (nmol) by the product of mg microsomal protein used times the incubation time (15 minutes). Average activity in the full activity control samples is calculated. Percent of control activity remaining in the presence of various inhibitor concentrations,

including the positive control, is calculated by dividing the aromatase activity at a given concentration by the average full activity control and multiplying by 100.

Nominally one might expect for an inhibitor the percent of control activity values to vary between approximately 0 percent near the high inhibition concentrations and approximately 100 percent near the low inhibition concentrations. However due to experimental variation individual observed percent of control values will sometimes extend below 0 percent or above 100 percent.

### 7.2 Model Fitting

The response curve will be fitted by weighted least squares nonlinear regression analysis with weights equal to 1/Y. Model fits will be carried out using a non-linear regression program such as Prism software (Version 3 or higher).

Concentration response trend curves will be fitted to the percent of control activity values within each of the repeat tubes at each test chemical concentration. Concentration is expressed on the log scale. In agreement with past convention, logarithms will be common logarithms (i.e., base 10). Let X denote the logarithm of the concentration of test chemical (e.g., if concentration = $10^{-5}$ then X = -5). Let

Y ≡ percent of control activity in the inhibitor tube
X ≡ logarithm (base 10) of the concentration
DAVG ≡ average DPMs across the repeat tubes with the same test chemical concentration
$\beta$ ≡ slope of the concentration response curve ($\beta$ will be negative)
$\mu \equiv \log_{10}IC_{50}$ ($IC_{50}$ is the concentration corresponding to percent of control activity equal to 50%)

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

$$Y = B + \frac{(T-B)}{1+10^{(\text{Log IC50}-X)\beta+\log[(T-B/50-B)-1]}}$$

Concentration response models will be fitted for each test run for each test chemical.

### 7.3 Graphical and Analysis of Variance Comparisons Among Concentration Response Curve Fits

For each run the individual percent of control values will be plotted versus logarithm of the test chemical concentration. The fitted concentration response curve will be superimposed on the plot. Individual plots will be prepared for each run.

Additional plots will be prepared to compare the percent of control activity values across runs. For each run the average percent of control values will be plotted versus logarithm of test chemical concentration on the same plot. Plotting symbols will distinguish among runs. The

fitted concentration response curves for each run will be superimposed on the plots. On a separate plot the average percent of control values for each run will be plotted versus logarithm of test chemical concentration. The average concentration response curve across runs will be superimposed on the same plot.

For each run treat ($\beta$, $\mu$) as a random variable with mean ($\beta_{avg}$, $\mu_{avg}$). Let X and Y ($0< Y <100$) denote logarithm of concentration and percent of control, as defined above. The average response curve is

$$Y = B + \frac{(T-B)}{1+10^{(\mu avg-X)\beta avg+\log[(T-B/50-B)-1]}} + \varepsilon$$

Slope ($\beta$) and $\log_{10}IC_{50}$ ($\mu$) will also be compared across runs based on one-way random effects analysis of variance, treating the runs as random effects. Plots will be prepared that display the parameters within each run with associated 95 percent confidence intervals based on the within-run standard error and, if three runs are required, the average across-run standard error with the associated 95 percent confidence interval incorporating run-to-run variation.

### 7.4 Quality Control--Analysis of Variance Comparisons of Full Enzyme Activity Control and Background Activity Control As Percent of Control

Within each run of each test chemical quadruplicate repetitions will be made of the full enzyme activity control (FEAC) and background activity control (BAC) control tubes. Half the repetitions will be carried out at the beginning of the run and half at the end. If the conditions are consistent throughout the test, the control tubes at the beginning should be equivalent to those at the end.

To assess whether this is the case the control responses will be adjusted for background DPMs, divided by the average of the (background adjusted) FEAC control values, and expressed as percent of control. The average of the four BAC controls within a run must necessarily be 0 percent and the average of the four FEAC controls within a run must necessarily be 100 percent.

### 7.5 Data Interpretation

Data from the assay will be used to classify chemicals according to their ability to inhibit aromatase. To be classed as an inhibitor, the data must fit the 4-parameter regression model to yield an inhibition curve and result in greater than 50% inhibition at the highest concentration. The value of the inhibition curve at each of three runs at the highest concentration should be averaged and compared with the following criteria. If the data do not fit the model the average activity of the data points at the highest concentration shall be used.

**Table 6. Data Interpretation Criteria**

| Criteria | | Classification |
|---|---|---|
| Data fit 4-parameter nonlinear regression model | Curve crosses 50% | Inhibitor |
| | Lower portion of curve is between 50% and 75% activity | Equivocal |
| Data do not fit the model | Data points at highest concentration range below 75% | |
| | Data points at the highest concentration are above 75% | Non-inhibitor |

### 7.6 Statistical Software

Concentration response curves will be fitted to the data using the non-linear regression analysis features in a commercial software package such as PRISM statistical analysis package, Version 3 or higher. Supplemental statistical analyses and displays such as summary tables, graphical displays, analysis of variance, and multiple comparisons can be carried out using PRISM, the SAS statistical analysis system, Version 8 or higher, or other general purpose statistical packages (e.g., SPSS), as convenient.

## 8.0 RETENTION OF RECORDS

All records that remain the responsibility of the testing laboratory will be retained in the archives for the life of the contract.

## 9.0 GOOD LABORATORY PRACTICES

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

## 10.0 REPORTS

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The data to be reported in the interim data summaries will include (but is not limited to) the following information: assay date and run number, technician code, chemical code and log chemical concentration, background corrected aromatase activity (for each control and test chemical repetition), percent of control activity, $IC_{50}$, slope and graphs of activity versus log chemical concentration.

In addition, draft and final reports will contain tables and graphs, as appropriate, containing the results of the statistical analyses described in Section 6 of this document.

## 11.0 STUDY RECORDS TO BE MAINTAINED

- All records that document the conduct of the laboratory experiments and results obtained, as well as the equipment and chemicals used
- Protocol and any Amendments
- List of any Protocol Deviations
- List of Standard Operating Procedures
- QAPP and any Amendments
- List of any QAPP Deviations

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**APPENDIX B**
**Supplementary Figures and Tables**

## Supplementary Material for Chapter 6

### Table B.6-1. Summary of Literature Results on Aromatase Testing

Chemicals tested in the Interlaboratory Validation Study
Chemicals tested in the supplemental study

**Azoles, Pyrimidines, Azole-like Fungicides**

| Chemical | Test System | IC50 (µM) | Ref |
|---|---|---|---|
| 1-Benzylimidazole | HT | 0.56 | Kragie |
| Bifonazole | Supersomes | 0.24 | Trosken |
| | HT | 0.007 | Kragie |
| | PM | 0.27 | Ayub |
| Biteranol | Supersomes | >300 | Trosken |
| Carbendazim | H295R/ R2C | Neg | Heneweer |
| Clotrimazole | Supersomes | 1.2 | Trosken |
| | HT | 0.018 | Kragie |
| | PM | 0.35 | Ayub |
| Cyproconazole | Supersomes | ~100 | Trosken |
| Diclobutrazole | H295R | Neg | Sanderson 2002 |
| Econazole | HT | 0.004 | Kragie |
| | PM | 0.023 | Ayub |
| | Supersomes | 2.22 | Battelle |
| | PM | 1.61 | Battelle |
| Epoxyconazole | Supersomes | ~100 | Trosken |
| | H295R | 4.5 | Heneweer |
| | R2C | 2 | Heneweer |
| Fadrazole | Supersomes | 0.66 | Trosken |
| Fenarimol | H295R | 80 | Sanderson 2002 |
| | PM | 10 | Vinggaard |
| | KGN | 2.0 | Ohno |
| | Supersomes | 5.63 | Battelle |
| | PM | 5.84 | Battelle |
| Fluconazole | Supersomes | >300 | Trosken |
| Flusilazole | Supersomes | 7.7 | Trosken |
| Hexaconazole | Supersomes | 96 | Trosken |
| Imazalil | Supersomes | 3.6 | Trosken |
| | H295R | 0.1 | Sanderson 2002 |
| | PM | 0.34 | Vinggaard |
| | KGN | 0.0044 | Ohno |

| Chemical | Test System | IC50 (µM) | Ref |
|---|---|---|---|
| Itraconazole | Supersomes | ~100 | Trosken |
| | HT | >100 | Kragie |
| Ketoconazole | Supersomes | 281 | Trosken |
| | HT | 0.9 | Stresser |
| | HT | 2.0 | Kragie |
| | Supersomes | 7.63 | Battelle |
| | PM | 6.47 | Battelle |
| Letrozole | Supersomes | 0.13 | Trosken |
| Metronidazole | HT | >100 | Kragie |
| Miconazole | Supersomes | 8.2 | Trosken |
| | HT | 0.036 | Kragie |
| | PM | 0.45 | Ayub |
| Mycobutanil | Supersomes | 47 | Trosken |
| Nuarimol | H295R | 100 | Sanderson 2002 |
| Penconazole | Supersomes | 47 | Trosken |
| Prochloraz | Supersomes | 0.44 | Trosken |
| | H295R | 0.1 | Sanderson 2002 |
| | PM | 0.04 | Vinggaard |
| | PM | 0.0284 | Battelle |
| | Supersomes | 0.0285 | Battelle |
| Propioconazole | Supersomes | 199 | Trosken |
| | KGN | 0.968 | Ohno |
| Ronidazole | HT | >1000 | Kragie |
| Sulfaphenazole | HT | 129 | Kragie |
| | PM | >100 | Kragie |
| Sulconazole | HT | 0.015 | Kragie |
| Tebuconazole | Supersomes | 609 | Trosken |
| Tetraconazole | HT | 0.22 | Kragie |
| Thiabendazole | HT | 195 | Kragie |
| | PM | >100 | Ayub |
| Tinidazole | HT | 824 | Kragie |
| Triadimefon | Supersomes | 483 | Trosken |
| | PM | 32 | Vinggaard |
| | KGN | 3.59 | Ohno |
| Triadimenol | Supersomes | 972 | Trosken |
| | PM | 21 | Vinggaard |
| Tricyclozole | H295R | Neg | Sanderson 2002 |

Supersomes = BD Gentest supersomes, HT = Supersomes high throughput assay, PM= placental microsomes, KGN=a human ovarian granulose-like tumor cell line, H295R=a human adrenocortical carcinoma cell line

**Other Substances/ Pesticides**

| Chemical | Test System | IC50 (µM) | Ref |
|---|---|---|---|
| Aminoglutethimide | KGN | 2.25 | Ohno |
| | HT | 0.77 | Stresser |
| | Supersomes | 4.46 | Battelle |
| | PM | 4.30 | Battelle |
| | H adipocytes | 7.4 | Campbell |
| Atrazine | H295R | Neg | Sanderson 2002 |
| | Supersomes | Neg | Battelle |
| | PM | Neg | Battelle |
| Dicofol | Supersomes | 25.00 | Battelle |
| | PM | 29.13 | Battelle |
| Dibenz[a,h,]anthracene | Supersomes | Neg | Battelle |
| | PM | Neg | Battelle |
| Imidacloprid | H295R/R2C | Neg | Heneweer |
| Lindane | PM | Neg | Battelle |
| | Supersomes | Neg | Battelle |
| 4-Nonylphenol | Supersomes | 18.87 | Battelle |
| | PM | 29.10 | Battelle |
| | KGN | Neg | Morinaga |
| Vinclozolin | H295R | Neg | Sanderson 2002 |

**Flavones**

| Chemical | Test System | IC50 (µM) | Ref |
|---|---|---|---|
| Apigenin | KGN | 2.58 | Ohno |
| | H295R | 20 | Sanderson 2004 |
| Biochanin A | H adipocytes | 113 | Campbell |
| Catechin | H295R | Neg. | Sanderson 2004 |
| Chrysin | KGN | 1.89 | Ohno |
| | H adipocytes | 4.6 | Campbell |
| | HT | 0.70 | Stresser |
| | Supersomes | 2.52 | Battelle |
| | PM | 2.50 | Battelle |
| | H295R | 7 | Sanderson 2004 |
| Epicatechin | H295R | Neg. | Sanderson 2004 |
| Flavanone | KGN | Weak | Ohno |
| | PM | 8.0 | Ibrahim |
| Flavone | KGN | Weak | Ohno |
| | H adipocytes | 68 | Campbell |
| | PM | 10 | Ibrahim |
| | H295R | Neg | Sanderson 2004 |

| Chemical | Test System | IC50 (µM) | Ref |
|---|---|---|---|
| Genestein | H295R | Neg | Sanderson 2004 |
| 7-Hydroxyflavanone | H295R | 65 | Sanderson 2004 |
| 7-Hydroxyflavone | KGN | 5.31 | Ohno |
| | PM | 0.5 | |
| | H295R | 4 | Sanderson 2004 |
| 7-Methoxyflavone | KGN | 1.18 | Ohno |
| | H295R | Neg | Sanderson 2004 |
| α-Napthoflavone | KGN | 0.412 | Ohno |
| Naringenin | KGN | 2.42 | Ohno |
| | H295R | 85 | Sanderson 2004 |
| Quercetin | H295R | Neg | Sanderson 2004 |
| Rotenone | H295R | 0.3 | Sanderson 2004 |

**Steroid Inhibitors**

| Chemical | Test System | IC50 (µM) | Ref |
|---|---|---|---|
| Exemestane | | 0.027 | |
| 4-Hydroxyandrostenedione | KGN | 0.00115 | Ohno |
| | HT | 0.031 | Stresser |
| | Supersomes | 0.0795 | Battelle |
| | PM | 0.0621 | Battelle |

**Chemicals Tested by Morinaga in the KGN System**

| Chemical | Chemical Group | Result |
|---|---|---|
| Benzo [a,h,]pyrene | Aromatic hydrocarbon | ↑↑ |
| Aldicarb | Carbamate | ↓ |
| Benomyl | Imidazole | ↑↑ |
| Carbaryl | Carbamate | Neg |
| Methomyl | Carbamate | Neg |
| Vinclozolin | Dicarboximide | Neg |
| Aldrin | Organochlorine | ↓ |
| Diedrin | Organochlorine | ↓ |
| Trans-Chlordane | Organochlorine | ↓ |
| Cis-Chlordane | Organochlorine | Neg |
| Heptachlor | Organochlorine | ↑↑ |
| Trans-nonachlor | Organochlorine | Neg |
| Oxychlordane | Organochlorine | Neg |
| p,p'-DDT | Organochlorine | Neg |
| p,p'-DDD | Organochlorine | ↓↓ |
| p,p'-DDE | Organochlorine | ↓↓ |

| Chemical | Chemical Group | Result |
|---|---|---|
| Endrin | Organochlorine | Neg |
| α-Endosulfan | Organochlorine | ↓ |
| β-Endoculfan | Organochlorine | ↓ |
| β-HCH | Organochlorine | Neg |
| γ-HCH | Organochlorine | Neg |
| Methoxychlor | Organochlorine | Neg |
| Cypermethrin | Pyrethroid | ↓↓ |
| Esfenvalerate | Pyrethroid | ? |
| Fenvalerate | Pyrethroid | ↓ |
| Atrazine | Triazine | Neg |
| Metribuzin | Triazine | ↓↓ |
| Simazine | Triazine | ↓ |
| 2,4-D | Chlorophenoxy | Neg |
| 2,4,5-T | Chlorophenoxy | Neg |
| Alachlor | Chloroacetonitrile | Neg |
| Amitrole | Triazole | Neg |
| 1,2-Dibromo-3-chloropropane | Organobromine | Neg |
| Hexachlorobenzene | Aromatic organochlorine | Neg |
| Kelthane (Dicofol) | Organochlorine | ↓↓ |
| Malathion | Organophosphate | Neg |
| Nitrofen | Diphenyl ether | Neg |
| Octachlorostyrene | Aromatic organochlorine | Neg |
| Pentachlorophenol | Phenol | ↓↓ |
| Trifluralin | Dinitroaniline | Neg |
| Dibutylphthalate | Phthalate | Neg |
| Dicyclohexylphthalate | Phthalate | ↓↓ |
| DEHP | Phthalate | ↓↓ |
| DEP | Phthalate | ↑ |
| DOA | Carboxylic acid ester | Neg |
| Bisphenol A | Phenol | ↓↓ |
| Nonylphenol | Phenol | Neg |
| p-n-Octylphenol | Phenol | ↑ |
| p-t-Octaphenol | Phenol | Neg |
| Tributyltin | Organotin | ↓↓ |
| Triphenyltin | Organotin | ↓↓ |
| Benzophenone | | Neg |
| n-Butylbenzene | Aromatic hydrocarbon | Neg |
| p-Nitrotoluene | Substitued aromatic hydrocarbon | Neg |

↑= increase in aromatase activity ($p< 0.05$), ↑↑= increase in aromatase activity ($p<0.01$) vs control

↓= decrease in aromatase activity ($p<0.05$), ↓↓= decrease in aromatase activity ($p<0.01$) vs control

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# Supplementary Material for Chapter 7

**Table B.7-1. Aromatase Activity Measured in the Placental Assay**

| Technician | Day | Replicate | Activity (nmol/mg/min) | Technician 3 determined Activity (nmol/mg/min )[a] | Mean and SD[b] |
|---|---|---|---|---|---|
| 1 | 3 | 1 | 0.0342 | 0.0411 | |
| | | 2 | 0.0351 | 0.0422 | |
| | | 3 | 0.0337 | 0.0422 | |
| | | Mean | 0.0343 | 0.0418 | 0.0381 |
| | | SD | 0.0007 | 0.0006 | 0.0041 |
| 1 | 4 | 1 | 0.0376 | 0.0467 | |
| | | 2 | 0.0392 | 0.0452 | |
| | | 3 | 0.0473 | 0.0439 | |
| | | Mean | 0.0414 | 0.0453 | 0.0433 |
| | | SD | 0.0052 | 0.0014 | 0.0040 |
| 1 | 5 | 1 | 0.0565 | 0.0515 | |
| | | 2 | 0.0529 | 0.0520 | |
| | | 3 | 0.0560 | 0.0480 | |
| | | Mean | 0.0551 | 0.0505 | 0.0528 |
| | | SD | 0.0020 | 0.0022 | 0.0031 |
| 2 | 3 | 1 | 0.0750 | 0.0673 | |
| | | 2 | 0.0723 | 0.0672 | |
| | | 3 | 0.0714 | 0.0633 | |
| | | Mean | 0.0729 | 0.0660 | 0.0694 |
| | | SD | 0.0018 | 0.0023 | 0.0042 |
| 2 | 4 | 1 | 0.0600 | 0.0534 | |
| | | 2 | 0.0598 | 0.0536 | |
| | | 3 | 0.0581 | 0.0471 | |
| | | Mean | 0.0593 | 0.0514 | 0.0553 |
| | | SD | 0.0010 | 0.0036 | 0.0050 |
| 2 | 5 | 1 | 0.0181 | 0.0370 | |
| | | 2 | 0.0185 | 0.0373 | |
| | | 3 | 0.0178 | 0.0354 | |
| | | Mean | 0.0181 | 0.0366 | 0.0273 |
| | | SD | 0.0003 | 0.0010 | 0.0101 |
| 3 | 3 | 1 | 0.0846 | | |
| | | 2 | 0.0896 | | |
| | | 3 | 0.0845 | | |
| | | Mean | 0.0862 | | |
| | | SD | 0.0029 | | |
| 3 | 4 | 1 | 0.0800 | | |
| | | 2 | 0.0826 | | |
| | | 3 | 0.0818 | | |
| | | Mean | 0.0815 | | |
| | | SD | 0.0013 | | |
| 3 | 5 | 1 | 0.0643 | | |
| | | 2 | 0.0650 | | |
| | | 3 | 0.0664 | | |
| | | Mean | 0.0652 | | |
| | | SD | 0.0011 | | |

[a]Activity determined by Technician 3 using the same protein samples as the subject Technician
[b]Mean and SD across activity determinations by Technician 3 and subject Technician on a given day.

**Table B.7-2. Aromatase Activity Measured in the Recombinant Assay**

| Technician | Day | Replicate | Activity (nmol/mg/min) | Technician 3 determined Activity (nmol/mg/min )[a] | Mean and SD[b] |
|---|---|---|---|---|---|
| 1 | 3 | 1 | 0.2623 | 0.2824 | |
| | | 2 | 0.2499 | 0.2888 | |
| | | 3 | 0.2498 | 0.2827 | |
| | | Mean | 0.2540 | 0.2846 | 0.2693 |
| | | SD | 0.0072 | 0.0036 | 0.0176 |
| 1 | 4 | 1 | 0.2124 | 0.2266 | |
| | | 2 | 0.2094 | 0.2389 | |
| | | 3 | 0.2118 | 0.2347 | |
| | | Mean | 0.2112 | 0.2327 | 0.2220 |
| | | SD | 0.0016 | 0.0054 | 0.0123 |
| 1 | 5 | 1 | 0.3260 | 0.3135 | |
| | | 2 | 0.2888 | 0.3134 | |
| | | 3 | 0.3110 | 0.3136 | |
| | | Mean | 0.3086 | 0.3135 | 0.3111 |
| | | SD | 0.0187 | 0.0001 | 0.0122 |
| 2 | 3 | 1 | 0.3792 | 0.3086 | |
| | | 2 | 0.3698 | 0.3078 | |
| | | 3 | 0.3461 | 0.2742 | |
| | | Mean | 0.3650 | 0.2969 | 0.3309 |
| | | SD | 0.0170 | 0.0196 | 0.0408 |
| 2 | 4 | 1 | 0.2996 | 0.2588 | |
| | | 2 | 0.2950 | 0.2647 | |
| | | 3 | 0.3196 | 0.2667 | |
| | | Mean | 0.3048 | 0.2634 | 0.2841 |
| | | SD | 0.0131 | 0.0041 | 0.0242 |
| 2 | 5 | 1 | 0.0837 | 0.2143 | |
| | | 2 | 0.0868 | 0.2229 | |
| | | 3 | 0.0914 | 0.1992 | |
| | | Mean | 0.0873 | 0.2121 | 0.1497 |
| | | SD | 0.0038 | 0.0120 | 0.0888 |
| 3 | 3 | 1 | 0.2759 | | |
| | | 2 | 0.2757 | | |
| | | 3 | 0.2746 | | |
| | | Mean | 0.2754 | | |
| | | SD | 0.0007 | | |
| 3 | 4 | 1 | 0.4364 | | |
| | | 2 | 0.4257 | | |
| | | 3 | 0.4182 | | |
| | | Mean | 0.4268 | | |
| | | SD | 0.0091 | | |
| 3 | 5 | 1 | 0.4096 | | |
| | | 2 | 0.3972 | | |
| | | 3 | 0.3897 | | |
| | | Mean | 0.3988 | | |
| | | SD | 0.0100 | | |

[a]Activity determined by Technician 3 using the same protein samples as the subject Technician
[b]Mean and SD across activity determinations by Technician 3 and subject Technician on a given day.

**Table B.7-3. Tests for Technician Variability**

| Type | Effect | Num DF | Den DF | F Value | Pr > F |
|---|---|---|---|---|---|
| Placental | Technician | 2 | 6 | 2.81 | 0.1378 |
| Recombinant | Technician | 2 | 6 | 1.24 | 0.3530 |

**Table B.7-4. Tests for Day-to-Day Variation within Technicians**

| Type | Cov Parm | Subject | Estimate | Standard Error | Z Value | Pr > \|Z\| |
|---|---|---|---|---|---|---|
| Placental | Day | Technician | 0.000347 | 0.000201 | 1.72 | 0.0424 |
| Placental | Residual | | 5.247E-6 | 1.749E-6 | 3.00 | 0.0013 |
| Recombinant | Day | Technician | 0.01003 | 0.005815 | 1.73 | 0.0422 |
| Recombinant | Residual | | 0.000118 | 0.000039 | 3.00 | 0.0013 |

**Table B.7-5. Tests for Technician Variability for Differences Between Techs Using Same Solutions**

| Type | Effect | Num DF | Den DF | F Value | Pr > F |
|---|---|---|---|---|---|
| Placental | Technician | 1 | 4 | 0.01 | 0.9142 |
| Recombinant | Technician | 1 | 4 | 0.05 | 0.8301 |

**Table B.7-6. Tests for Day-to-Day Variation Within Technicians for Differences Using Same Solutions**

| Type | Cov Parm | Subject | Estimate | Standard Error | Z Value | Pr > \|Z\| |
|---|---|---|---|---|---|---|
| Placental | Day | Technician | 0.000128 | 0.000093 | 1.37 | 0.0846 |
| Placental | Residual | | 0.000011 | 4.454E-6 | 2.45 | 0.0072 |
| Recombinant | Day | Technician | 0.005503 | 0.003925 | 1.40 | 0.0805 |
| Recombinant | Residual | | 0.000145 | 0.000059 | 2.45 | 0.0072 |

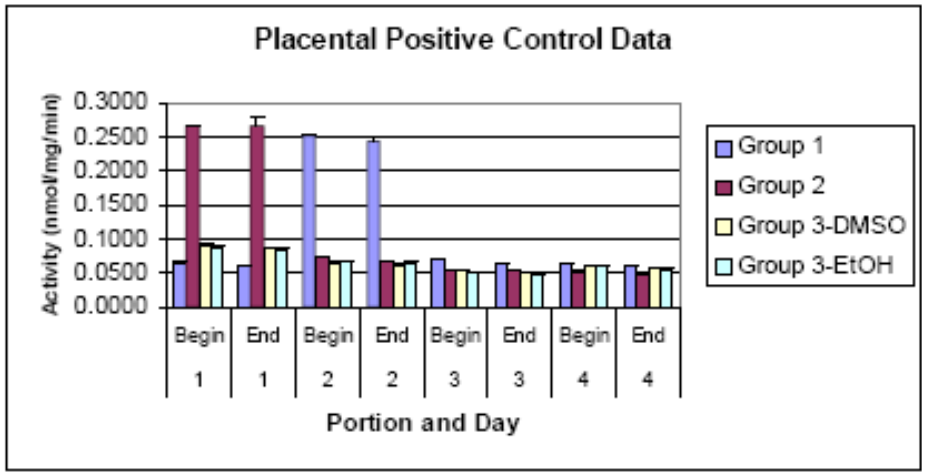


**Figure B.7-1. Mean Placental Positive Control Activities by Day and Portion**
n = 2 for each bar

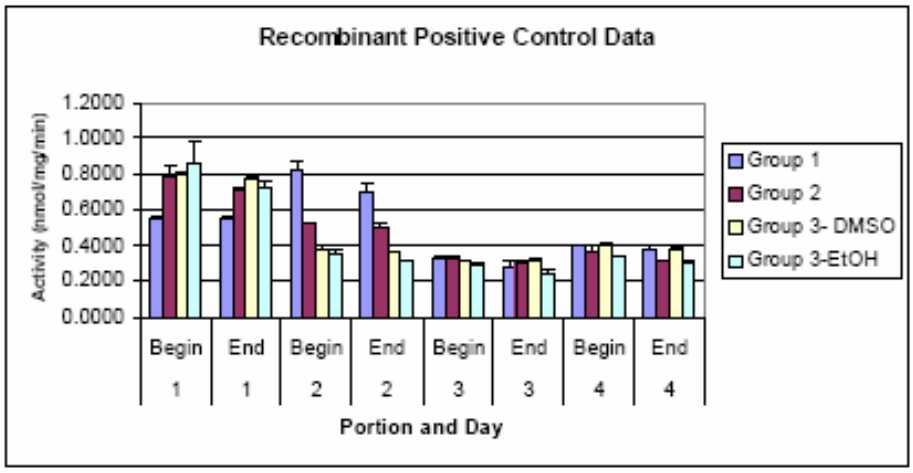


**Figure B.7-2. Mean Recombinant Positive Control Activities by Day and Portion**
n = 2 for each bar

## Table B.7-7. Mean and Standard Deviations of Control Activities (nmol/mg/min)

| Microsome Type | Control Type | Group | Portion of Batch | Batch 1 | | Batch 2 | | Batch 3 | | Batch 4 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Mean | Std Dev | Mean | Std Dev | Mean | Std Dev | Mean | Std Dev |
| Placental | Negative | 1 | Begin | -0.0013 | 0.0000 | -0.0004 | 0.0006 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |
| Placental | Negative | 1 | End | 0.0013 | 0.0008 | 0.0004 | 0.0001 | 0.0000 | 0.0000 | 0.0000 | 0.0001 |
| Placental | Negative | 2 | Begin | -0.0026 | 0.0018 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |
| Placental | Negative | 2 | End | 0.0026 | 0.0076 | 0.0000 | 0.0001 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |
| Placental | Negative | 3-DMSO | Begin | -0.0003 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |
| Placental | Negative | 3-DMSO | End | 0.0003 | 0.0001 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0001 |
| Placental | Negative | 3-EtOH | Begin | -0.0002 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0001 |
| Placental | Negative | 3-EtOH | End | 0.0002 | 0.0000 | 0.0000 | 0.0001 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |
| Placental | Positive | 1 | Begin | 0.0647 | 0.0021 | 0.2520 | 0.0016 | 0.0695 | 0.0001 | 0.0629 | 0.0009 |
| Placental | Positive | 1 | End | 0.0598 | 0.0020 | 0.2444 | 0.0063 | 0.0646 | 0.0003 | 0.0610 | 0.0015 |
| Placental | Positive | 2 | Begin | 0.2655 | 0.0005 | 0.0715 | 0.0016 | 0.0549 | 0.0005 | 0.0504 | 0.0010 |
| Placental | Positive | 2 | End | 0.2657 | 0.0142 | 0.0666 | 0.0012 | 0.0538 | 0.0005 | 0.0474 | 0.0016 |
| Placental | Positive | 3-DMSO | Begin | 0.0917 | 0.0028 | 0.0647 | 0.0022 | 0.0519 | 0.0003 | 0.0606 | 0.0002 |
| Placental | Positive | 3-DMSO | End | 0.0862 | 0.0011 | 0.0615 | 0.0021 | 0.0493 | 0.0008 | 0.0577 | 0.0010 |
| Placental | Positive | 3-EtOH | Begin | 0.0863 | 0.0015 | 0.0670 | 0.0009 | 0.0499 | 0.0014 | 0.0610 | 0.0008 |
| Placental | Positive | 3-EtOH | End | 0.0843 | 0.0005 | 0.0645 | 0.0029 | 0.0471 | 0.0006 | 0.0566 | 0.0009 |
| Recombinant | Negative | 1 | Begin | 0.0000 | 0.0003 | 0.0000 | 0.0001 | 0.0000 | 0.0001 | 0.0000 | 0.0001 |
| Recombinant | Negative | 1 | End | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0001 | 0.0000 | 0.0002 |
| Recombinant | Negative | 2 | Begin | -0.0001 | 0.0001 | 0.0000 | 0.0000 | -0.0001 | 0.0000 | 0.0000 | 0.0000 |
| Recombinant | Negative | 2 | End | 0.0001 | 0.0001 | 0.0000 | 0.0000 | 0.0001 | 0.0002 | 0.0000 | 0.0001 |
| Recombinant | Negative | 3-DMSO | Begin | 0.0000 | 0.0001 | 0.0000 | 0.0000 | 0.0001 | 0.0000 | 0.0002 | 0.0003 |
| Recombinant | Negative | 3-DMSO | End | 0.0000 | 0.0001 | 0.0000 | 0.0002 | -0.0001 | 0.0000 | -0.0002 | 0.0001 |
| Recombinant | Negative | 3-EtOH | Begin | 0.0001 | 0.0001 | 0.0000 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0000 |
| Recombinant | Negative | 3-EtOH | End | -0.0001 | 0.0002 | 0.0000 | 0.0001 | -0.0001 | 0.0001 | -0.0001 | 0.0001 |
| Recombinant | Positive | 1 | Begin | 0.5545 | 0.0023 | 0.8213 | 0.0478 | 0.3260 | 0.0116 | 0.3985 | 0.0040 |
| Recombinant | Positive | 1 | End | 0.5483 | 0.0181 | 0.7002 | 0.0547 | 0.2878 | 0.0213 | 0.3781 | 0.0259 |
| Recombinant | Positive | 2 | Begin | 0.7927 | 0.0510 | 0.5220 | 0.0028 | 0.3298 | 0.0050 | 0.3609 | 0.0393 |
| Recombinant | Positive | 2 | End | 0.7146 | 0.0019 | 0.4973 | 0.0249 | 0.3013 | 0.0131 | 0.3092 | 0.0001 |
| Recombinant | Positive | 3-DMSO | Begin | 0.8042 | 0.0033 | 0.3805 | 0.0185 | 0.3077 | 0.0141 | 0.4126 | 0.0053 |
| Recombinant | Positive | 3-DMSO | End | 0.7704 | 0.0141 | 0.3627 | 0.0033 | 0.3174 | 0.0084 | 0.3822 | 0.0066 |
| Recombinant | Positive | 3-EtOH | Begin | 0.8588 | 0.1277 | 0.3546 | 0.0207 | 0.2925 | 0.0055 | 0.3346 | 0.0051 |
| Recombinant | Positive | 3-EtOH | End | 0.7218 | 0.0467 | 0.3102 | 0.0121 | 0.2455 | 0.0180 | 0.2992 | 0.0154 |

**Table B.7-8. ANOVA Results for Control Data**

| Microsome Type | Control Type | Source | DF | Type I SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|---|---|
| Placental | Negative | Group | 3 | 8.634E-36 | 2.878E-36 | 0.00 | 1.0000 |
| Placental | Negative | Batch(Group) | 12 | 3.242E-35 | 2.702E-36 | 0.00 | 1.0000 |
| Placental | Negative | Portion | 1 | 5.868E-06 | 5.868E-06 | 2.96 | 0.0925 |
| Placental | Negative | Group*Portion | 3 | 4.030E-06 | 1.343E-06 | 0.68 | 0.5708 |
| Placental | Positive | Group | 3 | 5.147E-04 | 1.716E-04 | 84.89 | <.0001 |
| Placental | Positive | Batch(Group) | 10 | 7.091E-03 | 7.091E-04 | 350.86 | <.0001 |
| Placental | Positive | Portion | 1 | 1.541E-04 | 1.541E-04 | 76.26 | <.0001 |
| Placental | Positive | Group*Portion | 3 | 2.255E-06 | 7.516E-07 | 0.37 | 0.7738 |
| Recombinant | Negative | Group | 3 | 2.660E-37 | 8.865E-38 | 0.00 | 1.0000 |
| Recombinant | Negative | Batch(Group) | 12 | 1.652E-36 | 1.376E-37 | 0.00 | 1.0000 |
| Recombinant | Negative | Portion | 1 | 8.330E-09 | 8.330E-09 | 0.59 | 0.4481 |
| Recombinant | Negative | Group*Portion | 3 | 1.222E-07 | 4.072E-08 | 2.86 | 0.0474 |
| Recombinant | Positive | Group | 3 | 4.673E-02 | 1.558E-02 | 13.67 | <.0001 |
| Recombinant | Positive | Batch(Group) | 12 | 2.250E+00 | 1.875E-01 | 164.56 | <.0001 |
| Recombinant | Positive | Portion | 1 | 3.107E-02 | 3.107E-02 | 27.26 | <.0001 |
| Recombinant | Positive | Group*Portion | 3 | 4.644E-03 | 1.548E-03 | 1.36 | 0.2680 |

**Table B.7-9. Placental Aromatase Activity (nmol/mg/min) in the Presence of Various Test Chemicals**

| Chemical | Level | Concentration (M) | Log (concentration) | Day 1 | | Day 2 | | Day 3 | | Day 4 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Activity | % Full activity | Activity | % Full activity | Activity | % Full activity | Activity | % Full activity |
| Econazole | 1 | 1.00E-06 | -6.00 | -0.0012 | -1.88 | -0.0018 | -0.73 | 0.0004 | 0.56 | 0.0001 | 0.18 |
| Econazole | 2 | 5.00E-07 | -6.30 | -0.0012 | -1.88 | -0.0017 | -0.69 | 0.0005 | 0.68 | 0.0002 | 0.35 |
| Econazole | 3 | 2.50E-07 | -6.60 | -0.0008 | -1.34 | -0.0015 | -0.61 | 0.0008 | 1.18 | 0.0005 | 0.80 |
| Econazole | 4 | 1.00E-07 | -7.00 | -0.0001 | -0.24 | -0.0005 | -0.21 | 0.0018 | 2.73 | 0.0014 | 2.04 |
| Econazole | 5 | 5.00E-08 | -7.30 | 0.0010 | 1.61 | 0.0007 | 0.30 | 0.0034 | 5.10 | 0.0031 | 4.60 |
| Econazole | 6 | 2.50E-08 | -7.60 | 0.0032 | 5.17 | 0.0038 | 1.52 | 0.0063 | 9.33 | 0.0050 | 7.53 |
| Econazole | 7 | 1.00E-08 | -8.00 | 0.0089 | 14.23 | 0.0115 | 4.66 | 0.0134 | 19.98 | 0.0116 | 17.37 |
| Econazole | 8 | 1.00E-09 | -9.00 | 0.0422 | 67.83 | 0.0941 | 37.91 | 0.0506 | 75.49 | 0.0456 | 68.08 |
| Genistein | 1 | 1.00E-03* | -3.00 | 0.0414 | 66.55 | 0.0887 | 35.75 | 0.0508 | 75.85 | 0.0394 | 58.76 |
| Genistein | 2 | 5.00E-04* | -3.30 | 0.0431 | 69.18 | 0.1123 | 45.26 | 0.0565 | 84.33 | 0.0467 | 69.66 |
| Genistein | 3 | 2.50E-04 | -3.60 | 0.0471 | 75.64 | 0.1323 | 53.30 | 0.0589 | 84.94 | 0.0500 | 74.62 |
| Genistein | 4 | 1.00E-04 | -4.00 | 0.0564 | 90.65 | 0.1987 | 80.08 | 0.0605 | 90.29 | 0.0551 | 82.20 |
| Genistein | 5 | 5.00E-05 | -4.30 | 0.0591 | 94.95 | 0.2305 | 92.90 | 0.0685 | 102.27 | 0.0618 | 92.26 |
| Genistein | 6 | 2.50E-05 | -4.60 | 0.0589 | 94.55 | 0.2463 | 99.25 | 0.0705 | 105.14 | 0.0609 | 90.85 |
| Genistein | 7 | 1.00E-05 | -5.00 | 0.0603 | 96.81 | 0.2502 | 100.83 | 0.0682 | 101.75 | 0.0624 | 93.07 |
| Genistein | 8 | 1.00E-06 | -6.00 | 0.0606 | 97.29 | 0.2475 | 99.73 | 0.0674 | 100.50 | 0.0596 | 88.96 |
| Atrazine | 1 | 1.00E-03* | -3.00 | 0.0560 | 89.92 | 0.1550 | 62.47 | 0.0632 | 94.23 | 0.0555 | 82.81 |
| Atrazine | 2 | 1.00E-04 | -4.00 | 0.0607 | 97.51 | 0.2214 | 89.21 | 0.0674 | 100.56 | 0.0642 | 95.76 |
| Atrazine | 3 | 1.00E-05 | -5.00 | 0.0624 | 100.20 | 0.2527 | 101.83 | 0.0676 | 100.86 | 0.0610 | 90.98 |
| Atrazine | 4 | 1.00E-06 | -6.00 | 0.0621 | 99.77 | 0.2548 | 102.68 | 0.0656 | 97.83 | 0.0640 | 95.49 |
| Atrazine | 5 | 1.00E-07 | -7.00 | 0.0617 | 99.03 | 0.2539 | 102.33 | 0.0693 | 103.42 | 0.0635 | 94.69 |
| Atrazine | 6 | 1.00E-08 | -8.00 | 0.0621 | 99.74 | 0.2491 | 100.38 | 0.0682 | 101.71 | 0.0616 | 91.97 |
| Atrazine | 7 | 1.00E-09 | -9.00 | 0.0613 | 98.41 | 0.2501 | 100.78 | 0.0673 | 100.37 | 0.0611 | 91.16 |
| bis(2-ethylhexyl)phthalate | 1 | 1.00E-03 | -3.00 | 0.0528 | 84.87 | 0.2248 | 90.59 | 0.0638 | 95.26 | 0.0596 | 88.93 |
| bis(2-ethylhexyl)phthalate | 2 | 1.00E-04 | -4.00 | 0.0598 | 95.98 | 0.2478 | 99.85 | 0.0665 | 99.28 | 0.0608 | 90.74 |
| bis(2-ethylhexyl)phthalate | 3 | 1.00E-05 | -5.00 | 0.0165 | 26.47 | 0.2536 | 102.21 | 0.0674 | 100.56 | 0.0639 | 95.38 |
| bis(2-ethylhexyl)phthalate | 4 | 1.00E-06 | -6.00 | 0.0640 | 102.83 | 0.2610 | 105.15 | 0.0673 | 100.46 | 0.0631 | 94.09 |
| bis(2-ethylhexyl)phthalate | 5 | 1.00E-07 | -7.00 | 0.0633 | 101.67 | 0.2474 | 99.68 | 0.0674 | 100.56 | 0.0625 | 93.25 |
| bis(2-ethylhexyl)phthalate | 6 | 1.00E-08 | -8.00 | 0.0631 | 101.38 | 0.2596 | 104.61 | 0.0651 | 97.16 | 0.0607 | 90.61 |
| bis(2-ethylhexyl)phthalate | 7 | 1.00E-09 | -9.00 | 0.0614 | 98.58 | 0.2426 | 97.77 | 0.0639 | 95.27 | 0.0598 | 89.18 |

**Table B.7-9. (continued)**

| Chemical | Level | Concentration (M) | Log (concentration) | Day 1 | | Day 2 | | Day 3 | | Day 4 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Activity | % Full activity | Activity | % Full activity | Activity | % Full activity | Activity | % Full activity |
| Aminoglutethimide | 1 | 1.00E-03 | -3.00 | -0.0094 | -3.55 | 0.0005 | 0.75 | 0.0004 | 0.68 | 0.0003 | 0.65 |
| Aminoglutethimide | 2 | 1.00E-04 | -4.00 | -0.0036 | -1.37 | 0.0048 | 6.98 | 0.0038 | 7.07 | 0.0033 | 6.79 |
| Aminoglutethimide | 3 | 5.00E-05 | -4.30 | 0.0012 | 0.45 | 0.0087 | 12.63 | 0.0069 | 12.85 | 0.0066 | 13.43 |
| Aminoglutethimide | 4 | 2.50E-05 | -4.60 | 0.0104 | 3.91 | 0.0179 | 25.97 | 0.0125 | 23.07 | 0.0110 | 22.57 |
| Aminoglutethimide | 5 | 1.00E-05 | -5.00 | 0.0417 | 15.68 | 0.0304 | 44.04 | 0.0234 | 43.07 | 0.0229 | 46.88 |
| Aminoglutethimide | 6 | 1.00E-06 | -6.00 | 0.1817 | 68.39 | 0.0596 | 86.33 | 0.0477 | 87.84 | 0.0461 | 94.26 |
| Aminoglutethimide | 7 | 1.00E-07 | -7.00 | 0.2568 | 96.67 | 0.0693 | 100.39 | 0.0548 | 100.86 | 0.0514 | 105.12 |
| Aminoglutethimide | 8 | 1.00E-08 | -8.00 | 0.2640 | 99.39 | 0.0700 | 101.29 | 0.0557 | 102.43 | 0.0491 | 100.44 |
| Chrysin | 1 | 1.00E-03* | -3.00 | 0.0231 | 8.71 | 0.0223 | 32.22 | 0.0181 | 33.25 | 0.0160 | 32.64 |
| Chrysin | 2 | 1.00E-04* | -4.00 | 0.0256 | 9.62 | 0.0239 | 34.53 | 0.0195 | 35.91 | 0.0178 | 36.45 |
| Chrysin | 3 | 5.00E-05 | -4.30 | 0.0167 | 6.30 | 0.0142 | 20.60 | 0.0078 | 14.34 | 0.0126 | 25.87 |
| Chrysin | 4 | 2.50E-05 | -4.60 | 0.0075 | 2.84 | 0.0133 | 19.30 | 0.0104 | 19.22 | 0.0088 | 17.99 |
| Chrysin | 5 | 1.00E-05 | -5.00 | 0.0292 | 11.01 | 0.0254 | 36.71 | 0.0208 | 38.29 | 0.0179 | 36.67 |
| Chrysin | 6 | 1.00E-06 | -6.00 | 0.1660 | 62.50 | 0.0593 | 85.90 | 0.0499 | 91.83 | 0.0418 | 85.42 |
| Chrysin | 7 | 1.00E-07 | -7.00 | 0.2548 | 95.91 | 0.0667 | 96.56 | 0.0565 | 103.99 | 0.0477 | 97.56 |
| Chrysin | 8 | 1.00E-08 | -8.00 | 0.2717 | 102.28 | 0.0677 | 97.97 | 0.0553 | 101.79 | 0.0482 | 98.66 |
| Nonylphenol | 1 | 1.00E-03* | -3.00 | -0.0098 | -3.68 | 0.0000 | 0.06 | 0.0000 | 0.09 | -0.0001 | -0.15 |
| Nonylphenol | 2 | 1.00E-04* | -4.00 | -0.0001 | -0.02 | 0.0022 | 3.15 | 0.0020 | 3.62 | 0.0018 | 3.59 |
| Nonylphenol | 3 | 1.00E-05 | -5.00 | 0.1742 | 65.59 | 0.0594 | 85.99 | 0.0465 | 85.53 | 0.0429 | 87.82 |
| Nonylphenol | 4 | 1.00E-06 | -6.00 | 0.2572 | 96.83 | 0.0694 | 100.42 | 0.0578 | 106.43 | 0.0485 | 99.29 |
| Nonylphenol | 5 | 1.00E-07 | -7.00 | 0.2671 | 100.56 | 0.0669 | 96.93 | 0.0551 | 101.42 | 0.0473 | 96.72 |
| Nonylphenol | 6 | 1.00E-08 | -8.00 | 0.2803 | 105.53 | 0.0689 | 99.78 | 0.0580 | 106.76 | 0.0479 | 97.92 |
| Nonylphenol | 7 | 1.00E-09 | -9.00 | 0.2616 | 98.47 | 0.0690 | 99.88 | 0.0535 | 98.41 | 0.0479 | 97.92 |
| Lindane | 1 | 1.00E-03* | -3.00 | 0.2126 | 80.02 | 0.0611 | 88.50 | 0.0504 | 92.75 | 0.0378 | 77.37 |
| Lindane | 2 | 1.00E-04 | -4.00 | 0.2127 | 80.09 | 0.0662 | 95.82 | 0.0515 | 94.73 | 0.0442 | 90.41 |
| Lindane | 3 | 1.00E-05 | -5.00 | 0.2577 | 97.02 | 0.0655 | 94.87 | 0.0549 | 101.00 | 0.0478 | 97.76 |
| Lindane | 4 | 1.00E-06 | -6.00 | 0.2688 | 101.19 | 0.0666 | 96.47 | 0.0574 | 105.59 | 0.0472 | 96.61 |
| Lindane | 5 | 1.00E-07 | -7.00 | 0.2673 | 100.65 | 0.0656 | 94.91 | 0.0563 | 103.58 | 0.0448 | 91.73 |
| Lindane | 6 | 1.00E-08 | -8.00 | 0.2719 | 102.34 | 0.0669 | 96.85 | 0.0565 | 103.92 | 0.0445 | 91.13 |
| Lindane | 7 | 1.00E-09 | -9.00 | 0.2622 | 98.73 | 0.0672 | 97.36 | 0.0549 | 100.96 | 0.0477 | 97.55 |
| Dibenz[*a,h*]anthracene | 1 | 1.00E-04 | -4.00 | 0.0810 | 91.10 | 0.0614 | 97.27 | 0.0492 | 97.21 | 0.0585 | 99.02 |
| Dibenz[*a,h*]anthracene | 2 | 1.00E-05 | -5.00 | 0.0902 | 101.36 | 0.0646 | 102.41 | 0.0511 | 101.02 | 0.0581 | 98.22 |
| Dibenz[*a,h*]anthracene | 3 | 1.00E-06 | -6.00 | 0.0873 | 98.12 | 0.0646 | 102.42 | 0.0499 | 98.50 | 0.0604 | 102.10 |
| Dibenz[*a,h*]anthracene | 4 | 1.00E-07 | -7.00 | 0.0874 | 98.20 | 0.0643 | 101.98 | 0.0509 | 100.62 | 0.0596 | 100.85 |
| Dibenz[*a,h*]anthracene | 5 | 1.00E-08 | -8.00 | 0.0891 | 100.14 | 0.0644 | 102.04 | 0.0507 | 100.09 | 0.0596 | 100.77 |
| Dibenz[*a,h*]anthracene | 6 | 1.00E-09 | -9.00 | 0.0865 | 97.23 | 0.0635 | 100.70 | 0.0498 | 98.37 | 0.0580 | 98.08 |

| Chemical | Level | Concentration (M) | Log (concentration) | Day 1 | | Day 2 | | Day 3 | | Day 4 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Activity | % Full activity | Activity | % Full activity | Activity | % Full activity | Activity | % Full activity |
| Ketoconazole | 1 | 8.00E-04 | -3.10 | -0.0001 | -0.09 | 0.0001 | 0.19 | 0.0000 | 0.07 | 0.0002 | 0.34 |
| Ketoconazole | 2 | 5.00E-04 | -3.30 | 0.0016 | 1.93 | 0.0004 | 0.65 | 0.0002 | 0.33 | 0.0003 | 0.58 |
| Ketoconazole | 3 | 2.50E-04 | -3.60 | 0.0019 | 2.21 | 0.0010 | 1.46 | 0.0005 | 1.04 | 0.0009 | 1.55 |
| Ketoconazole | 4 | 1.00E-04 | -4.00 | 0.0081 | 9.48 | 0.0064 | 9.69 | 0.0042 | 8.56 | 0.0049 | 8.35 |
| Ketoconazole | 5 | 5.00E-05 | -4.30 | 0.0143 | 16.80 | 0.0116 | 17.69 | 0.0078 | 16.06 | 0.0111 | 18.81 |
| Ketoconazole | 6 | 2.50E-05 | -4.60 | 0.0269 | 31.50 | 0.0211 | 32.08 | 0.0146 | 30.05 | 0.0176 | 29.84 |
| Ketoconazole | 7 | 1.00E-05 | -5.00 | 0.0461 | 54.05 | 0.0347 | 52.80 | 0.0254 | 52.29 | 0.0292 | 49.69 |
| Ketoconazole | 8 | 1.00E-06 | -6.00 | 0.0789 | 92.50 | 0.0575 | 87.48 | 0.0454 | 93.64 | 0.0524 | 89.06 |
| 4-OH androstenedione | 1 | 1.00E-06 | -6.00 | 0.0030 | 3.54 | 0.0025 | 3.74 | 0.0020 | 4.19 | 0.0026 | 4.36 |
| 4-OH androstenedione | 2 | 5.00E-07 | -6.30 | 0.0058 | 6.77 | 0.0044 | 6.70 | 0.0038 | 7.75 | 0.0044 | 7.40 |
| 4-OH androstenedione | 3 | 2.50E-07 | -6.60 | 0.0116 | 13.60 | 0.0081 | 12.40 | 0.0072 | 14.80 | 0.0086 | 14.67 |
| 4-OH androstenedione | 4 | 1.00E-07 | -7.00 | 0.0237 | 27.79 | 0.0176 | 26.75 | 0.0148 | 30.42 | 0.0171 | 29.15 |
| 4-OH androstenedione | 5 | 5.00E-08 | -7.30 | 0.0399 | 46.78 | 0.0297 | 45.14 | 0.0242 | 49.86 | 0.0286 | 48.69 |
| 4-OH androstenedione | 6 | 2.50E-08 | -7.60 | 0.0332 | 38.96 | 0.0411 | 62.44 | 0.0343 | 70.72 | 0.0404 | 68.76 |
| 4-OH androstenedione | 7 | 1.00E-08 | -8.00 | 0.0729 | 85.44 | 0.0527 | 80.13 | 0.0432 | 88.97 | 0.0492 | 83.62 |
| 4-OH androstenedione | 8 | 1.00E-09 | -9.00 | 0.0846 | 99.23 | 0.0584 | 88.80 | 0.0499 | 102.75 | 0.0587 | 98.43 |

* Data were not used because of test substance insolubility in the assay mixture at this concentration.

**Table B.7-10. Recombinant Aromatase Activity (nmol/mg/min) in the Presence of Various Test Chemicals**

| Chemical | Level | Concentration (M) | Log (concentration) | Day 1 | | Day 2 | | Day 3 | | Day 4 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Activity | % Full activity | Activity | % Full activity | Activity | % Full activity | Activity | % Full activity |
| Econazole | 1 | 1.00E-06 | -6.00 | 0.0014 | 0.26 | 0.0014 | 0.19 | 0.0009 | 0.31 | 0.0011 | 0.28 |
| Econazole | 2 | 5.00E-07 | -6.30 | 0.0023 | 0.41 | 0.0016 | 0.21 | 0.0016 | 0.53 | 0.0015 | 0.38 |
| Econazole | 3 | 2.50E-07 | -6.60 | 0.0048 | 0.87 | 0.0042 | 0.55 | 0.0028 | 0.93 | 0.0033 | 0.84 |
| Econazole | 4 | 1.00E-07 | -7.00 | 0.0138 | 2.50 | 0.0102 | 1.34 | 0.0071 | 2.30 | 0.0069 | 1.77 |
| Econazole | 5 | 5.00E-08 | -7.30 | 0.0215 | 3.90 | 0.0204 | 2.68 | 0.0122 | 3.96 | 0.0141 | 3.62 |
| Econazole | 6 | 2.50E-08 | -7.60 | 0.0435 | 7.89 | 0.0327 | 4.29 | 0.0306 | 9.98 | 0.0256 | 6.58 |
| Econazole | 7 | 1.00E-08 | -8.00 | 0.1031 | 18.70 | 0.1004 | 13.20 | 0.0606 | 19.74 | 0.0631 | 16.24 |
| Econazole | 8 | 1.00E-09 | -9.00 | 0.3940 | 71.46 | 0.5893 | 77.47 | 0.2512 | 81.84 | 0.2851 | 73.42 |
| Genistein | 1 | 1.00E-03* | -3.00 | 0.2423 | 43.95 | 0.3424 | 45.01 | 0.1620 | 52.80 | 0.1619 | 41.71 |
| Genistein | 2 | 5.00E-04* | -3.30 | 0.2983 | 54.11 | 0.5291 | 69.55 | 0.1903 | 62.00 | 0.1957 | 50.40 |
| Genistein | 3 | 2.50E-04 | -3.60 | 0.4047 | 73.40 | 0.6409 | 84.24 | 0.2510 | 81.80 | 0.2804 | 72.21 |
| Genistein | 4 | 1.00E-04 | -4.00 | 0.4875 | 88.42 | 0.6792 | 89.28 | 0.3004 | 97.90 | 0.3024 | 77.87 |
| Genistein | 5 | 5.00E-05 | -4.30 | 0.4836 | 87.70 | 0.7720 | 101.48 | 0.3131 | 102.01 | 0.3738 | 96.27 |
| Genistein | 6 | 2.50E-05 | -4.60 | 0.5436 | 98.58 | 0.8465 | 111.28 | 0.3085 | 100.51 | 0.3837 | 98.82 |
| Genistein | 7 | 1.00E-05 | -5.00 | 0.5334 | 96.74 | 0.6406 | 84.20 | 0.3288 | 107.15 | 0.3880 | 99.92 |
| Genistein | 8 | 1.00E-06 | -6.00 | 0.6050 | 109.72 | 0.7189 | 94.48 | 0.3043 | 99.15 | 0.4139 | 106.59 |
| Atrazine | 1 | 1.00E-03* | -3.00 | 0.4423 | 80.21 | 0.5360 | 70.45 | 0.2144 | 69.87 | 0.2763 | 71.16 |
| Atrazine | 2 | 1.00E-04 | -4.00 | 0.5167 | 93.70 | 0.6788 | 89.23 | 0.2966 | 96.66 | 0.3895 | 100.31 |
| Atrazine | 3 | 1.00E-05 | -5.00 | 0.5813 | 105.43 | 0.7655 | 100.63 | 0.3183 | 103.72 | 0.3758 | 96.77 |
| Atrazine | 4 | 1.00E-06 | -6.00 | 0.5600 | 101.55 | 0.8320 | 83.08 | 0.2982 | 96.51 | 0.3814 | 98.23 |
| Atrazine | 5 | 1.00E-07 | -7.00 | 0.5418 | 98.27 | 0.7069 | 92.92 | 0.3078 | 100.29 | 0.3732 | 96.11 |
| Atrazine | 6 | 1.00E-08 | -8.00 | 0.5565 | 100.93 | 0.7243 | 95.22 | 0.3005 | 97.93 | 0.3979 | 102.48 |
| Atrazine | 7 | 1.00E-09 | -9.00 | 0.5264 | 95.46 | 0.8338 | 109.60 | 0.2857 | 93.08 | 0.4115 | 105.98 |
| bis(2-ethylhexyl)phthalate | 1 | 1.00E-03 | -3.00 | 0.4542 | 82.37 | 0.5829 | 76.62 | 0.2935 | 95.64 | 0.3822 | 98.43 |
| bis(2-ethylhexyl)phthalate | 2 | 1.00E-04 | -4.00 | 0.5740 | 104.10 | 0.8447 | 111.06 | 0.3060 | 99.69 | 0.4055 | 104.44 |
| bis(2-ethylhexyl)phthalate | 3 | 1.00E-05 | -5.00 | 0.5415 | 98.21 | 0.6084 | 79.97 | 0.3159 | 102.94 | 0.4330 | 111.51 |
| bis(2-ethylhexyl)phthalate | 4 | 1.00E-06 | -6.00 | 0.5550 | 100.65 | 0.7357 | 96.71 | 0.3006 | 97.92 | 0.3738 | 96.25 |
| bis(2-ethylhexyl)phthalate | 5 | 1.00E-07 | -7.00 | 0.5320 | 96.48 | 0.6432 | 84.54 | 0.2720 | 88.63 | 0.3672 | 94.56 |
| bis(2-ethylhexyl)phthalate | 6 | 1.00E-08 | -8.00 | 0.5348 | 96.98 | 0.6658 | 87.52 | 0.2926 | 95.34 | 0.3479 | 89.60 |
| bis(2-ethylhexyl)phthalate | 7 | 1.00E-09 | -9.00 | 0.5448 | 98.80 | 0.6621 | 87.04 | 0.2788 | 90.85 | 0.3584 | 92.31 |

(continued)

| Chemical | Level | Concentration (M) | Log (concentration) | Day 1 | | Day 2 | | Day 3 | | Day 4 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Activity | % Full activity | Activity | % Full activity | Activity | % Full activity | Activity | % Full activity |
| Aminoglutethimide | 1 | 1.00E-03 | -3.00 | 0.0026 | 0.34 | 0.0024 | 0.47 | 0.0017 | 0.55 | 0.0021 | 0.63 |
| Aminoglutethimide | 2 | 1.00E-04 | -4.00 | 0.0297 | 3.94 | 0.0273 | 5.35 | 0.0161 | 5.10 | 0.0208 | 6.20 |
| Aminoglutethimide | 3 | 5.00E-05 | -4.30 | 0.0548 | 7.28 | 0.0454 | 8.91 | 0.0330 | 10.48 | 0.0361 | 10.79 |
| Aminoglutethimide | 4 | 2.50E-05 | -4.60 | 0.1061 | 14.08 | 0.0960 | 18.85 | 0.0652 | 20.67 | 0.0560 | 16.71 |
| Aminoglutethimide | 5 | 1.00E-05 | -5.00 | 0.2515 | 33.38 | 0.1700 | 33.35 | 0.1075 | 34.07 | 0.1071 | 31.97 |
| Aminoglutethimide | 6 | 1.00E-06 | -6.00 | 0.6070 | 80.54 | 0.4007 | 78.62 | 0.2779 | 88.08 | 0.2830 | 84.48 |
| Aminoglutethimide | 7 | 1.00E-07 | -7.00 | 0.7206 | 95.61 | 0.5079 | 99.67 | 0.3239 | 102.67 | 0.3304 | 98.60 |
| Aminoglutethimide | 8 | 1.00E-08 | -8.00 | 0.7253 | 96.24 | 0.5162 | 101.28 | 0.3202 | 101.47 | 0.3495 | 104.32 |
| Chrysin | 1 | 1.00E-03* | -3.00 | 0.1507 | 20.00 | 0.1286 | 25.23 | 0.0790 | 25.04 | 0.0803 | 23.98 |
| Chrysin | 2 | 1.00E-04* | -4.00 | 0.0655 | 8.69 | 0.0663 | 13.01 | 0.0646 | 20.47 | 0.0477 | 14.25 |
| Chrysin | 3 | 5.00E-05 | -4.30 | 0.0600 | 7.97 | 0.0600 | 11.77 | 0.0504 | 15.97 | 0.0483 | 14.40 |
| Chrysin | 4 | 2.50E-05 | -4.60 | 0.0776 | 10.30 | 0.0646 | 12.67 | 0.0480 | 15.22 | 0.0490 | 14.62 |
| Chrysin | 5 | 1.00E-05 | -5.00 | 0.1975 | 26.21 | 0.1368 | 26.85 | 0.0997 | 31.61 | 0.0955 | 28.49 |
| Chrysin | 6 | 1.00E-06 | -6.00 | 0.6060 | 80.41 | 0.3886 | 76.26 | 0.2467 | 78.19 | 0.2900 | 86.57 |
| Chrysin | 7 | 1.00E-07 | -7.00 | 0.8014 | 106.34 | 0.5112 | 100.30 | 0.3176 | 100.65 | 0.3345 | 99.83 |
| Chrysin | 8 | 1.00E-08 | -8.00 | 0.7909 | 104.95 | 0.5036 | 98.82 | 0.3303 | 104.69 | 0.3401 | 101.52 |
| Nonylphenol | 1 | 1.00E-03* | -3.00 | 0.0001 | 0.01 | 0.0003 | 0.06 | 0.0001 | 0.03 | 0.0000 | 0.01 |
| Nonylphenol | 2 | 1.00E-04* | -4.00 | 0.0044 | 0.58 | 0.0048 | 0.95 | 0.0025 | 0.78 | 0.0023 | 0.69 |
| Nonylphenol | 3 | 1.00E-05 | -5.00 | 0.5447 | 72.27 | 0.3648 | 71.57 | 0.2642 | 83.72 | 0.2297 | 68.57 |
| Nonylphenol | 4 | 1.00E-06 | -6.00 | 0.7642 | 101.41 | 0.5206 | 102.14 | 0.3299 | 104.55 | 0.3305 | 98.66 |
| Nonylphenol | 5 | 1.00E-07 | -7.00 | 0.7246 | 96.15 | 0.5256 | 103.14 | 0.3090 | 97.92 | 0.3424 | 102.20 |
| Nonylphenol | 6 | 1.00E-08 | -8.00 | 0.7361 | 97.67 | 0.5047 | 99.04 | 0.3130 | 99.21 | 0.3256 | 97.18 |
| Nonylphenol | 7 | 1.00E-09 | -9.00 | 0.7326 | 97.21 | 0.4807 | 94.33 | 0.2988 | 94.70 | 0.3403 | 101.57 |
| Lindane | 1 | 1.00E-03* | -3.00 | 0.5671 | 75.24 | 0.3961 | 77.73 | 0.2403 | 76.17 | 0.2659 | 79.37 |
| Lindane | 2 | 1.00E-04 | -4.00 | 0.6349 | 84.25 | 0.4666 | 91.55 | 0.2622 | 83.10 | 0.2994 | 89.37 |
| Lindane | 3 | 1.00E-05 | -5.00 | 0.6856 | 90.98 | 0.4820 | 94.57 | 0.2905 | 92.08 | 0.3078 | 91.87 |
| Lindane | 4 | 1.00E-06 | -6.00 | 0.7034 | 93.33 | 0.4827 | 94.71 | 0.3045 | 96.51 | 0.3225 | 96.25 |
| Lindane | 5 | 1.00E-07 | -7.00 | 0.7234 | 95.99 | 0.4934 | 96.81 | 0.2960 | 93.82 | 0.3361 | 100.31 |
| Lindane | 6 | 1.00E-08 | -8.00 | 0.7397 | 98.15 | 0.5001 | 98.13 | 0.3156 | 100.01 | 0.3040 | 90.73 |
| Lindane | 7 | 1.00E-09 | -9.00 | 0.7294 | 96.78 | 0.5065 | 99.38 | 0.3226 | 102.25 | 0.3057 | 91.23 |
| Dibenz[*a,h*]anthracene | 1 | 1.00E-04 | -4.00 | 0.6839 | 86.86 | 0.3218 | 86.60 | 0.2915 | 93.28 | 0.3482 | 97.61 |
| Dibenz[*a,h*]anthracene | 2 | 1.00E-05 | -5.00 | 0.8088 | 102.73 | 0.3633 | 97.77 | 0.3191 | 102.11 | 0.3957 | 99.58 |
| Dibenz[*a,h*]anthracene | 3 | 1.00E-06 | -6.00 | 0.7737 | 98.26 | 0.3441 | 92.60 | 0.3773 | 120.73 | 0.4113 | 103.50 |
| Dibenz[*a,h*]anthracene | 4 | 1.00E-07 | -7.00 | 0.9105 | 115.65 | 0.3681 | 99.05 | 0.3308 | 105.84 | 0.4353 | 109.53 |
| Dibenz[*a,h*]anthracene | 5 | 1.00E-08 | -8.00 | 0.9131 | 115.97 | 0.3547 | 95.46 | 0.3150 | 100.80 | 0.4178 | 105.14 |
| Dibenz[*a,h*]anthracene | 6 | 1.00E-09 | -9.00 | 0.8045 | 102.19 | 0.3430 | 92.31 | 0.2928 | 93.70 | 0.3416 | 85.95 |

| Chemical | Level | Concentration (M) | Log (concentration) | Day 1 | | Day 2 | | Day 3 | | Day 4 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Activity | % Full activity | Activity | % Full activity | Activity | % Full activity | Activity | % Full activity |
| Ketoconazole | 1 | 8.00E-04 | -3.10 | 0.0006 | 0.08 | 0.0003 | 0.10 | -0.0001 | -0.03 | 0.0002 | 0.08 |
| Ketoconazole | 2 | 5.00E-04 | -3.30 | 0.0042 | 0.53 | 0.0026 | 0.79 | 0.0002 | 0.06 | 0.0018 | 0.58 |
| Ketoconazole | 3 | 2.50E-04 | -3.60 | 0.0083 | 1.05 | 0.0025 | 0.74 | 0.0010 | 0.37 | 0.0019 | 0.60 |
| Ketoconazole | 4 | 1.00E-04 | -4.00 | 0.0280 | 3.54 | 0.0219 | 6.59 | 0.0147 | 5.47 | 0.0180 | 5.67 |
| Ketoconazole | 5 | 5.00E-05 | -4.30 | 0.0655 | 8.29 | 0.0435 | 13.07 | 0.0342 | 12.72 | 0.0367 | 11.57 |
| Ketoconazole | 6 | 2.50E-05 | -4.60 | 0.0958 | 12.12 | 0.0772 | 23.24 | 0.0600 | 22.31 | 0.0805 | 25.40 |
| Ketoconazole | 7 | 1.00E-05 | -5.00 | 0.2439 | 30.86 | 0.1433 | 43.12 | 0.1194 | 44.40 | 0.1308 | 41.28 |
| Ketoconazole | 8 | 1.00E-06 | -6.00 | 0.6739 | 85.27 | 0.2644 | 79.55 | 0.2390 | 88.84 | 0.2955 | 93.25 |
| 4-OH androstenedione | 1 | 1.00E-06 | -6.00 | 0.0251 | 3.18 | 0.0298 | 8.97 | 0.0201 | 7.46 | 0.0397 | 12.54 |
| 4-OH androstenedione | 2 | 5.00E-07 | -6.30 | 0.0465 | 5.89 | 0.0443 | 13.32 | 0.0362 | 13.46 | 0.0518 | 16.36 |
| 4-OH androstenedione | 3 | 2.50E-07 | -6.60 | 0.0789 | 9.98 | 0.0648 | 19.49 | 0.0597 | 22.21 | 0.1010 | 31.87 |
| 4-OH androstenedione | 4 | 1.00E-07 | -7.00 | 0.1413 | 17.88 | 0.1248 | 37.50 | 0.0984 | 35.84 | 0.1627 | 51.35 |
| 4-OH androstenedione | 5 | 5.00E-08 | -7.30 | 0.2300 | 29.10 | 0.1587 | 47.75 | 0.1454 | 54.04 | 0.2178 | 68.71 |
| 4-OH androstenedione | 6 | 2.50E-08 | -7.60 | 0.3845 | 48.65 | 0.1962 | 59.02 | 0.1914 | 71.17 | 0.2467 | 77.85 |
| 4-OH androstenedione | 7 | 1.00E-08 | -8.00 | 0.5280 | 66.81 | 0.2541 | 76.43 | 0.2550 | 94.81 | 0.2800 | 88.35 |
| 4-OH androstenedione | 8 | 1.00E-09 | -9.00 | 0.6729 | 85.15 | 0.2739 | 82.39 | 0.2970 | 110.40 | 0.3036 | 95.79 |

**Table B.7-11. 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 B.7-12: 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 |

# Supplementary Material for Chapter 8

## Table B.8-1. 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 runs 1 to 4 and for runs 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 runs for Laboratories A and B and all four runs for Laboratory C;
   - including all three runs for Laboratory B, all three runs for Laboratory A but excluding an outlier for full enzyme activity control, and runs 2 to 4 for Laboratory C.
4. CV is calculated for the average results for $Log_{10}IC_{50}$ and slope parameters.

**Table B.8-2. 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 | $5.8692x10^{-24}$ ($1.0298x10^{-24}$, $2.3117x10^{-22}$) |
| **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 runs 1 to 4 and for runs 2 to 4. Laboratory A had results with and without an outlier for the full enzyme activity controls

# Supplementary Material for Chapter 9

**Table B.9-1. Placental Assay: Effect of Aminoglutethimide on Aromatase Activity by Laboratory**

| Laboratory | Reference Chemical Log Conc (M) | Number of Replicates | Percent of Control | | | |
|---|---|---|---|---|---|---|
| | | | Mean | sd | SEM | %CV |
| RTI | -3.00 | 3 | -0.12 | 0.88 | 0.51 | 736.32 |
| | -4.00 | 3 | 3.60 | 1.21 | 0.70 | 33.52 |
| | -5.00 | 3 | 28.43 | 0.70 | 0.40 | 2.45 |
| | -5.30 | 3 | 43.07 | 2.25 | 1.30 | 5.23 |
| | -6.00 | 3 | 74.82 | 3.51 | 2.03 | 4.69 |
| | -7.00 | 3 | 93.74 | 4.85 | 2.80 | 5.17 |
| | -8.00 | 3 | 96.28 | 3.79 | 2.19 | 3.93 |
| | -9.00 | 3 | 95.82 | 3.76 | 2.17 | 3.92 |
| Battelle | -3.00 | 3 | 0.36 | 0.26 | 0.15 | 72.68 |
| | -4.00 | 3 | 4.48 | 0.68 | 0.39 | 15.11 |
| | -5.00 | 3 | 32.53 | 2.68 | 1.55 | 8.24 |
| | -5.30 | 2 | 49.12 | NC[a] | NC | NC |
| | -5.60 | 3 | 64.88 | 1.74 | 1.00 | 2.68 |
| | -6.00 | 3 | 80.48 | 2.28 | 1.31 | 2.83 |
| | -7.00 | 3 | 94.72 | 3.32 | 1.92 | 3.51 |
| | -8.00 | 3 | 98.68 | 2.01 | 1.16 | 2.04 |
| | -9.00 | 1 | 99.66 | NC | NC | NC |
| In Vitro | -3.00 | 3 | 0.21 | 0.04 | 0.02 | 20.8 |
| | -4.00 | 3 | 4.45 | 1.22 | 0.70 | 27.4 |
| | -5.00 | 3 | 28.50 | 6.73 | 3.89 | 23.6 |
| | -6.00 | 3 | 80.04 | 6.72 | 3.88 | 8.4 |
| | -7.00 | 3 | 94.80 | 5.03 | 2.90 | 5.3 |
| | -8.00 | 3 | 99.32 | 6.62 | 3.82 | 6.7 |
| | -9.00 | 3 | 100.92 | 7.82 | 4.51 | 7.8 |
| | -10.00[b] | 3 | 98.75 | 10.11 | 5.84 | 10.2 |
| WIL | -3.00 | 3 | 0.23 | 0.06 | 0.03 | 26.45 |
| | -4.00 | 3 | 4.07 | 0.63 | 0.36 | 15.43 |
| | -4.30 | 3 | 8.26 | 0.94 | 0.54 | 11.41 |
| | -5.00 | 3 | 28.25 | 3.41 | 1.97 | 12.07 |
| | -6.00 | 3 | 80.84 | 3.97 | 2.29 | 4.91 |
| | -7.00 | 3 | 97.38 | 2.12 | 1.23 | 2.18 |
| | -8.00 | 3 | 100.09 | 2.49 | 1.44 | 2.48 |
| | -9.00 | 3 | 98.71 | 2.77 | 1.60 | 2.81 |

a. NC – value **N**ot **C**alculated when n ≤2.
b. See text for explanation for this dilution.

**Table B.9-2. Recombinant Assay: Effect of Aminoglutethimide on Aromatase Activity (Percent of Control) by Laboratory**

| Laboratory | Reference Chemical Log Conc (M) | Number of Replicates | Percent of Control | | | |
|---|---|---|---|---|---|---|
| | | | Mean | sd | SEM | %CV |
| RTI | -3.00 | 3 | 0.30 | 0.21 | 0.12 | 68.23 |
| | -4.00 | 3 | 4.47 | 0.36 | 0.21 | 7.98 |
| | -5.00 | 3 | 32.20 | 1.70 | 0.98 | 5.27 |
| | -5.30 | 3 | 47.05 | 1.14 | 0.66 | 2.42 |
| | -6.00 | 3 | 77.80 | 3.01 | 1.74 | 3.87 |
| | -7.00 | 3 | 91.09 | 5.48 | 3.17 | 6.02 |
| | -8.00 | 3 | 92.84 | 5.82 | 3.36 | 6.27 |
| | -9.00 | 3 | 93.44 | 4.01 | 2.31 | 4.29 |
| Battelle | -3.00 | 3 | 0.51 | 0.12 | 0.07 | 23.94 |
| | -4.00 | 3 | 5.41 | 0.79 | 0.45 | 14.57 |
| | -5.00 | 3 | 34.82 | 3.44 | 1.98 | 9.87 |
| | -5.30 | 2 | 52.33 | NC[a] | NC | NC |
| | -5.60 | 3 | 67.62 | 2.83 | 1.64 | 4.19 |
| | -6.00 | 3 | 82.10 | 2.44 | 1.41 | 2.97 |
| | -7.00 | 3 | 94.77 | 3.55 | 2.05 | 3.74 |
| | -8.00 | 3 | 97.88 | 3.02 | 1.75 | 3.09 |
| | -9.00 | 1 | 103.34 | NC | NC | NC |
| In Vitro | -3.00 | 3 | 0.34 | 0.11 | 0.06 | 31.4 |
| | -4.00 | 3 | 4.40 | 0.22 | 0.13 | 5.0 |
| | -5.00 | 3 | 32.17 | 1.36 | 0.79 | 4.2 |
| | -6.00 | 3 | 85.74 | 3.05 | 1.76 | 3.6 |
| | -7.00 | 3 | 106.05 | 2.77 | 1.60 | 2.6 |
| | -8.00 | 3 | 110.06 | 6.24 | 3.60 | 5.7 |
| | -9.00 | 3 | 111.16 | 1.76 | 1.01 | 1.6 |
| | -10.00 | 3 | 109.98 | 5.99 | 3.46 | 5.4 |
| WIL | -3.00 | 3 | 0.38 | 0.11 | 0.06 | 27.75 |
| | -4.00 | 3 | 4.21 | 1.17 | 0.67 | 27.66 |
| | -4.30 | 3 | 7.99 | 2.60 | 1.50 | 32.57 |
| | -5.00 | 3 | 29.12 | 7.71 | 4.45 | 26.46 |
| | -6.00 | 3 | 78.42 | 5.37 | 3.10 | 6.85 |
| | -7.00 | 3 | 96.78 | 1.84 | 1.06 | 1.90 |
| | -8.00 | 3 | 99.54 | 0.36 | 0.21 | 0.37 |
| | -9.00 | 3 | 97.54 | 1.04 | 0.60 | 1.07 |

**Table B.9-3. Placental Assay: Effect of Atrazine on Aromatase Activity by Laboratory**

| Laboratory | Reference Chemical Log Conc (M) | Number of Replicates | Percent of Control | | | |
|---|---|---|---|---|---|---|
| | | | Mean | sd | SEM | %CV |
| RTI | -3.00 | 3 | 78.41 | 3.73 | 2.15 | 4.75 |
| | -3.30 | 2 | 78.54 | NC[a] | NC | NC |
| | -3.60 | 2 | 85.75 | NC | NC | NC |
| | -4.00 | 3 | 90.87 | 4.12 | 2.38 | 4.53 |
| | -4.30 | 2 | 94.17 | NC | NC | NC |
| | -5.00 | 3 | 95.71 | 1.75 | 1.01 | 1.83 |
| | -6.00 | 3 | 95.44 | 1.68 | 0.97 | 1.76 |
| | -7.00 | 3 | 96.64 | 2.01 | 1.16 | 2.08 |
| | -8.00 | 1 | 94.14 | NC | NC | NC |
| | -9.00 | 1 | 94.48 | NC | NC | NC |
| | -10.00 | 1 | 93.12 | NC | NC | NC |
| Battelle | -3.00 | 3 | 83.05 | 2.11 | 1.22 | 2.54 |
| | -3.05 | 2 | 83.63 | NC | NC | NC |
| | -3.10 | 2 | 81.99 | NC | NC | NC |
| | -3.12 | 2 | 84.21 | NC | NC | NC |
| | -3.30 | 2 | 84.41 | NC | NC | NC |
| | -4.00 | 3 | 93.74 | 1.00 | 0.58 | 1.07 |
| | -5.00 | 3 | 96.89 | 4.56 | 2.63 | 4.70 |
| | -6.00 | 3 | 97.35 | 2.79 | 1.61 | 2.86 |
| | -7.00 | 1 | 97.59 | NC | NC | NC |
| | -8.00 | 1 | 97.89 | NC | NC | NC |
| | -9.00 | 1 | 95.60 | NC | NC | NC |
| | -10.00 | 1 | 93.71 | NC | NC | NC |
| In Vitro | -4.00 | 3 | 89.31 | 5.83 | 3.37 | 6.5 |
| | -4.48 | 3 | 89.83 | 3.47 | 2.00 | 3.9 |
| | -5.00 | 3 | 95.04 | 3.68 | 2.12 | 3.9 |
| | -6.00 | 3 | 92.80 | 5.16 | 2.98 | 5.6 |
| | -7.00 | 3 | 89.21 | 6.33 | 3.65 | 7.1 |
| | -8.00 | 3 | 89.24 | 5.73 | 3.31 | 6.4 |
| | -9.00 | 3 | 90.74 | 6.98 | 4.03 | 7.7 |
| | -10.00 | 3 | 89.98 | 6.60 | 3.81 | 7.3 |
| WIL | -3.00 | 3 | 82.48 | 6.09 | 3.51 | 7.38 |
| | -4.00 | 3 | 94.17 | 2.55 | 1.47 | 2.71 |
| | -5.00 | 3 | 99.18 | 2.76 | 1.59 | 2.78 |
| | -6.00 | 3 | 98.43 | 3.40 | 1.96 | 3.45 |
| | -7.00 | 3 | 98.09 | 0.18 | 0.10 | 0.18 |
| | -8.00 | 3 | 99.69 | 3.76 | 2.17 | 3.77 |
| | -9.00 | 3 | 97.92 | 2.35 | 1.36 | 2.40 |
| | -10.00 | 3 | 97.65 | 1.33 | 0.77 | 1.36 |

a. NC – value **N**ot **C**alculated when n <2

**Table B.9-4. Recombinant Assay: Effect of Atrazine on Aromatase Activity (Percent of Control) by Laboratory**

| Laboratory | Reference Chemical Log Conc (M) | Number of Replicates | Percent of Control | | | |
|---|---|---|---|---|---|---|
| | | | Mean | sd | SEM | %CV |
| RTI | -3.00 | 3 | 85.74 | 4.50 | 2.60 | 5.25 |
| | -3.30 | 2 | 87.59 | 4.18 | 2.95 | 4.77 |
| | -3.60 | 2 | 90.43 | 1.81 | 1.28 | 2.00 |
| | -4.00 | 3 | 96.23 | 1.61 | 0.93 | 1.68 |
| | -4.30 | 2 | 95.94 | 5.39 | 3.81 | 5.62 |
| | -5.00 | 3 | 96.71 | 1.73 | 1.00 | 1.79 |
| | -6.00 | 3 | 96.44 | 3.71 | 2.14 | 3.85 |
| | -7.00 | 3 | 98.92 | 7.32 | 4.23 | 7.40 |
| | -8.00 | 1 | 96.03 | NC | NC | NC |
| | -9.00 | 1 | 95.46 | NC | NC | NC |
| | -10.00 | 1 | 90.15 | NC | NC | NC |
| Battelle | -3.00 | 3 | 92.09 | 5.81 | 3.35 | 6.31 |
| | -3.05 | 2 | 90.66 | NC | NC | NC |
| | -3.10 | 2 | 88.29 | NC | NC | NC |
| | -3.12 | 2 | 89.41 | NC | NC | NC |
| | -3.30 | 2 | 90.15 | NC | NC | NC |
| | -4.00 | 3 | 101.05 | 8.86 | 5.12 | 8.77 |
| | -5.00 | 3 | 105.36 | 5.11 | 2.95 | 4.85 |
| | -6.00 | 3 | 104.89 | 6.64 | 3.83 | 6.33 |
| | -7.00 | 1 | 110.10 | NC | NC | NC |
| | -8.00 | 1 | 107.15 | NC | NC | NC |
| | -9.00 | 1 | 105.84 | NC | NC | NC |
| | -10.00 | 1 | 105.10 | NC | NC | NC |
| In Vitro | -4.00 | 3 | 87.86 | 3.23 | 1.87 | 3.7 |
| | -4.48 | 3 | 92.14 | 2.13 | 1.23 | 2.3 |
| | -5.00 | 3 | 92.70 | 2.61 | 1.51 | 2.8 |
| | -6.00 | 3 | 88.93 | 4.33 | 2.50 | 4.9 |
| | -7.00 | 3 | 92.41 | 0.45 | 0.26 | 0.5 |
| | -8.00 | 3 | 89.87 | 3.30 | 1.90 | 3.7 |
| | -9.00 | 3 | 91.89 | 3.42 | 1.97 | 3.7 |
| | -10.00 | 3 | 91.36 | 4.39 | 2.54 | 4.8 |
| WIL | -3.00 | 3 | 86.34 | 2.93 | 1.69 | 3.40 |
| | -4.00 | 3 | 97.24 | 1.96 | 1.13 | 2.02 |
| | -5.00 | 3 | 101.36 | 2.82 | 1.63 | 2.78 |
| | -6.00 | 3 | 100.67 | 3.15 | 1.82 | 3.13 |
| | -7.00 | 3 | 100.90 | 1.04 | 0.60 | 1.03 |
| | -8.00 | 3 | 101.19 | 2.77 | 1.60 | 2.74 |
| | -9.00 | 3 | 100.83 | 1.73 | 1.00 | 1.72 |
| | -10.00 | 3 | 99.21 | 1.68 | 0.97 | 1.69 |

**Table B.9-5. Placental Assay: Effect of Chrysin on Aromatase Acitivity by Laboratory**

| Laboratory | Reference Chemical Log Conc (M) | Number of Replicates | Percent of Control | | | |
|---|---|---|---|---|---|---|
| | | | Mean | sd | SEM | %CV |
| RTI | -4.00 | 3 | 22.63 | 0.51 | 0.29 | 2.24 |
| | -5.00 | 3 | 22.79 | 0.04 | 0.03 | 0.19 |
| | -5.30 | 2 | 35.45 | NC[a] | NC | NC |
| | -5.60 | 2 | 50.48 | NC | NC | NC |
| | -6.00 | 3 | 70.35 | 0.68 | 0.39 | 0.96 |
| | -6.30 | 3 | 80.70 | 1.23 | 0.71 | 1.53 |
| | -7.00 | 3 | 91.71 | 2.45 | 1.41 | 2.67 |
| | -8.00 | 3 | 94.14 | 3.45 | 1.99 | 3.66 |
| | -9.00 | 1 | 95.41 | NC | NC | NC |
| | -10.00 | 1 | 93.30 | NC | NC | NC |
| Battelle | -4.00 | 3 | 20.52 | 1.43 | 0.82 | 6.95 |
| | -5.00 | 3 | 26.24 | 1.37 | 0.79 | 5.21 |
| | -5.30 | 2 | 39.52 | NC | NC | NC |
| | -5.60 | 2 | 53.73 | NC | NC | NC |
| | -6.00 | 3 | 73.68 | 2.88 | 1.66 | 3.91 |
| | -6.60 | 1 | 86.99 | NC | NC | NC |
| | -7.00 | 3 | 87.18 | 7.00 | 4.04 | 8.03 |
| | -8.00 | 3 | 92.31 | 3.04 | 1.75 | 3.29 |
| | -9.00 | 3 | 93.43 | 1.08 | 0.63 | 1.16 |
| | -10.00 | 1 | 90.23 | NC | NC | NC |
| In Vitro | -4.00 | 1 | 17.90 | NC | NC | NC |
| | -4.48 | 3 | 8.19 | 2.67 | 1.54 | 32.6 |
| | -5.00 | 3 | 21.94 | 4.03 | 2.33 | 18.3 |
| | -5.48 | 2 | 47.21 | NC | NC | NC |
| | -6.00 | 3 | 71.99 | 7.00 | 4.04 | 9.7 |
| | -7.00 | 3 | 86.10 | 6.92 | 4.00 | 8.0 |
| | -8.00 | 3 | 91.05 | 12.36 | 7.14 | 13.6 |
| | -9.00 | 3 | 88.36 | 15.37 | 8.87 | 17.4 |
| | -10.00 | 3 | 93.90 | 9.50 | 5.48 | 10.1 |
| WIL | -4.00 | 3 | 13.91 | 2.14 | 1.23 | 15.37 |
| | -5.00 | 3 | 26.13 | 2.37 | 1.37 | 9.09 |
| | -5.30 | 3 | 40.01 | 1.91 | 1.10 | 4.78 |
| | -5.60 | 2 | 56.70 | NC | NC | NC |
| | -6.00 | 3 | 75.70 | 0.65 | 0.38 | 0.86 |
| | -7.00 | 3 | 93.05 | 5.65 | 3.26 | 6.07 |
| | -8.00 | 3 | 91.74 | 9.41 | 5.43 | 10.26 |
| | -9.00 | 1 | 102.08 | NC | NC | NC |
| | -10.00 | 3 | 82.75 | 15.61 | 9.01 | 18.86 |

a. NC – value **N**ot **C**alculated when n ≤2.

**Table B.9-6. Recombinant Assay: Effect of Chrysin on Aromatase Activity (Percent of Control) by Laboratory**

| Laboratory | Reference Chemical Log Conc (M) | Number of Replicates | Percent of Control | | | |
|---|---|---|---|---|---|---|
| | | | Mean | sd | SEM | %CV |
| RTI | -4.00 | 3 | 24.83 | 2.23 | 1.29 | 8.98 |
| | -5.00 | 3 | 26.89 | 2.03 | 1.17 | 7.53 |
| | -5.30 | 3 | 40.68 | 4.28 | 2.47 | 10.52 |
| | -5.60 | 3 | 56.60 | 3.97 | 2.29 | 7.01 |
| | -6.00 | 3 | 71.20 | 1.79 | 1.04 | 2.52 |
| | -6.30 | 3 | 82.17 | 2.83 | 1.64 | 3.45 |
| | -7.00 | 3 | 91.73 | 3.51 | 2.03 | 3.82 |
| | -8.00 | 3 | 91.77 | 1.74 | 1.00 | 1.90 |
| Battelle | -4.00 | 3 | 23.97 | 2.88 | 1.66 | 12.00 |
| | -5.00 | 3 | 25.84 | 1.09 | 0.63 | 4.23 |
| | -5.30 | 2 | 37.53 | NC[a] | NC | NC |
| | -5.60 | 2 | 53.06 | NC | NC | NC |
| | -6.00 | 3 | 59.56 | 10.39 | 6.00 | 17.45 |
| | -6.60 | 1 | 64.93 | NC | NC | NC |
| | -7.00 | 3 | 80.97 | 8.05 | 4.65 | 9.94 |
| | -8.00 | 3 | 85.30 | 10.33 | 5.96 | 12.11 |
| | -9.00 | 3 | 87.37 | 12.58 | 7.26 | 14.40 |
| | -10.00 | 1 | 75.02 | NC | NC | NC |
| In Vitro | -4.00 | 1 | 19.16 | NC | NC | NC |
| | -4.48 | 3 | 18.13 | 11.18 | 6.45 | 61.6 |
| | -5.00 | 3 | 25.19 | 4.54 | 2.62 | 18.0 |
| | -5.48 | 2 | 67.65 | NC | NC | NC |
| | -6.00 | 3 | 71.48 | 4.09 | 2.36 | 5.7 |
| | -7.00 | 3 | 90.10 | 8.09 | 4.67 | 9.0 |
| | -8.00 | 3 | 92.16 | 5.60 | 3.23 | 6.1 |
| | -9.00 | 3 | 86.88 | 9.97 | 5.76 | 11.5 |
| | -10.00 | 3 | 91.13 | 9.94 | 5.74 | 10.9 |
| WIL | -4.00 | 3 | 15.39 | 1.74 | 1.01 | 11.33 |
| | -5.00 | 3 | 30.27 | 1.07 | 0.62 | 3.53 |
| | -5.30 | 3 | 45.51 | 2.53 | 1.46 | 5.56 |
| | -5.60 | 2 | 61.92 | 3.12 | 2.21 | 5.04 |
| | -6.00 | 3 | 78.63 | 3.43 | 1.98 | 4.36 |
| | -7.00 | 3 | 96.10 | 3.48 | 2.01 | 3.63 |
| | -8.00 | 3 | 95.68 | 3.59 | 2.07 | 3.75 |
| | -9.00 | 1 | 100.36 | NC | NC | NC |
| | -10.00 | 3 | 88.87 | 8.50 | 4.91 | 9.56 |

**Table B.9-7. Placental Assay: Effect of Dicofol on Aromatase Activity by Laboratory**

| Laboratory | Reference Chemical Log Conc (M) | Number of Replicates | Percent of Control | | | |
|---|---|---|---|---|---|---|
| | | | Mean | sd | SEM | %CV |
| RTI | -3.00 | 3 | 2.78 | 1.06 | 0.61 | 37.97 |
| | -3.30 | 2 | 5.59 | NC[a] | NC | NC |
| | -3.60 | 2 | 12.05 | NC | NC | NC |
| | -4.00 | 3 | 28.66 | 9.69 | 5.60 | 33.82 |
| | -5.00 | 3 | 66.79 | 2.14 | 1.23 | 3.20 |
| | -6.00 | 3 | 90.12 | 2.78 | 1.61 | 3.08 |
| | -7.00 | 3 | 96.91 | 2.58 | 1.49 | 2.66 |
| | -8.00 | 3 | 99.69 | 2.39 | 1.38 | 2.40 |
| | -9.00 | 1 | 100.89 | NC | NC | NC |
| | -10.00 | 1 | 100.13 | NC | NC | NC |
| Battelle | -3.00 | 3 | 2.65 | 0.40 | 0.23 | 15.01 |
| | -4.00 | 3 | 22.04 | 1.32 | 0.76 | 6.00 |
| | -4.30 | 2 | 33.03 | NC | NC | NC |
| | -5.00 | 3 | 67.69 | 12.77 | 7.38 | 18.87 |
| | -6.00 | 3 | 91.41 | 1.45 | 0.83 | 1.58 |
| | -7.00 | 3 | 97.52 | 1.11 | 0.64 | 1.14 |
| | -8.00 | 3 | 97.74 | 0.58 | 0.33 | 0.59 |
| | -9.00 | 3 | 96.25 | 0.52 | 0.30 | 0.54 |
| | -10.00 | 1 | 98.05 | NC | NC | NC |
| In Vitro | -4.00 | 3 | 44.60 | 10.27 | 5.93 | 23.0 |
| | -4.48 | 3 | 61.52 | 11.31 | 6.53 | 18.4 |
| | -5.00 | 3 | 68.81 | 4.05 | 2.34 | 5.9 |
| | -6.00 | 3 | 89.42 | 2.31 | 1.33 | 2.6 |
| | -7.00 | 3 | 95.67 | 6.32 | 3.65 | 6.6 |
| | -8.00 | 3 | 91.54 | 6.27 | 3.62 | 6.9 |
| | -9.00 | 3 | 94.49 | 1.71 | 0.99 | 1.8 |
| | -10.00 | 3 | 96.35 | 5.70 | 3.29 | 5.9 |
| WIL | -3.00 | 3 | 3.14 | 2.73 | 1.58 | 87.00 |
| | -3.52 | 2 | 13.57 | NC | NC | NC |
| | -4.00 | 3 | 31.83 | 4.96 | 2.87 | 15.60 |
| | -4.52 | 2 | 60.61 | NC | NC | NC |
| | -5.00 | 3 | 66.33 | 6.72 | 3.88 | 10.13 |
| | -5.52 | 2 | 84.67 | NC | NC | NC |
| | -6.00 | 3 | 93.07 | 3.26 | 1.88 | 3.50 |
| | -7.00 | 1 | 99.34 | NC | NC | NC |
| | -8.00 | 3 | 100.47 | 2.37 | 1.37 | 2.36 |
| | -9.00 | 1 | 101.22 | NC | NC | NC |
| | -10.00 | 1 | 98.88 | NC | NC | NC |

a. NC – value **N**ot **C**alculated when n ≤2.

**Table B.9-8. Recombinant Assay: Effect of Dicofol on Aromatase Activity Percent of Control by Laboratory**

| Laboratory | Reference Chemical Log Conc (M) | Number of Replicates | Percent of Control | | | |
|---|---|---|---|---|---|---|
| | | | Mean | sd | SEM | %CV |
| RTI | -3.00 | 3 | 2.32 | 0.12 | 0.07 | 5.09 |
| | -3.30 | 2 | 6.25 | 0.96 | 0.68 | 15.37 |
| | -3.60 | 2 | 12.42 | 0.15 | 0.10 | 1.19 |
| | -4.00 | 3 | 25.69 | 3.02 | 1.75 | 11.77 |
| | -5.00 | 3 | 76.12 | 3.24 | 1.87 | 4.25 |
| | -6.00 | 3 | 96.78 | 5.83 | 3.37 | 6.02 |
| | -7.00 | 3 | 95.64 | 4.90 | 2.83 | 5.12 |
| | -8.00 | 3 | 101.80 | 6.53 | 3.77 | 6.41 |
| | -9.00 | 1 | 98.19 | NC[a] | NC | NC |
| | -10.00 | 1 | 93.21 | NC | NC | NC |
| Battelle | -3.00 | 3 | 2.35 | 0.44 | 0.25 | 18.76 |
| | -4.00 | 3 | 16.54 | 0.72 | 0.42 | 4.37 |
| | -4.30 | 2 | 22.95 | NC | NC | NC |
| | -5.00 | 3 | 61.72 | 6.09 | 3.51 | 9.86 |
| | -6.00 | 3 | 92.58 | 9.20 | 5.31 | 9.94 |
| | -7.00 | 3 | 91.02 | 4.53 | 2.62 | 4.98 |
| | -8.00 | 3 | 94.43 | 3.14 | 1.81 | 3.32 |
| | -9.00 | 3 | 95.84 | 5.04 | 2.91 | 5.26 |
| | -10.00 | 1 | 93.55 | NC | NC | NC |
| In Vitro | -4.00 | 3 | 38.10 | 8.34 | 4.81 | 21.9 |
| | -4.48 | 3 | 47.10 | 14.21 | 8.21 | 30.2 |
| | -5.00 | 3 | 56.91 | 5.31 | 3.06 | 9.3 |
| | -6.00 | 3 | 87.98 | 2.24 | 1.29 | 2.5 |
| | -7.00 | 3 | 89.97 | 3.39 | 1.96 | 3.8 |
| | -8.00 | 3 | 85.46 | 6.76 | 3.90 | 7.9 |
| | -9.00 | 3 | 89.45 | 1.82 | 1.05 | 2.0 |
| | -10.00 | 3 | 91.58 | 3.53 | 2.04 | 3.9 |
| WIL | -3.00 | 3 | 3.29 | 0.64 | 0.37 | 19.48 |
| | -3.52 | 2 | 12.10 | 0.36 | 0.26 | 3.00 |
| | -4.00 | 3 | 27.92 | 3.01 | 1.74 | 10.77 |
| | -4.52 | 2 | 59.51 | 3.26 | 2.31 | 5.48 |
| | -5.00 | 3 | 69.51 | 2.60 | 1.50 | 3.74 |
| | -5.52 | 2 | 86.35 | 1.43 | 1.01 | 1.65 |
| | -6.00 | 3 | 94.72 | 2.00 | 1.16 | 2.11 |
| | -7.00 | 1 | 96.65 | NC | NC | NC |
| | -8.00 | 3 | 101.38 | 2.37 | 1.37 | 2.34 |
| | -9.00 | 1 | 103.12 | NC | NC | NC |
| | -10.00 | 1 | 102.13 | NC | NC | NC |

**Table B.9-9. Placental Assay: Effect of Dibenz[a,h,]anthracene on Aromatase Activity**

| Laboratory | Reference Chemical Log Conc (M) | Number of Replicates | Percent of Control | | | |
|---|---|---|---|---|---|---|
| | | | Mean | sd | SEM | %CV |
| RTI | -4.00 | 3 | 96.42 | 7.74 | 4.47 | 8.03 |
| | -5.00 | 3 | 102.08 | 1.98 | 1.14 | 1.94 |
| | -6.00 | 3 | 102.64 | 1.39 | 0.80 | 1.36 |
| | -6.30 | 3 | 101.70 | 2.60 | 1.50 | 2.55 |
| | -7.00 | 3 | 103.11 | 2.01 | 1.16 | 1.95 |
| | -8.00 | 3 | 103.57 | 2.42 | 1.40 | 2.34 |
| | -9.00 | 3 | 102.74 | 1.61 | 0.93 | 1.57 |
| | -10.00 | 3 | 100.57 | 3.67 | 2.12 | 3.65 |
| Battelle | -4.00 | 3 | 102.10 | 3.63 | 2.10 | 3.56 |
| | -5.00 | 3 | 101.95 | 4.40 | 2.54 | 4.31 |
| | -6.00 | 3 | 104.78 | 4.23 | 2.44 | 4.04 |
| | -6.60 | 3 | 104.17 | 4.04 | 2.33 | 3.88 |
| | -7.00 | 3 | 101.95 | 2.49 | 1.44 | 2.44 |
| | -8.00 | 3 | 100.82 | 1.80 | 1.04 | 1.78 |
| | -9.00 | 3 | 101.38 | 3.11 | 1.80 | 3.07 |
| | -10.00 | 3 | 96.68 | 6.30 | 3.64 | 6.51 |
| In Vitro | -4.48 | 3 | 83.46 | 10.19 | 5.88 | 12.2 |
| | -5.00 | 3 | 92.47 | 7.57 | 4.37 | 8.2 |
| | -5.48 | 3 | 88.28 | 4.91 | 2.83 | 5.6 |
| | -6.00 | 3 | 81.59 | 9.83 | 5.68 | 12.1 |
| | -7.00 | 3 | 93.05 | 4.11 | 2.37 | 4.4 |
| | -8.00 | 3 | 89.33 | 5.92 | 3.42 | 6.6 |
| | -9.00 | 3 | 84.17 | 7.36 | 4.25 | 8.7 |
| | -10.00 | 3 | 87.10 | 8.50 | 4.91 | 9.8 |
| WIL | -4.00 | 3 | 103.78 | 5.47 | 3.16 | 5.27 |
| | -5.00 | 3 | 106.46 | 7.62 | 4.40 | 7.15 |
| | -5.30 | 3 | 105.04 | 6.07 | 3.50 | 5.78 |
| | -6.00 | 3 | 103.54 | 5.28 | 3.05 | 5.10 |
| | -7.00 | 3 | 106.22 | 6.28 | 3.63 | 5.92 |
| | -8.00 | 3 | 103.75 | 4.30 | 2.48 | 4.15 |
| | -9.00 | 3 | 108.87 | 5.27 | 3.05 | 4.85 |
| | -10.00 | 3 | 105.87 | 5.01 | 2.89 | 4.73 |

**Table B.9-10. Recombinant Assay: Effect of Dibenz(a,h)anthracene on Aromatase Activity (Percent of Control) by Laboratory**

| Laboratory | Reference Chemical Log Conc (M) | Number of Replicates | Percent of Control | | | |
|---|---|---|---|---|---|---|
| | | | Mean | sd | SEM | %CV |
| RTI | -4.00 | 3 | 93.49 | 4.34 | 2.51 | 4.64 |
| | -5.00 | 3 | 96.24 | 4.60 | 2.66 | 4.78 |
| | -6.00 | 3 | 94.46 | 3.78 | 2.18 | 4.00 |
| | -6.30 | 3 | 94.48 | 2.45 | 1.42 | 2.60 |
| | -7.00 | 3 | 95.72 | 5.10 | 2.95 | 5.33 |
| | -8.00 | 3 | 94.76 | 7.00 | 4.04 | 7.39 |
| | -9.00 | 3 | 96.30 | 2.13 | 1.23 | 2.22 |
| | -10.00 | 3 | 94.64 | 0.76 | 0.44 | 0.80 |
| Battelle | -4.00 | 3 | 106.44 | 4.51 | 2.60 | 4.24 |
| | -5.00 | 3 | 103.50 | 7.26 | 4.19 | 7.01 |
| | -6.00 | 3 | 106.03 | 8.37 | 4.83 | 7.89 |
| | -6.60 | 3 | 102.86 | 5.10 | 2.94 | 4.95 |
| | -7.00 | 3 | 101.57 | 6.97 | 4.02 | 6.86 |
| | -8.00 | 3 | 101.88 | 4.48 | 2.59 | 4.40 |
| | -9.00 | 3 | 102.11 | 5.70 | 3.29 | 5.58 |
| | -10.00 | 3 | 102.58 | 3.39 | 1.95 | 3.30 |
| In Vitro | -4.00 | 2 | 88.24 | NC[a] | NC | NC |
| | -4.48 | 3 | 89.56 | 1.52 | 0.88 | 1.7 |
| | -5.00 | 3 | 91.20 | 1.24 | 0.72 | 1.4 |
| | -5.48 | 1 | 94.50 | NC | NC | NC |
| | -6.00 | 3 | 89.80 | 1.26 | 0.73 | 1.4 |
| | -7.00 | 3 | 90.80 | 4.67 | 2.69 | 5.1 |
| | -8.00 | 3 | 89.93 | 3.51 | 2.03 | 3.9 |
| | -9.00 | 3 | 84.35 | 6.17 | 3.56 | 7.3 |
| | -10.00 | 3 | 85.26 | 3.50 | 2.02 | 4.1 |
| WIL | -4.00 | 3 | 95.75 | 2.09 | 1.21 | 2.18 |
| | -5.00 | 3 | 103.31 | 1.06 | 0.61 | 1.03 |
| | -5.30 | 3 | 104.28 | 1.44 | 0.83 | 1.38 |
| | -6.00 | 3 | 104.26 | 1.64 | 0.95 | 1.57 |
| | -7.00 | 3 | 95.06 | 8.21 | 4.74 | 8.63 |
| | -8.00 | 3 | 105.61 | 1.47 | 0.85 | 1.39 |
| | -9.00 | 3 | 103.93 | 0.85 | 0.49 | 0.82 |
| | -10.00 | 3 | 103.47 | 2.35 | 1.36 | 2.27 |

**Table B.9-11. Placental Assay: Effect of Econazole on Aromatase Activity (Percent of Control) by Laboratory**

| Laboratory | Reference Chemical Log Conc (M) | Number of Replicates | Percent of Control | | | |
|---|---|---|---|---|---|---|
| | | | Mean | sd | SEM | %CV |
| RTI | -3.00 | 1 | 0.09 | NC[a] | NC | NC |
| | -4.00 | 1 | 0.06 | NC | NC | NC |
| | -5.00 | 1 | 0.01 | NC | NC | NC |
| | -6.00 | 3 | 0.10 | 0.08 | 0.05 | 83.80 |
| | -7.00 | 3 | 1.34 | 0.09 | 0.05 | 7.08 |
| | -8.00 | 3 | 12.20 | 1.02 | 0.59 | 8.33 |
| | -8.30 | 2 | 22.32 | NC | NC | NC |
| | -9.00 | 3 | 59.60 | 1.42 | 0.82 | 2.39 |
| | -9.30 | 2 | 76.05 | NC | NC | NC |
| | -9.60 | 2 | 86.43 | NC | NC | NC |
| | -10.00 | 3 | 93.28 | 1.12 | 0.65 | 1.21 |
| Battelle | -3.00 | 1 | -0.34 | NC | NC | NC |
| | -4.00 | 3 | -0.36 | 0.16 | 0.09 | 45.47 |
| | -5.00 | 3 | -0.23 | 0.13 | 0.08 | 58.41 |
| | -6.00 | 3 | -0.25 | 0.09 | 0.05 | 37.41 |
| | -7.00 | 3 | 1.65 | 0.41 | 0.23 | 24.52 |
| | -8.00 | 3 | 14.93 | 2.30 | 1.33 | 15.42 |
| | -8.60 | 2 | 44.40 | NC | NC | NC |
| | -9.00 | 3 | 58.89 | 14.21 | 8.20 | 24.13 |
| | -10.00 | 3 | 80.39 | 22.31 | 12.88 | 27.75 |
| In Vitro | -4.00 | 1 | 0.04 | NC | NC | NC |
| | -4.48 | 1 | -0.35 | NC | NC | NC |
| | -5.00 | 3 | -0.21 | 0.33 | 0.19 | 154.5 |
| | -6.00 | 3 | -0.15 | 0.14 | 0.08 | 95.0 |
| | -7.00 | 3 | 1.48 | 0.45 | 0.26 | 30.3 |
| | -8.00 | 3 | 10.77 | 0.29 | 0.17 | 2.7 |
| | -8.48 | 2 | 29.15 | NC | NC | NC |
| | -9.00 | 3 | 50.39 | 7.81 | 4.51 | 15.5 |
| | -9.48 | 2 | 72.39 | NC | NC | NC |
| | -10.00 | 3 | 90.62 | 6.76 | 3.90 | 7.5 |
| WIL | -3.00 | 1 | 0.27 | NC | NC | NC |
| | -4.00 | 1 | -0.18 | NC | NC | NC |
| | -5.00 | 3 | 0.36 | 0.62 | 0.36 | 171.69 |
| | -6.00 | 3 | 0.19 | 0.14 | 0.08 | 74.56 |
| | -7.00 | 3 | 1.59 | 0.04 | 0.02 | 2.62 |
| | -8.00 | 3 | 13.54 | 1.72 | 0.99 | 12.68 |
| | -8.52 | 2 | 35.32 | NC | NC | NC |
| | -9.00 | 3 | 64.00 | 2.08 | 1.20 | 3.25 |
| | -9.52 | 2 | 89.83 | NC | NC | NC |
| | -10.00 | 3 | 95.48 | 3.28 | 1.89 | 3.44 |

**Table B.9-12. Recombinant Assay: Effect of Econazole on Aromatase Activity (Percent of Control) by Laboratory**

| Laboratory | Reference Chemical Log Conc (M) | Number of Replicates | Percent of Control | | | |
|---|---|---|---|---|---|---|
| | | | Mean | sd | SEM | %CV |
| RTI | -6.00 | 3 | 0.26 | 0.04 | 0.02 | 13.59 |
| | -7.00 | 3 | 2.12 | 0.13 | 0.08 | 6.34 |
| | -8.00 | 3 | 17.61 | 1.30 | 0.75 | 7.38 |
| | -8.30 | 3 | 31.41 | 2.49 | 1.44 | 7.94 |
| | -9.00 | 3 | 73.59 | 2.13 | 1.23 | 2.89 |
| | -9.30 | 3 | 87.85 | 4.31 | 2.49 | 4.91 |
| | -9.60 | 3 | 93.78 | 1.98 | 1.14 | 2.11 |
| | -10.00 | 3 | 97.77 | 2.80 | 1.62 | 2.86 |
| Battelle | -3.00 | 1 | 0.14 | NC[a] | NC | NC |
| | -4.00 | 3 | 0.07 | 0.03 | 0.02 | 45.33 |
| | -5.00 | 3 | -0.01 | 0.08 | 0.05 | -998.02 |
| | -6.00 | 3 | 0.20 | 0.07 | 0.04 | 34.69 |
| | -7.00 | 3 | 1.79 | 0.26 | 0.15 | 14.79 |
| | -8.00 | 3 | 15.43 | 2.42 | 1.40 | 15.69 |
| | -8.60 | 2 | 49.15 | NC | NC | NC |
| | -9.00 | 3 | 68.20 | 10.73 | 6.19 | 15.73 |
| | -10.00 | 3 | 90.12 | 8.14 | 4.70 | 9.03 |
| In Vitro | -4.00 | 1 | -0.01 | NC | NC | NC |
| | -4.48 | 1 | -0.06 | NC | NC | NC |
| | -5.00 | 3 | 0.20 | 0.35 | 0.20 | 178.0 |
| | -6.00 | 3 | 0.25 | 0.17 | 0.10 | 69.0 |
| | -7.00 | 3 | 1.30 | 0.18 | 0.11 | 14.2 |
| | -8.00 | 3 | 12.92 | 2.52 | 1.46 | 19.5 |
| | -8.48 | 2 | 32.94 | NC | NC | NC |
| | -9.00 | 3 | 58.72 | 3.43 | 1.98 | 5.8 |
| | -9.48 | 2 | 84.05 | NC | NC | NC |
| | -10.00 | 3 | 90.26 | 6.85 | 3.95 | 7.6 |
| WIL | -3.00 | 1 | 0.01 | NC | NC | NC |
| | -4.00 | 1 | 0.00 | NC | NC | NC |
| | -5.00 | 3 | 0.04 | 0.03 | 0.02 | 89.54 |
| | -6.00 | 3 | 0.21 | 0.02 | 0.01 | 10.92 |
| | -7.00 | 3 | 1.83 | 0.11 | 0.06 | 5.96 |
| | -8.00 | 3 | 16.17 | 0.60 | 0.35 | 3.73 |
| | -8.52 | 2 | 43.76 | 3.26 | 2.30 | 7.44 |
| | -9.00 | 3 | 74.78 | 2.74 | 1.58 | 3.67 |
| | -9.52 | 2 | 94.03 | 2.72 | 1.92 | 2.89 |
| | -10.00 | 3 | 98.68 | 1.72 | 0.99 | 1.74 |

**Table B.9-13. Placental Assay: Effect of Fenarimol on Aromatase Activity Percent of Control by Laboratory**

| Laboratory | Reference Chemical Log Conc (M) | Number of Replicates | Percent of Control | | | |
|---|---|---|---|---|---|---|
| | | | Mean | sd | SEM | %CV |
| RTI | -3.00 | 3 | 1.23 | 0.49 | 0.28 | 40.17 |
| | -4.00 | 3 | 6.09 | 0.40 | 0.23 | 6.61 |
| | -4.60 | 2 | 20.26 | NC[a] | NC | NC |
| | -5.00 | 3 | 35.61 | 2.52 | 1.45 | 7.08 |
| | -5.30 | 2 | 52.63 | NC | NC | NC |
| | -6.00 | 3 | 78.26 | 5.81 | 3.36 | 7.43 |
| | -7.00 | 3 | 91.47 | 8.60 | 4.96 | 9.40 |
| | -8.00 | 3 | 92.96 | 8.56 | 4.94 | 9.21 |
| | -9.00 | 1 | 83.54 | NC | NC | NC |
| | -10.00 | 1 | 81.48 | NC | NC | NC |
| Battelle | -3.00 | 3 | 1.35 | 0.21 | 0.12 | 15.73 |
| | -4.00 | 3 | 6.01 | 0.20 | 0.11 | 3.30 |
| | -5.00 | 3 | 37.69 | 0.31 | 0.18 | 0.83 |
| | -5.60 | 2 | 69.43 | NC | NC | NC |
| | -6.00 | 3 | 84.28 | 3.29 | 1.90 | 3.91 |
| | -7.00 | 3 | 97.43 | 2.62 | 1.51 | 2.69 |
| | -8.00 | 3 | 97.82 | 0.57 | 0.33 | 0.59 |
| | -9.00 | 3 | 98.72 | 1.10 | 0.64 | 1.12 |
| | -10.00 | 1 | 98.91 | NC | NC | NC |
| In Vitro | -4.48 | 3 | 14.85 | 3.20 | 1.85 | 21.6 |
| | -5.00 | 3 | 31.51 | 1.05 | 0.61 | 3.3 |
| | -5.48 | 3 | 55.23 | 4.98 | 2.88 | 9.0 |
| | -6.00 | 3 | 80.46 | 4.95 | 2.86 | 6.2 |
| | -7.00 | 3 | 92.25 | 4.65 | 2.68 | 5.0 |
| | -8.00 | 3 | 95.88 | 5.60 | 3.23 | 5.8 |
| | -9.00 | 3 | 96.89 | 3.75 | 2.17 | 3.9 |
| | -10.00 | 3 | 95.60 | 3.76 | 2.17 | 3.9 |
| WIL | -3.00 | 3 | -5.91 | 12.06 | 6.96 | 204.0 |
| | -4.00 | 3 | -1.11 | 11.74 | 6.78 | 1060 |
| | -4.52 | 2 | 9.44 | NC | NC | NC |
| | -5.00 | 3 | 32.44 | 10.38 | 5.99 | 32.00 |
| | -5.52 | 2 | 57.22 | NC | NC | NC |
| | -6.00 | 3 | 79.91 | 7.85 | 4.53 | 9.82 |
| | -7.00 | 3 | 86.36 | 12.12 | 7.00 | 14.03 |
| | -8.00 | 1 | 105.45 | NC | NC | NC |
| | -9.00 | 3 | 92.35 | 9.35 | 5.40 | 10.13 |
| | -10.00 | 1 | 97.62 | NC | NC | NC |

a. NC = value Not Calculated when n <2

**Table B.9-14. Recombinant Assay: Effect of Fenarimol on Aromatase Activity (Percent of Control) by Laboratory**

| Laboratory | Reference Chemical Log Conc (M) | Number of Replicates | Percent of Control | | | |
|---|---|---|---|---|---|---|
| | | | Mean | sd | SEM | %CV |
| RTI | -3.00 | 3 | 0.95 | 0.08 | 0.05 | 8.38 |
| | -4.00 | 3 | 6.29 | 0.12 | 0.07 | 1.96 |
| | -4.60 | 2 | 19.64 | 0.48 | 0.34 | 2.44 |
| | -5.00 | 3 | 35.58 | 1.94 | 1.12 | 5.44 |
| | -5.30 | 2 | 49.42 | 0.70 | 0.49 | 1.41 |
| | -6.00 | 3 | 80.47 | 2.62 | 1.51 | 3.26 |
| | -7.00 | 3 | 90.28 | 1.74 | 1.00 | 1.93 |
| | -8.00 | 3 | 90.15 | 0.47 | 0.27 | 0.52 |
| | -9.00 | 1 | 91.26 | NC[a] | NC | NC |
| | -10.00 | 1 | 89.84 | NC | NC | NC |
| Battelle | -3.00 | 3 | 1.45 | 0.12 | 0.07 | 8.17 |
| | -4.00 | 3 | 5.66 | 0.10 | 0.06 | 1.84 |
| | -5.00 | 3 | 36.03 | 1.38 | 0.80 | 3.84 |
| | -5.60 | 2 | 68.00 | NC | NC | NC |
| | -6.00 | 3 | 81.88 | 0.73 | 0.42 | 0.89 |
| | -7.00 | 3 | 97.75 | 2.63 | 1.52 | 2.69 |
| | -8.00 | 3 | 100.56 | 0.30 | 0.17 | 0.30 |
| | -9.00 | 3 | 97.49 | 0.82 | 0.48 | 0.84 |
| | -10.00 | 1 | 99.01 | NC | NC | NC |
| In Vitro | -4.48 | 3 | 20.45 | 11.70 | 6.75 | 57.2 |
| | -5.00 | 3 | 32.23 | 4.13 | 2.38 | 12.8 |
| | -5.48 | 3 | 53.86 | 11.42 | 6.59 | 21.2 |
| | -6.00 | 3 | 79.07 | 9.25 | 5.34 | 11.7 |
| | -7.00 | 3 | 95.86 | 11.40 | 6.58 | 11.9 |
| | -8.00 | 3 | 94.10 | 14.22 | 8.21 | 15.1 |
| | -9.00 | 3 | 95.51 | 13.20 | 7.62 | 13.8 |
| | -10.00 | 3 | 94.54 | 10.39 | 6.00 | 11.0 |
| WIL | -3.00 | 3 | 8.16 | 12.69 | 7.33 | 155.58 |
| | -4.00 | 3 | 27.40 | 37.29 | 21.53 | 136.13 |
| | -4.52 | 2 | 55.70 | 49.79 | 35.21 | 89.40 |
| | -5.00 | 3 | 59.17 | 35.57 | 20.54 | 60.12 |
| | -5.52 | 2 | 81.53 | 20.60 | 14.57 | 25.27 |
| | -6.00 | 3 | 90.00 | 5.81 | 3.35 | 6.45 |
| | -7.00 | 3 | 98.80 | 3.41 | 1.97 | 3.45 |
| | -8.00 | 1 | 103.78 | NC | NC | NC |
| | -9.00 | 3 | 96.36 | 7.57 | 4.37 | 7.85 |
| | -10.00 | 1 | 98.95 | NC | NC | NC |

**Table B.9-15. Placental Assay: Effect of Ketoconazole on Aromatase Activity (Percent of Control) by Laboratory**

| Laboratory | Reference Chemical Log Conc (M) | Number of Replicates | Percent of Control | | | |
|---|---|---|---|---|---|---|
| | | | Mean | sd | SEM | %CV |
| RTI | -4.00 | 3 | 4.70 | 0.97 | 0.56 | 20.69 |
| | -4.30 | 2 | 11.08 | NC[a] | NC | NC |
| | -4.60 | 2 | 21.02 | NC | NC | NC |
| | -5.00 | 3 | 39.35 | 1.46 | 0.84 | 3.71 |
| | -5.30 | 2 | 55.64 | NC | NC | NC |
| | -6.00 | 3 | 85.46 | 3.73 | 2.15 | 4.36 |
| | -7.00 | 3 | 98.20 | 2.35 | 1.36 | 2.39 |
| | -8.00 | 3 | 98.98 | 1.45 | 0.84 | 1.46 |
| | -9.00 | 1 | 96.15 | NC | NC | NC |
| | -10.00 | 1 | 95.39 | NC | NC | NC |
| | -11.00 | 1 | 94.48 | NC | NC | NC |
| Battelle | -4.00 | 2 | 5.54 | NC | NC | NC |
| | -4.60 | 2 | 19.79 | NC | NC | NC |
| | -5.00 | 3 | 37.87 | 1.31 | 0.76 | 3.46 |
| | -5.30 | 2 | 55.00 | NC | NC | NC |
| | -5.60 | 2 | 70.19 | NC | NC | NC |
| | -6.00 | 3 | 84.51 | 0.64 | 0.37 | 0.76 |
| | -6.60 | 2 | 91.15 | NC | NC | NC |
| | -7.00 | 3 | 95.87 | 1.84 | 1.06 | 1.92 |
| | -8.00 | 1 | 97.50 | NC | NC | NC |
| | -9.00 | 1 | 98.57 | NC | NC | NC |
| | -10.00 | 1 | 98.90 | NC | NC | NC |
| | -11.00 | 1 | 100.9 | NC | NC | NC |
| | -12.00[b] | 1 | 98.71 | NC | NC | NC |
| In Vitro | -4.00 | 3 | 29.14 | 44.12 | 25.47 | 151.4 |
| | -4.52 | 1 | 33.01 | NC | NC | NC |
| | -5.00 | 3 | 39.85 | 1.86 | 1.07 | 4.7 |
| | -5.48 | 2 | 63.05 | NC | NC | NC |
| | -6.00 | 3 | 83.62 | 4.36 | 2.52 | 5.2 |
| | -7.00 | 3 | 106.74 | 3.96 | 2.29 | 3.7 |
| | -8.00 | 3 | 104.53 | 3.22 | 1.86 | 3.1 |
| | -9.00 | 3 | 100.56 | 6.02 | 3.48 | 6.0 |
| | -10.00 | 3 | 106.46 | 9.01 | 5.20 | 8.5 |
| WIL | -4.00 | 3 | 5.31 | 1.82 | 1.05 | 34.20 |
| | -4.30 | 2 | 12.59 | NC | NC | NC |
| | -5.00 | 3 | 40.07 | 2.36 | 1.37 | 5.90 |
| | -5.30 | 2 | 58.53 | NC | NC | NC |
| | -6.00 | 3 | 89.05 | 2.74 | 1.58 | 3.08 |
| | -7.00 | 3 | 101.21 | 3.28 | 1.89 | 3.24 |
| | -8.00 | 3 | 103.31 | 2.68 | 1.55 | 2.59 |
| | -9.00 | 1 | 105.95 | NC | NC | NC |
| | -10.00 | 3 | 101.71 | 2.68 | 1.55 | 2.64 |
| | -11.00 | 1 | 104.59 | NC | NC | NC |

**Table B.9-16. Recombinant Assay: Effect of Ketoconazole on Aromatase Activity (Percent of Control) by Laboratory**

| Laboratory | Reference Chemical Log Conc (M) | Number of Replicates | Percent of Control | | | |
|---|---|---|---|---|---|---|
| | | | Mean | sd | SEM | %CV |
| RTI | -4.00 | 3 | 8.13 | 1.01 | 0.59 | 12.47 |
| | -4.30 | 2 | 14.60 | 0.39 | 0.28 | 2.67 |
| | -4.60 | 2 | 27.38 | 0.84 | 0.60 | 3.08 |
| | -5.00 | 3 | 45.54 | 2.02 | 1.17 | 4.44 |
| | -5.30 | 2 | 60.82 | 2.88 | 2.04 | 4.74 |
| | -6.00 | 3 | 86.61 | 1.48 | 0.85 | 1.70 |
| | -7.00 | 3 | 92.04 | 3.73 | 2.15 | 4.05 |
| | -8.00 | 3 | 96.45 | 1.86 | 1.07 | 1.93 |
| | -9.00 | 1 | 99.02 | NC[a] | NC | NC |
| | -10.00 | 1 | 94.84 | NC | NC | NC |
| | -11.00 | 1 | 95.35 | NC | NC | NC |
| Battelle | -4.00 | 2 | 7.10 | NC | NC | NC |
| | -4.60 | 2 | 25.44 | NC | NC | NC |
| | -5.00 | 3 | 44.05 | 4.77 | 2.75 | 10.82 |
| | -5.30 | 2 | 63.44 | NC | NC | NC |
| | -5.60 | 2 | 79.55 | NC | NC | NC |
| | -6.00 | 3 | 87.96 | 7.29 | 4.21 | 8.29 |
| | -6.60 | 2 | 101.75 | NC | NC | NC |
| | -7.00 | 3 | 99.79 | 7.34 | 4.24 | 7.36 |
| | -8.00 | 1 | 91.26 | NC | NC | NC |
| | -9.00 | 1 | 95.64 | NC | NC | NC |
| | -10.00 | 1 | 94.72 | NC | NC | NC |
| | -11.00 | 1 | 94.49 | NC | NC | NC |
| | -12.00[b] | 1 | 91.39 | NC | NC | NC |
| In Vitro | -4.00 | 3 | 3.59 | 0.91 | 0.52 | 25.4 |
| | -4.52 | 1 | 17.88 | NC | NC | NC |
| | -5.00 | 3 | 29.48 | 5.27 | 3.04 | 17.9 |
| | -5.48 | 2 | 37.41 | NC | NC | NC |
| | -6.00 | 3 | 87.88 | 9.76 | 5.64 | 11.1 |
| | -7.00 | 3 | 100.93 | 13.18 | 7.61 | 13.1 |
| | -8.00 | 3 | 94.99 | 11.61 | 6.70 | 12.2 |
| | -9.00 | 3 | 105.05 | 9.20 | 5.31 | 8.8 |
| | -10.00 | 3 | 102.94 | 10.59 | 6.12 | 10.3 |
| WIL | -4.00 | 3 | 5.20 | 1.30 | 0.75 | 25.04 |
| | -4.30 | 2 | 11.63 | 3.75 | 2.65 | 32.26 |
| | -5.00 | 3 | 40.30 | 6.22 | 3.59 | 15.44 |
| | -5.30 | 2 | 54.53 | 10.18 | 7.20 | 18.67 |
| | -6.00 | 3 | 86.48 | 4.96 | 2.87 | 5.74 |
| | -7.00 | 3 | 100.44 | 1.19 | 0.69 | 1.19 |
| | -8.00 | 3 | 102.87 | 0.57 | 0.33 | 0.55 |
| | -9.00 | 1 | 104.52 | NC | NC | NC |
| | -10.00 | 3 | 101.52 | 2.55 | 1.47 | 2.51 |
| | -11.00 | 1 | 103.08 | NC | NC | NC |

**Table B.9-17. Placental Assay: Effect of Nonylphenol on Aromatase Activity (Percent of Control) by Laboratory**

| Laboratory | Reference Chemical Log Conc (M) | Number of Replicates | Percent of Control | | | |
|---|---|---|---|---|---|---|
| | | | Mean | sd | SEM | %CV |
| RTI | -3.00 | 3 | 0.07 | 0.06 | 0.03 | 79.51 |
| | -4.00 | 3 | 6.78 | 0.31 | 0.18 | 4.52 |
| | -4.30 | 2 | 22.87 | NC[a] | NC | NC |
| | -4.60 | 2 | 46.63 | NC | NC | NC |
| | -5.00 | 3 | 86.28 | 1.56 | 0.90 | 1.81 |
| | -5.30 | 2 | 95.10 | NC | NC | NC |
| | -6.00 | 3 | 98.18 | 1.15 | 0.66 | 1.17 |
| | -7.00 | 3 | 98.30 | 2.20 | 1.27 | 2.24 |
| | -8.00 | 1 | 100.10 | NC | NC | NC |
| | -9.00 | 1 | 99.48 | NC | NC | NC |
| | -10.00 | 1 | 99.58 | NC | NC | NC |
| Battelle | -3.00 | 3 | 0.39 | 0.17 | 0.10 | 43.48 |
| | -4.00 | 3 | 14.30 | 2.35 | 1.35 | 16.41 |
| | -4.30 | 2 | 29.96 | NC | NC | NC |
| | -4.60 | 2 | 47.26 | NC | NC | NC |
| | -5.00 | 3 | 89.25 | 6.45 | 3.72 | 7.23 |
| | -6.00 | 3 | 97.87 | 2.47 | 1.43 | 2.53 |
| | -7.00 | 3 | 98.18 | 7.16 | 4.13 | 7.29 |
| | -8.00 | 3 | 94.80 | 2.69 | 1.55 | 2.84 |
| | -9.00 | 1 | 90.22 | NC | NC | NC |
| | -10.00 | 1 | 94.73 | NC | NC | NC |
| In Vitro | -4.00 | 3 | 15.62 | 1.24 | 0.72 | 7.9 |
| | -4.48 | 3 | 40.85 | 1.32 | 0.76 | 3.2 |
| | -5.00 | 3 | 83.72 | 4.13 | 2.38 | 4.9 |
| | -6.00 | 3 | 90.94 | 4.95 | 2.86 | 5.4 |
| | -7.00 | 3 | 90.62 | 0.60 | 0.35 | 0.7 |
| | -8.00 | 3 | 90.10 | 5.94 | 3.43 | 6.6 |
| | -9.00 | 3 | 88.18 | 4.28 | 2.47 | 4.8 |
| | -10.00 | 3 | 86.12 | 1.71 | 0.99 | 2.0 |
| WIL | -3.00 | 3 | 0.32 | 0.14 | 0.08 | 42.27 |
| | -4.00 | 3 | 7.84 | 0.17 | 0.10 | 2.13 |
| | -4.30 | 2 | 22.46 | NC | NC | NC |
| | -5.00 | 3 | 87.97 | 0.45 | 0.26 | 0.52 |
| | -6.00 | 3 | 101.63 | 3.19 | 1.84 | 3.14 |
| | -7.00 | 3 | 100.58 | 2.97 | 1.71 | 2.95 |
| | -8.00 | 3 | 101.38 | 2.99 | 1.73 | 2.95 |
| | -9.00 | 3 | 101.02 | 3.47 | 2.00 | 3.44 |
| | -10.00 | 1 | 104.55 | NC | NC | NC |

a NC = value Not Calculated when n <2

**Table B.9-18. Recombinant Assay: 4-Nonylphenol on Aromatase Activity (Percent of Control) by Laboratory**

| Laboratory | Reference Chemical Log Conc (M) | Number of Replicates | Percent of Control | | | |
|---|---|---|---|---|---|---|
| | | | Mean | sd | SEM | %CV |
| RTI | -3.00 | 3 | 0.10 | 0.01 | 0.01 | 13.72 |
| | -4.00 | 3 | 3.57 | 1.58 | 0.91 | 44.22 |
| | -4.30 | 2 | 20.67 | 0.90 | 0.64 | 4.37 |
| | -4.60 | 2 | 40.62 | 0.12 | 0.09 | 0.30 |
| | -5.00 | 3 | 85.47 | 1.61 | 0.93 | 1.89 |
| | -5.30 | 2 | 101.41 | 6.94 | 4.91 | 6.85 |
| | -6.00 | 3 | 101.14 | 3.60 | 2.08 | 3.55 |
| | -7.00 | 3 | 100.53 | 3.00 | 1.73 | 2.98 |
| | -8.00 | 1 | 93.65 | NC | NC | NC |
| | -9.00 | 1 | 98.57 | NC | NC | NC |
| | -10.00 | 1 | 102.63 | NC | NC | NC |
| Battelle | -3.00 | 3 | 0.78 | 0.25 | 0.15 | 32.10 |
| | -4.00 | 3 | 4.04 | 1.20 | 0.69 | 29.58 |
| | -4.30 | 2 | 10.84 | NC | NC | NC |
| | -4.60 | 2 | 35.75 | NC | NC | NC |
| | -5.00 | 3 | 78.70 | 7.40 | 4.27 | 9.40 |
| | -6.00 | 3 | 93.93 | 12.03 | 6.94 | 12.80 |
| | -7.00 | 3 | 95.99 | 6.62 | 3.82 | 6.90 |
| | -8.00 | 3 | 101.60 | 7.65 | 4.42 | 7.53 |
| | -9.00 | 1 | 106.82 | NC | NC | NC |
| | -10.00 | 1 | 105.53 | NC | NC | NC |
| In Vitro | -4.00 | 3 | 4.38 | 2.34 | 1.35 | 53.3 |
| | -4.48 | 3 | 27.11 | 8.09 | 4.67 | 29.8 |
| | -5.00 | 3 | 60.67 | 5.15 | 2.97 | 8.5 |
| | -6.00 | 3 | 95.01 | 0.81 | 0.47 | 0.9 |
| | -7.00 | 3 | 95.27 | 1.34 | 0.77 | 1.4 |
| | -8.00 | 3 | 91.21 | 3.63 | 2.10 | 4.0 |
| | -9.00 | 3 | 91.17 | 0.87 | 0.50 | 1.0 |
| | -10.00 | 3 | 90.00 | 5.55 | 3.20 | 6.2 |
| WIL | -3.00 | 3 | 0.13 | 0.04 | 0.02 | 31.39 |
| | -4.00 | 3 | 1.79 | 0.21 | 0.12 | 11.89 |
| | -4.30 | 2 | 12.15 | 2.56 | 1.81 | 21.09 |
| | -5.00 | 3 | 67.82 | 4.46 | 2.57 | 6.57 |
| | -6.00 | 3 | 98.02 | 2.77 | 1.60 | 2.83 |
| | -7.00 | 3 | 99.68 | 5.67 | 3.27 | 5.68 |
| | -8.00 | 3 | 99.04 | 5.13 | 2.96 | 5.18 |
| | -9.00 | 3 | 97.47 | 2.56 | 1.48 | 2.63 |
| | -10.00 | 1 | 97.65 | NC | NC | NC |

**Table B.9-19. Placental Assay: Effect of Prochloraz on Aromatase Activity (Percent of Control) by Laboratory**

| Laboratory | Reference Chemical Log Conc (M) | Number of Replicates | Percent of Control | | | |
|---|---|---|---|---|---|---|
| | | | Mean | sd | SEM | %CV |
| RTI | -3.00 | 1 | 0.01 | NC[a] | NC | NC |
| | -4.00 | 1 | -0.07 | NC | NC | NC |
| | -5.00 | 3 | 0.13 | 0.13 | 0.07 | 95.06 |
| | -6.00 | 3 | 2.41 | 0.48 | 0.28 | 20.04 |
| | -7.00 | 3 | 17.79 | 1.51 | 0.87 | 8.47 |
| | -7.60 | 2 | 47.64 | NC | NC | NC |
| | -8.00 | 3 | 66.59 | 4.11 | 2.37 | 6.17 |
| | -8.30 | 2 | 84.11 | NC | NC | NC |
| | -9.00 | 3 | 95.97 | 5.01 | 2.89 | 5.22 |
| | -10.00 | 3 | 100.77 | 6.25 | 3.61 | 6.20 |

| Laboratory | Reference Chemical Log Conc (M) | Number of Replicates | Percent of Control | | | |
|---|---|---|---|---|---|---|
| | | | Mean | sd | SEM | %CV |
| Battelle | -4.00 | 1 | 0.07 | NC | NC | NC |
| | -5.00 | 3 | 0.14 | 0.11 | 0.07 | 79.54 |
| | -6.00 | 3 | 2.68 | 0.47 | 0.27 | 17.70 |
| | -7.00 | 3 | 21.52 | 3.05 | 1.76 | 14.20 |
| | -7.60 | 2 | 52.31 | NC | NC | NC |
| | -8.00 | 3 | 70.44 | 4.08 | 2.35 | 5.79 |
| | -8.60 | 2 | 88.90 | NC | NC | NC |
| | -9.00 | 3 | 94.70 | 0.45 | 0.26 | 0.47 |
| | -10.00 | 3 | 97.38 | 1.34 | 0.77 | 1.37 |
| | -11.00[a] | 1 | 96.71 | NC | NC | NC |
| In Vitro | -4.00 | 3 | 1.13 | 1.14 | 0.66 | 100.1 |
| | -5.00 | 3 | 0.15 | 0.19 | 0.11 | 125.3 |
| | -6.00 | 3 | 2.07 | 0.70 | 0.40 | 33.6 |
| | -7.00 | 3 | 15.68 | 2.28 | 1.32 | 14.6 |
| | -7.48 | 1 | 78.51 | NC | NC | NC |
| | -8.00 | 3 | 65.02 | 3.42 | 1.97 | 5.3 |
| | -8.48 | 2 | 82.65 | NC | NC | NC |
| | -9.00 | 3 | 87.78 | 10.10 | 5.83 | 11.5 |
| | -10.00 | 3 | 96.36 | 7.99 | 4.61 | 8.3 |
| WIL | -3.00 | 1 | 0.06 | NC | NC | NC |
| | -4.00 | 1 | -0.21 | NC | NC | NC |
| | -5.00 | 3 | -6.80 | 11.97 | 6.91 | 176.1 |
| | -6.00 | 3 | -4.14 | 11.63 | 6.71 | 281.1 |
| | -7.00 | 3 | 15.72 | 9.12 | 5.27 | 58.03 |
| | -7.52 | 2 | 42.22 | NC | NC | NC |
| | -8.00 | 3 | 72.91 | 2.87 | 1.66 | 3.94 |
| | -8.52 | 2 | 90.14 | NC | NC | NC |
| | -9.00 | 3 | 99.40 | 5.06 | 2.92 | 5.10 |
| | -10.00 | 3 | 103.27 | 3.61 | 2.08 | 3.49 |

**Table B.9-20. Recombinant Assay: Effect of Prochloraz on Aromatase Activity (Percent of Control) by Laboratory**

| Laboratory | Reference Chemical Log Conc (M) | Number of Replicates | Percent of Control | | | |
|---|---|---|---|---|---|---|
| | | | Mean | sd | SEM | %CV |
| RTI | -3.00 | 1 | -0.05 | NC[a] | NC | NC |
| | -4.00 | 1 | -0.01 | NC | NC | NC |
| | -5.00 | 3 | 0.43 | 0.14 | 0.08 | 31.04 |
| | -6.00 | 3 | 3.35 | 0.21 | 0.12 | 6.26 |
| | -7.00 | 3 | 24.92 | 1.45 | 0.83 | 5.80 |
| | -7.60 | 2 | 53.79 | 1.34 | 0.95 | 2.49 |
| | -8.00 | 3 | 72.33 | 1.41 | 0.81 | 1.94 |
| | -8.30 | 2 | 83.06 | 2.72 | 1.92 | 3.28 |
| | -9.00 | 3 | 92.99 | 1.56 | 0.90 | 1.68 |
| | -10.00 | 3 | 94.24 | 2.45 | 1.42 | 2.60 |
| Battelle | -4.00 | 1 | 0.12 | NC | NC | NC |
| | -5.00 | 3 | 0.35 | 0.04 | 0.03 | 12.83 |
| | -6.00 | 3 | 3.32 | 0.48 | 0.28 | 14.54 |
| | -7.00 | 3 | 23.98 | 2.62 | 1.51 | 10.91 |
| | -7.60 | 2 | 57.80 | NC | NC | NC |
| | -8.00 | 3 | 73.97 | 5.12 | 2.96 | 6.92 |
| | -8.60 | 2 | 92.89 | NC | NC | NC |
| | -9.00 | 3 | 93.13 | 6.71 | 3.88 | 7.21 |
| | -10.00 | 3 | 97.66 | 1.12 | 0.65 | 1.15 |
| | -11.00[b] | 1 | 100.41 | NC | NC | NC |
| In Vitro | -4.00 | 3 | 0.24 | 0.32 | 0.18 | 133.6 |
| | -5.00 | 3 | 0.41 | 0.12 | 0.07 | 29.4 |
| | -6.00 | 3 | 2.31 | 0.51 | 0.30 | 22.1 |
| | -7.00 | 3 | 18.81 | 2.20 | 1.27 | 11.7 |
| | -7.48 | 1 | 82.69 | NC | NC | NC |
| | -8.00 | 3 | 67.45 | 8.45 | 4.88 | 12.5 |
| | -8.48 | 2 | 87.75 | NC | NC | NC |
| | -9.00 | 3 | 94.62 | 13.09 | 7.56 | 13.8 |
| | -10.00 | 3 | 98.37 | 13.52 | 7.81 | 1.37 |
| WIL | -3.00 | 1 | 0.08 | NC | NC | NC |
| | -4.00 | 1 | 0.22 | NC | NC | NC |
| | -5.00 | 3 | 4.12 | 6.43 | 3.71 | 156.03 |
| | -6.00 | 3 | 20.05 | 28.60 | 16.51 | 142.65 |
| | -7.00 | 3 | 48.59 | 42.67 | 24.64 | 87.83 |
| | -7.52 | 2 | 76.98 | 31.32 | 22.15 | 40.69 |
| | -8.00 | 3 | 86.92 | 13.47 | 7.78 | 15.50 |
| | -8.52 | 2 | 100.92 | 5.95 | 4.21 | 5.89 |
| | -9.00 | 3 | 104.56 | 1.40 | 0.81 | 1.34 |
| | -10.00 | 3 | 104.79 | 4.51 | 2.60 | 4.30 |