INTEGRATED SUMMARY REPORT

for the

VALIDATION OF AN ANDROGEN RECEPTOR BINDING ASSAY AS A POTENTIAL SCREEN IN THE ENDOCRINE DISRUPTOR SCREENING PROGRAM

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TABLE OF CONTENTS

Page

TABLE OF CONTENTS
(CONTINUED)

Page

List of Tables

TABLE OF CONTENTS
(continued)

Page

List of Figures

TABLE OF CONTENTS
(continued)

Page

TABLE OF CONTENTS
(continued)

Page

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1.0 EXECUTIVE SUMMARY

The Endocrine Disruptor Screening Program (EDSP) of the US EPA was charged with developing a screening program to determine whether certain substances may have estrogenic effects in humans. Upon the advice of the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), EPA expanded the program to include estrogen, androgen, and thyroid systems. The EDSP consists of two tiers: Tier I to identify the potential of chemicals to interact with the estrogen, androgen, or thyroid hormone systems; and Tier II to identify and characterize the adverse effects resulting from that interaction and the exposures required to produce them. A negative result in Tier I would be sufficient to put a chemical aside as having low to no potential to cause endocrine disruption. An *in vitro* rat ventral prostate cytosol androgen receptor (AR) binding assay was optimized and validated as a principal component of the Tier I testing requirement to identify chemicals that have the potential to bind to the AR.

Criteria for validation of *in vitro* methods designed to replace animal tests include:

- Scientific and regulatory rationale for the test method
- A clear relationship of the endpoints determined by the test method
- A formal detailed protocol
- Evaluation of within-test, intralaboratory, and interlaboratory evaluation of variability, and how these parameters vary with time
- Performance demonstration using a series of coded reference chemicals.

The purpose of this Integrated Summary Report is to summarize in a single coherent document all of the relevant information from the scientific literature and EPA's validation program that supports the conclusion that the AR binding assay using receptors from rat prostate cytosol is valid for the purpose of identifying chemicals that have the potential to bind to the AR.

The text is organized into 13 chapters. Chapter 2 outlines the scope of the document, the intended audience, and the topics covered in the remaining chapters. Chapter 3 presents the scientific rationale for the androgen assay and a brief history of *in vitro* AR binding assay development. Chapter 4 presents a description of the test method, summarizing a detailed protocol that is presented in Appendix A. Chapter 5 discusses the statistical modeling approaches and data analysis. Chapter 6 presents the reference chemicals and chemicals tested in the various validation exercises, as described in Chapters 7 through 10. Chapter 7 describes the assay development and optimization in the lead laboratory. The interlaboratory validation studies are presented in Chapters 8 (preliminary) and 9. Appendix B also details the interlaboratory parameter variability. Chapter 10 details the experimental results of the supplemental validation studies conducted in the lead laboratory. Chapter 11 presents a discussion of the performance criteria employed for determining acceptable assay runs and data interpretation procedures to classify test chemicals as binders or non-binders. Compliance with the validation criteria and general conclusions are covered in Chapter 12. Chapter 13 contains a list of relevant references.

AR binding assays generally use a soluble, testosterone-binding protein complex from the rat ventral prostate similar to the estrogen receptor (ER) binding assay. Testosterone is the principal

endogenous androgenic substance. *In vitro* AR binding assays are based on the conservation of the AR ligand binding domain among vertebrate species, so that substances that bind to AR derived from one species are expected to bind to the AR from other vertebrate species. The assay measures the presence and strength of AR binding of test substances and is performed in two phases. The initial phase is a saturation binding assay, which measures the affinity of the particular AR preparation for the reference androgen. The results from the saturation binding assay are then used as a reference value for the competitive binding assay of the unknown substance.

The assays measure the affinity (Kd) of radiolabeled androgen for the AR and the concentration at which the unlabeled androgen displaces half the specific binding of radiolabeled androgen to the AR (IC_{50}). In *in vitro* AR binding assays, there are substances that do not decrease the binding of the radiolabeled, reference androgen by at least 50%; the IC_{50} values for these substances are generally reported as being greater than the highest concentration tested, or the substances are classified as not binding. Because of the potential for variation in IC_{50} values among AR binding assays using different test protocols, the generally accepted method for presenting and comparing the assay results is to compute the relative binding affinity (RBA) of the test substance against a reference androgen. The RBA is calculated as

$$IC_{50(\text{reference androgen})}/IC_{50(\text{test substance})} \text{ x } 100.$$

The assay evaluates the inhibition of AR binding of methyltrienolone (R1881) in rat ventral prostate cytosol. A saturation assay is first performed to characterize the receptor activity. Competitive binding of test compounds and a weak positive control (dexamethasone) to the AR is measured using a radio tracer (radiolabeled [^{3}H]-R1881).

For each test run the four parameter concentration response model is fitted to the concentration response data for each chemical by nonlinear regression analysis using PRISM or SAS. 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. Included in the analysis is a criterion to classify chemicals as binders, non-binders or equivocal.

The assay development and optimization experiments were designed to identify the optimal factors and conditions for the AR assay. This was conducted by (1) confirming the performance of the AR assay, (2) evaluating the performance of the competitive binding assay with three unknown chemicals, and (3) performing a series of competitive assays with 16 unknown chemical samples to expand the database for determining the effectiveness of the assay.

In evaluations of the optimized protocol at the lead laboratory, no statistically significant differences were found between technicians or day of assay for the saturation or competitive assays. The standard curve was run several times during this task, with an average log IC_{50} of -9.03 log M, and a coefficient of variation of 11.2%. The individual runs are evenly distributed about the mean and are consistent with previously reported IC_{50}s.

In the test with the 16 coded, unknown chemicals, the logIC_{50} values ranged from -8.89 to -3.08 log M with RBA of means from 72.111 to 0.001% for the chemicals that bound to the AR. The R^2 values for the individual curve fits were above 0.9 for all except the two weakest binders, for which the highest experimental concentrations did not achieve full displacement of the radiolabel. Additionally, the run-to-run variability was quite small; the logIC_{50} standard deviations were less than 10% of the IC_{50} values.

Preliminary interlaboratory studies were conducted to evaluate the intra- and interlaboratory variability of results among five independent laboratories. Participating laboratories were supplied with a detailed protocol for AR saturation and competitive binding assays. This validation was conducted in two stages, first using a supplied "standard" cytosol preparation, then using cytosol prepared by individual laboratories.

Each of the five participating laboratories conducted three independent replicate saturation assays with three replicate runs of each concentration. The goodness-of-fit ranged from 0.57 to 1.00 with a mean value of 0.96 for the 15 runs. The range of B_{max} (fmole/100 μg) values was 6.67 to 15.6 with a mean value of 11.0. The range of K_d (nM) values was 0.685 to 1.57 with a mean value of 0.978. The intra-laboratory CVs for B_{max} ranged from 3.4% to 27% with a mean of 11% and for K_d ranged from 3.0% to 22% with a mean of 10.0%. The interlaboratory variability of the two saturation binding measurements was 16% and 25% for B_{max} and K_d, respectively. The variability in these measurements was fairly large and can be explained by the variability in the fitted one-site binding curves that resulted from each laboratory's interpretation and reproduction of the saturation assay protocol.

Competitive assays were conducted with the standard, R1881, and weak positive control, dexamethasone. The range of logIC_{50} values for the standard was -8.9 to -8.6 with a mean value of -8.8. The range of IC_{50} values for the weak positive control was -4.6 to -4.0 with a mean value of -4.4. The resulting RBAs ranged from 0.0017% to 0.0097% with a mean value of 0.0046%. The inter-laboratory variability of the three competitive binding measurements was 13.1%, 16.7%, and 14.7% for the standard and weak positive log IC_{50} values and RBA, respectively.

For the second part of the preliminary validation, all five laboratories attempted to prepare cytosol for the assay. Three laboratories successfully prepared the cytosol and performed acceptable saturation and competitive assays. One laboratory was not able to obtain cytosol with appropriate activity and was limited to several saturation binding assays, which did not demonstrate appropriate binding activity. Another lab exhausted their funds and did not complete any of the work for this task.

The range of B_{max} (fmole/100 μg) values was 4.81 to 16.8 with a median value of 9.25. The range of K_d (nM) values was 0.660 to 2.88 with a median value of 0.928. The intra-laboratory CVs for B_{max} ranged from 4.4% to 11% with a median of 6.9% and for K_d ranged from 2.9% to 24% with a median of 8.3%. The inter-laboratory variability of the saturation binding measurements from the four laboratories was 53% and 61% for B_{max} and K_d, respectively. The variability in these measurements was large and can be explained by the variability in the fitted

one-site binding curves, which resulted from each laboratory's interpretation and reproduction of the saturation assay protocol.

The range of log IC_{50} values for the standard ranged from -9.2 to -8.7 with a mean value of -8.9. The range of log IC_{50} (log M) values for the weak positive control was -4.3 to -4.0 with a mean value of -4.2. The resulting RBAs ranged from 0.0010% to 0.0033% with a mean value of 0.0024%. The intralaboratory CVs for RBA ranged from 1% to 46% with a median of 5%. The variability in these measurements can be inferred by the variability in the fitted one-site competitive curves.

Four laboratories tested 10 chemicals (as coded unknowns) in the main interlaboratory validation study, whose binding affinities spanned the spectrum of activity from strong binder to non-binder. The objective of these studies was to establish the variability of results among independent laboratories when using cytosol prepared by each of the individual laboratories. Each participating laboratory conducted three independent runs of the competitive binding assay with a standard, a weak positive control, and 10 test chemicals.

The range of log IC_{50} values for the standard was -8.9 to -8.2 with a mean value of -8.6. The range of log IC_{50} values for the weak positive control was -4.5 to -3.0 with a mean value of -4.2. The resulting RBAs ranged from 0.0004% to 0.0100% with a median value of 0.0039%. The intralaboratory CV for RBA was 27%.

Many of the test runs converged and had better fits after excluding values at the highest test concentrations. In some cases the laboratories made notations that indicated solubility limitations (precipitation). Even in cases where solubility was not limiting, it is possible that such high chemical concentrations can cause physical or biological changes to the system, which are not directly related to the binding activity of the AR. DEHP and atrazine were found to be nonbinders.

The objective for the supplemental validation studies was to test approximately 30 test chemicals in one laboratory to expand the data base of binding information on chemicals for the validation of the AR binding assay. Test substances included steroids, non-steroidal antiandrogens, synthetic androgens and antiandrogens, chlorinated pesticides, PAHs, flavanoids, phenols, and heterocyclic compounds. Test chemicals were tested as coded unknowns.

The K_d and B_{max} values were consistent with those in earlier validation work. For the R1881 standard the range of logIC_{50} (log M) values was -8.9 to -8.7 with a median value of -8.8. The goodness of fit for all data sets was greater than 0.983, indicating an adequate model fit. For the competitive binding assays with the weak positive dexamethasone (18 runs) the range of $\log_{10}IC_{50}$ (log M) values was -4.6 to -4.3 with a median value of -4.4. The goodness of fit for all data sets was greater than 0.952, indicating an adequate model fit. Phenobarbital, phorbol 12-myristate 13-acetate, and 2,4,5-trichlorophenoxyacetic acid did not exhibit any significant inhibition of the binding of [^{3}H] R1881 to the androgen receptor. The other 27 compounds exhibited some degree of AR activity. Most of these chemicals were selected with some indications that they were binders and most were found to be. Others were clearly non-binders in the assay with a few that were equivocal. However, since authoritative data were not available

for many of these chemicals, they could not be used to determine the specificity or selectivity of the assay.

It is clear from the data from these experiments that laboratory proficiency in the assay is challenged by the weak positive control and that more proficient laboratories are able to obtain consistent results across multiple runs of the assay. Laboratories had little trouble with the strong binders which produced full binding curves. As expected from the results with the weak positive control, laboratories had more difficulty with the weaker binders. To obtain a high quality binding curve for weak binders, it is necessary to provide good quality data at high concentrations in order to define the bottom of the binding curve.

Various options for performance criteria were explored for both standards and test chemicals. Performance criteria were developed for the strong and weak positive controls only, as consistent results on the positive controls and especially on the weak positive control, are good indicators that the laboratory is proficient in conducting the assay. The performance criteria should be met for each run; however, it is important that test chemicals be subjected to a reasonableness test as well.

EPA investigated several different options for data interpretation criteria. There is no perfect system. Although some believe there should be a complete binding curve for positive chemicals, EPA concluded that this was too restrictive and would miss a substantial number of weak positives. EPA concludes that the criteria 50% or greater displacement of the binding curve to define binders and a maximum of 25% displacement to define a non-binder with equivocal chemicals in between these values provides a reasonable balance between false negatives and false positive results.

2.0 INTRODUCTION

2.1 Purpose of the EDSP

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 (USEPA, 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 (USEPA 1998a). 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. (EDSTAC, 1998)

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 2-1.

Table 2-1. Assays Recommended for Consideration for the Tier 1 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.

Assay	Nature of Assay and Reasons for Inclusion
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.

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 2-2 as alternatives.

Table 2-2. Alternative Assays for Tier 1

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.

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

2.5 Validation

As noted in Section 2.1, 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 U.S. 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 that were developed to screen chemicals for endocrine activity rather than to replace existing assays.

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; the information needed 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 (USEPA, 2006a).

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 reduce 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 1 screens and Tier 2 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 1 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 1 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 for existing *in vivo* or *in vitro* 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 compounds that was recommended by the SAP (SAP, 1999) is expected to allow a more thorough assessment of the performance of the Tier 1 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 relevant information from the scientific literature and EPA's validation program that supports the conclusion that the androgen receptor binding assay using receptors from rat prostate cytosol is valid for the purpose of identifying chemicals that have the potential to bind to the androgen receptor. The documents supporting the validation of the assay 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 by each of the participating laboratories. Subsequent chapters will describe the scientific basis of the androgen receptor binding assay (Chapter 3); the test method (Chapter 4); how data are modeled, analyzed, and interpreted (Chapter 5); the chemicals used in the various studies and the rationale for their selection (Chapter 6); the optimization of the assay protocol (Chapter 7); studies using controls in the laboratories participating in the interlaboratory studies (Chapter 8); the interlaboratory validation studies and a comparison of laboratory performance with 10 reference substances (Chapter 9); the study of an additional 27 chemicals to test the protocol with more diverse chemicals (Chapter 10); the development of performance criteria (Chapter 11); and a statement of compliance with validation criteria (Chapter 12).

3.0 SCIENTIFIC BASIS OF THE ANDROGEN ASSAY

The information in this section was adapted from ICCVAM (2003a,b).

3.1 Rationale for the Use of *In Vitro* AR Binding Assays

The biological action of androgens is mediated through their interaction with the androgen receptor (AR). Androgens are male sex hormones that also have a role in female development and physiology. They exert their actions through a series of steps in cells in different tissues of the body. The principal endogenous androgens in humans and other mammals are testosterone and 5α-dihydrotestosterone (DHT). Androgen binds to the AR in the cell which subsequently dimerizes (Wong et al., 1993). This conformational change initiates a cascade of events; the hormone-receptor complex can be phosphorylated and interact with AR-associated transcriptional factors in the cell, including activators, repressors, and modulators (Culig et al., 2000; Sharma et al., 2000; Haendler et al., 2001; Geserick et al., 2003; Verrijdt et al., 2003, 2006; Yu et al., 2005; Wang et al., 2005). The activated receptor complex is transported to the nucleus where it can bind to specific DNA regulatory sequences of androgen-responsive genes (androgen response elements, or AREs) that are located upstream from, or within, the intron regions of the genes under androgen control. Binding to the AREs activates, or inactivates, their associated genes and leads to the initiation or inhibition of cellular processes. The specific AREs that are affected depend on the specific cofactors that are bound to the AR-receptor complex, and these are a function of the cell type and tissue, and the structure of the ARE (e.g., Culig et al., 2000; Sharma et al., 2000; Haendler et al., 2001; Heinlein & Chang 2002; Geserick et al., 2003; Hodgson et al., 2005; Verrijdt et al., 2003, 2006; Yu et al., 2005; Wang et al., 2005). The androgens have no hormonal activity in the absence of a functional AR. Single gene mutations in the ligand-binding domain that render the AR unable to bind androgens result in androgen insensitivity syndrome in humans, and the affected individuals have no male characteristics (Rosa et al., 2002).

The AR has a high degree of homology with other members of the steroid hormone receptor family. There is a high degree of sequence conservation in the cysteine-rich DNA-binding domain, with less conservation in the carboxyl-terminal, androgen-binding domain. Based on these domains, the AR is closely related to the progesterone, glucocorticoid, and mineralocorticoid receptors (Tilley et al., 1989; Rogerson et al., 2007). Kelce et al. (1998) reported that there is 100% homology between the human and rat ligand-binding domains of the AR, and Rosa et al. (2002) showed that the mouse has 100% homology, and Xenopus has 98%. In the *N*-terminal domain of the AR, which is responsible for transactivation, there is 97% homology (34/35 amino acids) between humans and baboons and macaques, 91% homology between humans and rats and mice, and less with non-mammalian species such as Xenopus (71%) and rainbow trout (60%) (Betney and McEwan, 2003). Therefore, substances that bind the AR from these organisms are presumed to be capable of producing androgenic effects in humans and other vertebrates.

The AR plays a pivotal role in the development and maintenance of the male and female reproductive systems because it is the primary receptor for endogenous androgens that initiate the transcription of messenger RNA, and ultimately protein synthesis in androgen-target cells. Because of its role in translating circulating androgen levels to physiological responses, the AR

can be considered the key molecule in male sexual differentiation, maintenance of the male sexual characteristics, spermatocyte production, prostate gland development and growth, and a number of non-sexual functions, and is a major factor in the onset and maintenance of prostate cancer. There is evidence that androgens may also indirectly influence the expression of genes that do not contain AREs by modulating the activity of secondary transcription factors, mediating the expression of other growth factors, or by affecting the production of other hormones (Heemers et al., 2006). In addition to being present in the male reproductive tissues and sex accessory glands, ARs are located in non-sex-related tissues, including hair cells (Jave-Suarez et al., 2004), skin (Mowszowicz et al., 1981), connective tissue and bone marrow, including female bone marrow (Liegibel et al., 2003; Mantalaris, 2001), neural cells (Kritzer, 2004; O'Bryant & Jordan, 2005; Tabori et al., 2005), cardiac myocytes (Schock et al., 2006), the pituitary and hypothalamus glands (Perez-Palacios et al., 1983), and are also implicated in obesity (Pasquali, 2006). Although male external genitalia lack ER, the female external genitalia contain AR (Kalloo et al., 1993), as does the human ovary (Edmonson et al., 2002).

Because AR binding is the initiating step in the cascade of androgen-related effects, a number of *in vitro* AR-binding assays have been developed to measure interference or competition with this binding step. These screening assays measure the receptor-binding affinity of chemicals by their ability to displace bound reference androgen, usually testosterone or DHT. Such interference with normal androgen binding has the potential to interfere (i.e., compete) with normal androgen activity *in vivo* by acting as an agonist and producing androgenic effects, or as an antagonist, interfering with the actions of natural androgens. However, the AR-binding assays only have the ability to determine if a substance binds to the receptor and cannot distinguish between agonist and antagonist activity.

Because of the conservation of the ligand binding site of the AR across mammalian phylogenetic lines (described above), substances that bind the AR and activate or inhibit AR-induced transcriptional activation are expected to have the same activity across vertebrate species. As a result, AR-binding assays can be conducted using ARs from a variety of cell types and animal sources.

3.2 Historical Background of the Development of *In Vitro* AR Binding Assays

The AR binding assays currently in use can be traced back to the late 1960s when Anderson and Liao (1968), Bruchovsky and Wilson (1968), and Fang et al. (1969) demonstrated that DHT, a metabolite of testosterone, bound to a nuclear protein in rat ventral prostate tissue. Subsequently, other investigators (Mainwaring, 1969a,b; Unhjem et al., 1969) isolated a soluble, testosterone-binding protein complex from the rat ventral prostate gland, from prostate tissue slices *in vitro*, and from prostate cytosol. This androgen-protein complex is associated with nuclear chromatin, and its size and physicochemical behavior suggested that it was similar to the estrogen receptor (ER) (Noteboom and Gorski, 1965; Toft and Gorski, 1966).

Testosterone, which is produced by the Leydig cells of the testes, is the principal endogenous androgenic substance. It is metabolized to its more active metabolite, DHT, by steroid 5α-reductase (Δ^4-3-ketosteroid-5α-oxidoreductase), which is located in the microsomal and nuclear fractions of the cell. DHT appears to be the favored ligand *in vivo*, primarily as a result of its ability to stabilize the receptor complex more effectively than testosterone. DHT and testosterone

have similar *in vitro* equilibrium dissociation constants of approximately 2–5 x 10^{-10} M (Wilson and French, 1976; Lubahn et al., 1988a).

Some researchers have reported that the AR protein is relatively unstable *in vitro*. It is highly sensitive to pH and temperature, and rapidly degrades in the absence of ligand. Wilson and French (1976) found that cytosolic AR from rat testis or epididymis degraded rapidly ($t_{½}$ = 15–25 minutes at 23°C) when not bound to a ligand. Kemppainen et al. (1992) also reported rapid degradation of the AR expressed in transiently transfected COS cells ($t_{½}$ = 1 hour at 37°C) in the absence of ligand. However, stability of the receptor was greatly enhanced at lower temperatures, at a basic pH (e.g., pH = 8 at 0°C), and in the presence of testosterone, DHT, or a synthetic androgen, such as 17β-hydroxy-17α-methyl-estra-4,9,11-trien-3-one (methyltrienolone; R1881) (Wilson and French, 1976; Kemppainen et al., 1992).

The *in vitro* AR binding assays are based on the conservation of the AR ligand binding domain among vertebrate species, so that substances that bind to AR derived from one species are expected to bind to the AR from other vertebrate species. However, the relative binding affinities of receptors from different species for the same ligand, or the same AR in different matrices may be different, as is evident from the differences in binding affinities calculated from binding assays using different AR receptors or cell carriers (ICCVAM, 2003a).

The assays to measure the presence and strength of AR binding of test substances are performed in two phases. The initial phase is a saturation binding assay which measures the affinity of the particular AR preparation for the reference androgen. The results from the saturation binding assay are then used as a reference value for the competitive binding assay of the unknown substance. The historical development of the assay is described briefly below, and both assays are described more completely in Chapter 4.

The assays measure the affinity of radiolabeled androgen (Kd) for the AR, the affinity of unlabeled, reference androgen (Ki) for the AR, and the concentration at which the unlabeled androgen displaces half the specific binding of radiolabeled androgen to the AR (IC_{50}). The Kd, which is measured in concentration units, is the equilibrium dissociation constant of the radiolabeled androgen-AR complex and represents the concentration of labeled reference androgen that will bind to half the binding sites at equilibrium in the absence of competitors. A low Kd represents high affinity and a high Kd represents low affinity. The Ki is the analogous constant for the unlabeled ligand. The IC_{50} values, which represent the competition of the radio-labeled androgen and unlabeled (test) ligand for the receptor sites, depend on a number of factors, such as the specific assay system used, binding affinity of the unlabeled ligand for the AR, androgen concentration, AR concentration, and experimental conditions (e.g., pH, exposure duration). In *in vitro* AR binding assays, there are substances that, because of biological inactivity, low solubility, or other considerations, do not decrease the binding of the radiolabeled, reference androgen by at least 50%. The IC_{50} values for these substances are generally reported as being greater than the highest concentration tested, or the substances are classified as not binding.

The procedures used to calculate the binding parameters are essentially variations on the method published by Scatchard (1949), who developed models for the binding of small molecules to

proteins and for extrapolating binding data. In a "Scatchard plot", a straight line indicates that a single class of binding site is present; if competing binding sites are present, the line will deviate from linearity. The intercept on the abscissa indicates the number of binding sites available; the association constant is the ratio of the intercepts on the abscissa and ordinate (Puca and Bresciani, 1969). Scatchard plots are widely used in receptor binding studies, but are generally used today to characterize saturation binding assay results.

Baulieu and Raynaud (1970) proposed using an alternative procedure for approximating the binding parameters of small molecules in protein mixtures. They developed a nonlinear function by plotting the log of the bound fraction to the log of the total ligand, and demonstrated that this procedure was able to quantitatively distinguish between specific and nonspecific binding (i.e., to sites other than the AR) in a tissue extract that contained a mixture of specific and nonspecific receptors.

Because of the potential for variation in IC_{50} values among AR binding assays, the generally accepted method for presenting and comparing the assay results is to compute the relative binding affinity (RBA) of the test substance against a reference androgen. The RBA is calculated as $IC_{50(\text{reference androgen})}/IC_{50(\text{test substance})}$ x 100. DHT has generally been used as the reference androgen for calculating the RBA value, but testosterone and synthetic androgens, such as R1881, have also been used. An examination of the literature shows that the RBA values cover approximately seven orders of magnitude (ICCVAM, 2003a); there is insufficient information available as to which levels of AR-binding activity are biologically meaningful, and there is no general agreement regarding the distinction between the RBA values needed to distinguish endocrine disruptors from non-disruptors.

3.3 Mechanistic Basis of *In Vitro* AR Binding Assays

The AR is a transcriptional regulatory protein belonging to the nuclear hormone receptor superfamily. The human AR gene was cloned and sequenced by Lubahn et al. (1988a) and Chang et al. (1988). It is located on the long arm of the X-chromosome as a single copy and encodes a protein of 110-114 kD (Lubahn et al. 1988a,b; Brown, et al., 1989; Tilley et al., 1989). The AR contains 919 amino acids and is localized in the soluble nuclear fraction of androgen target cells; the protein plays a major role in controlling the transcriptional activation and/or repression of androgen-responsive genes (Culig et al., 2000). The AR contains two discrete domains that are necessary for its role as a transcription factor—a ligand-binding domain in the *C*-terminal region, and a DNA-binding domain located approximately centrally in the receptor. The DNA-binding domain contains two zinc finger motifs, which are associated with DNA-binding activity. AR isolated from different rat tissues are identical in structure and function (Wilson and French, 1976).

Two AR subtypes (A and B) have been identified in humans (Wilson and McPhaul, 1994), in rainbow trout (Takeo and Yamashita, 1999), and Japanese eel (Ikeuchi et al., 1999, 2001). The human forms differ in their molecular weights because the A-form lacks the *N*-terminus region found in the B-form. The ratio of expression of AR-A to AR-B in normal human genital fibroblasts is approx. 1:10 (Wilson and McPhaul, 1994); the proportion of AR-A protein to total AR protein in other tissues range from less than 1% to 26%; the actual proportion depended on the tissue being examined and its state of development, i.e., fetal vs. adult (Wilson and McPhaul,

1996). Unlike the two estrogen receptor (ER) subtypes that have different binding characteristics (Kuiper et al., 1996, 1998; Gaido et al., 1999), there do not appear to be any major functional differences in the binding activities of ligands to the two AR subtypes (Gao and McPhaul, 1998); differences reported in early studies may have been the consequence of the lower expression of AR-A (Liegibel et al., 2003; Gao and McPhaul, 1998), or differences in the binding affinity of the AR-A and B-ligand complexes to the ARE (Liegibel et al., 2003).

The current concern for AR-mediated endocrine disruption is that certain xenobiotic substances may mimic or block the action of the natural ligand, testosterone or DHT, by competing with it for the AR-binding site. This interference with the binding of the natural ligand, or the displacement of bound testosterone or DHT, has the potential to produce an androgen-like effect or interfere with normal, physiological, androgen-mediated processes.

Factors that affect ligand binding to the AR include:

- Affinity for the AR. This affinity depends on the rates of the association and disassociation of the ligand with the receptor. The natural ligand, DHT, has a low equilibrium constant because of its rapid association rate, about 5.3×10^{-7} M^{-1} h^{-1} , and slow disassociation rate, $t_{1/2}$ = 38 hours at 0ºC, for AR in rat prostate cytosol (Wilson and French, 1976).
- Systemic half-life of the ligand. The half-life will depend on the rate of metabolism of the ligand to an active intermediate, or metabolic inactivation of an active substance, and to the clearance of the ligand and its metabolites from the organism.
- Concentration of the ligand. Weakly binding ligands may produce an effect if they are administered at high enough concentrations, and strongly binding ligands would be ineffective if they do not reach androgen-sensitive tissues. High concentrations of ligands may also produce [physicochemical effects] that can mimic binding in the assay.

In addition to ligand binding to displace the reference androgen, the reference androgen may also be released by changes in the conformation of the AR resulting from non-specific chemical effects such as protein denaturation, or pH changes that may affect ionization of the AR or the natural ligand. Such procedure-related factors would lead to ligand displacement, and would mimic the competitive binding effect, but would not result in an active ligand-AR complex.

AR binding assays are most often conducted with a cell-free AR preparation obtained from androgen-responsive tissues or cells from rodents or humans (e.g., ventral prostate, foreskin fibroblasts). Although AR binding assays have changed little since they were initially developed, some of the more recent procedures have incorporated new technology, for example, including the use of recombinant AR proteins in place of AR isolated from tissues or cells (Bauer et al., 2000; Freyberger and Ahr, 2004), or using immobilized AR preparations (Bauer et al., 2002). In addition, a number of laboratories have begun to define AR-induced agonistic and antagonistic effects by changes in gene expression (e.g., Ohsako et al., 2003; Altieri et al., 2006; Mu et al., 2006).

3.4 Relationship of *In Vitro* AR-Binding Activity to *In Vivo* Androgenic or Antiandrogenic Activity

In vitro AR binding assays have been proposed as a way to screen large numbers of chemicals for potential androgen disruption in intact organisms (USEPA 1997; 1998a,b; 1999). The ability of a substance to induce the release of reference androgen in an *in vitro* binding assay is sufficient to identify the substance as having potential androgenic activity. As noted above in **Section 3.3**, the *in vitro* binding assay, as typically performed, may also respond to non-specific interference with the binding of the reference androgen and lead to the calculation of an RBA, although no ligand-AR complex is formed. The assay is therefore used as a screen for identifying and prioritizing potential AR-binding substances that can then be further examined for their functional effects in other *in vitro* and/or *in vivo* tests. Such subsequent tests could include the examination of the binding kinetics (Ki) of the reaction to determine if the reference androgen release was through a competitive binding with the reference androgen, or by a functional assay, such as those that measure transcriptional activation in intact cells or endocrine effects *in vivo* (Kelce et al., 1995; Lambright et al., 2000; ICCVAM 2003a,b, Yamasaki et al., 2004; Charles et al., 2005; Owens et al., 2007).

At present, there is limited information to relate the *in vitro* binding affinity, as measured by the assays described in **Section 3.2**, to the ability of the AR-ligand interaction to produce *in vivo* effects. Among the factors that may lead to differences between responses *in vitro* and *in vivo* is the absence of mammalian metabolic activation *in vitro*. Many chemicals are biologically inactive in their native form, but can be metabolized to more (or less) active substances by enzymes *in vivo*. As a consequence, the *in vitro* assays may not test the same chemicals present in the body, i.e., the metabolites and other breakdown products of the test chemical. In addition, the test chemical-AR complex formed *in vivo* needs to further interact with other cellular factors (described in **Section 3.1**) to produce the biologically active complex. These additional factors are not present in the i*n vitro* assay systems, where only the ability of the parent substance to bind AR and/or displace the reference androgen is measured.

Potential binding activity is inferred for a substance by its ability to compete with a reference androgen for binding to the AR, although the AR binding activity provides no information as to whether the substance would act as an agonist or antagonist *in vivo*. Assays such as the *in vitro* transcriptional assay or the *in vivo* Hershberger assay can be used to determine whether a substance that binds to the AR is an agonist or antagonist, or may have both activities.

3.5 Intended Role of *In Vitro* AR-Binding Assays in an Endocrine Disruptor Screening Program

An *in vitro* AR binding assay is one component of the proposed Endocrine Disruptor Tier 1 screening battery, which also comprises *in vitro* and *in vivo* assays for androgen, estrogen, and thyroid hormone activity (USEPA, 1998a,b; Gray et al., 2002; O'Connor et al., 2002). The main purpose of the assay is as a screen for potential AR binders. Therefore, a positive response in an AR-binding assay, by itself, does not demonstrate biological activity or predict subsequent cellular effects, only that the substance is capable of reacting with the AR binding site or interfering with the binding of the natural androgen. For this reason, the *in vitro* AR binding assay will be used in conjunction with other assays in the Tier-1 battery (USEPA, 1998a,b;

OECD, 2007). The other proposed Tier-1 components of the androgen screening battery include the steroidogenesis assay, the rodent Hershberger assay, and possibly a male pubertal assay or 15-day assay in the intact adult male.

3.6 Assay Limitations

As noted above, the AR binding assay has several limitations. It can only detect binding to the receptor and, therefore, cannot predict transcriptional activation or distinguish between chemicals that act as androgens and those that block the receptor and act as antiandrogens. Chemicals that are poorly solubilized will not be able to be tested, and chemicals that denature the receptor will appear as false positives. There is no metabolic capability to the assay, which is both an advantage and a disadvantage. It is useful in maximizing the sensitivity of the assay for weak binders since they are not degraded, but it will fail to detect chemicals that need metabolic conversion to bind to the receptor. In addition, the assay uses animal tissue and is not amenable for high-throughput screening.

4.0 DESCRIPTION OF THE TEST METHOD

The following is a general description of the test method; a detailed protocol is provided as Appendix A. The test method evaluates the inhibition of androgen receptor (AR) binding of R1881 in rat ventral prostate cytosol by known chemicals. Rat ventral prostate cytosol is used as a biological source of the androgen receptor. The assay is designed as a screening assay to reduce the need for extensive whole animal exposure studies. A saturation assay is first performed to characterize the receptor activity. Competitive binding of test compounds and a positive control (dexamethasone) to the AR are measured using a radio tracer (Radiolabeled [^{3}H]-R1881).

4.1 Assay Design

The assay consists of two sets of experiments, the saturation and the competitive binding assays. The saturation assay measures the affinity of radioactive ligand for the receptor and is required to demonstrate sufficient AR specificity and activity. It is quantified by the calculated values of AR binding affinity (K_d, nM) and maximum specific binding number (B_{max}, fmoles of R1881/100 µg of protein). Briefly, the saturation assay tests a range of mixtures of labeled and unlabeled R1881 to measure the total, nonspecific and specific [^{3}H]-R1881 binding. K_d and B_{max} may be calculated through non-linear regression or using a Scatchard plot. The purpose of the saturation binding assay is to characterize the cytosol preparation and ensure that the AR activity is sufficient for the competitive assay.

The competitive assay measures the affinity of unlabeled ligand in competition with high affinity radioligand (R1881). It is quantified by the calculated values of the 50% inhibition concentration (IC_{50}, M) and frequently by relative binding affinity (RBA, %). The competitive assay measures the binding of [^{3}H]-R1881 in the presence of unlabelled R1881 and a wide range of test chemical concentrations. The data is then normalized to the percent of R1881 binding and an IC_{50} is fit for unlabelled R1881 and test chemical using a nonlinear curve fit. Based on the IC_{50} values of R1881 and the test compounds, the test compounds can be evaluated for their AR binding potential and a RBA generated based on the ratio of IC_{50} values observed. RBA is simply the ratio $IC_{50,\ standard}/IC_{50,\ test}$ and is designed to assess the relative binding affinity of the standard R1881 to the test compound (in log units, the $\log_{10}RBA = (\log_{10}IC_{50,\ standard} - \log_{10}IC_{50,\ test})$.

4.2 Assay Components

4.2.1 Reference Androgen – Methyltrienolone (R1881)

Methyltrienolone (R1881, 17β-hydroxy-17α-methyl-estra-4,9,11-trien-3-one, CAS 965-93-5) is a synthetic steroid that binds with high affinity to the androgen receptor of rat prostate. R1881 stock solutions (30 mM) are prepared in absolute ethanol then further diluted in ethanol for the saturation assays at 10^{-5} and 10^{-6} M. The final unlabeled R1881 concentration in the assay tubes are 0.025, 0.05 0.07, 0.10, 0.15, 0.25, 0.5 and 1.0 µM.

Dilutions of the R1881 stock solutions are also prepared in ethanol for competitive assays. The final assay target concentrations for R1881 are 10^{-6} to 10^{-11} M. The total volume of solvent used in each assay is no more than 3.33% of the total assay volume.

4.2.2 Marker/Tracer Preparation – Radiolabeled Methyltrienolone (R1881)

The marker/tracer solution is prepared from the radiolabeled R1881. Dilutions of the [^{3}H] R1881 stock are prepared in ethanol for the saturation assays at 10^{-7} and 10^{-8} M. In the saturation assay, final [^{3}H] R1881 concentration in the assay tubes are 0.25, 0.5 0.7, 1.0, 1.5, 2.5, 5.0 and 10.0 nM.

For competitive assays, the [^{3}H]-R1881 is diluted with ethanol to achieve a substrate solution at a concentration of 10 nM. The substrate solution (30 µL) is added to the incubation mixtures to achieve a final concentration of 1 nM [^{3}H]-R1881 in the 300 µL volume for the assay.

4.2.3 Positive Control – Dexamethasone

Dexamethasone (CAS 50-02-2) is used as a positive control (weak positive) in the competitive binding assay. Dexamethasone stock solutions (30 mM) are prepared in absolute ethanol with final target concentrations for dexamethasone in assay tubes of 10^{-3} to 10^{-10} M. The total volume of solvent used in each assay is no more than 3.33% of the total assay volume.

4.3 Rat Ventral Prostate Cytosol

The rat prostate cytosol is prepared following specific protocols. Briefly, the ventral prostate tissues are collected from Sprague-Dawley male rats (85 to 100 days of age) castrated 24 hours prior to being humanely killed. For the study, weighed and trimmed prostate tissues are placed in ice-cold buffer prepared with Tris, Ethylenediaminetetraacetic acid and Glycerol (TEDG) with phenylmethylsulfonyl fluoride (PMSF) with final extraction volume equaling a ratio of 0.1 g of tissue per 1.0 ml TEDG buffer with PMSF. The tissues are homogenized and the cytosol pooled, aliquoted and stored at –80 ºC.

The protein concentration of the cytosol preparation is determined for each batch of the cytosol. In brief, a six-point curve is prepared, ranging from 0.2 to 2.0 mg protein / mL. The protein standards are made from bovine serum albumin (BSA) and protein content determined using a protein assay kit. Absorbance (600 nm) is measured using a plate reader. The protein concentration of the cytosol sample is determined by extrapolation of the absorbance value using the standard curve developed using the protein standard.

4.4 Saturation Radioligand Binding Assay

Androgen receptor saturation binding experiments measure total, non-specific, and specific binding of increasing concentrations of [^{3}H]-R1881 under conditions of equilibrium. Final [^{3}H] R1881 concentrations are 0.25, 0.5 0.7, 1.0, 1.5, 2.5, 5.0 and 10.0 nM. The saturation binding experiments are conducted as three independent replicates. Total binding (fmoles of R1881) is calculated by converting the DPM from samples containing only [^{3}H]-R1881. Nonspecific binding is calculated by converting the DPM from tubes containing [^{3}H]-R1881 + 100-fold molar excess of radioinert R1881, assuming that the excess of radioinert R1881 will occupy all of the available androgen receptor binding sites. Specific binding is calculated as the difference between the nonspecific binding and total binding at each of the tested doses.

In general, when evaluating data from AR saturation assays, the following points should be considered.

- As increasing concentrations of [^{3}H]-R1881 were used, does the specific binding curve reach a plateau? Maximum specific binding must be reached, indicating saturation of AR with ligand.
- Does the data produce a linear Scatchard plot (a plot of bound/free ligand as a function of specific binding)?
- Is the K_d within an acceptable range? The values for K_d in the EPA validation program ranged from 0.8121 to 0.9698 nM.
- Is non-specific binding excessive? The non-specific binding for the assay optimization tasks ranged from 8.1 to 10.0%, well within the criteria. The value for non-specific binding should be less than 50% of the total binding.

The responses to these questions are presented in Section 7.1.1 below.

4.5 Competitive Binding Assay (Inhibition of Androgen Receptor Binding of [^{3}H]-R1881 by Test substance)

An androgen receptor competitive binding assay measures the binding of a single concentration of [^{3}H]-R1881 in the presence of increasing concentrations of a test substance. These experiments test the androgen receptor binding of [^{3}H]-R1881 in the presence of multiple concentrations of a test substance. The inhibition experiments are conducted as three independent replicates. Standard curves, containing varying amounts of radioinert R1881, are also constructed (Table 4-1).

Control samples are included for each replicate experiment. These include:

- Vehicle or ethanol control (substrate, buffer, vehicle [used for preparation of test substance solutions], and cytosol)
- Non-specific background control (substrate, buffer, R1881, and cytosol) (see Table 4-2).

Six repetitions of each type of control are included with each replicate experiment and treated the same as the other samples. The control sets are split so that three tubes (of each control type) were run at the beginning and three at the end of each replicate set.

In general, the assay should demonstrate that increasing concentrations of unlabeled R1881 can compete with a single concentration of [^{3}H]-R1881 for binding to the AR. The curve generated in this step is referred to as the 'standard curve' since it is the standard to which the binding of the unknown is related. Specific questions to evaluate are as follows:

- As a safeguard against ligand depletion, was the total maximal binding no greater than 10 – 25 % of the amount of [^{3}H]-R1881 added per assay tube?
- Are the K_i and IC_{50} values for unlabeled R1881 reasonable? The IC_{50} value for unlabeled R1881 should be approximately equal to the molar concentration of [^{3}H]-R1881 used in the assay tube plus the K_d (determined by nonlinear analysis and Scatchard plot of data obtained from saturation radioligand binding assays).

- Are the K_i, IC_{50}, and RBA values for the substance used to validate the performance of the assay reasonable based on published and historical data?
- Is the negative control substance unable to inhibit binding of the [^{3}H]-R1881?

Table 4-1. Standard Curve

Standards	Initial R1881 Concentration (Molar)	*Final R1881 Concentration (Molar) in AR assay tube
Negative Control	0	
0	0 (EtOH)	0
NSB	1×10^{-5}	1×10^{-6}
S1	3×10^{-6}	1×10^{-7}
S2	3×10^{-7}	1×10^{-8}
S3	3×10^{-8}	1×10^{-9}
S4	3×10^{-9}	1×10^{-10}
S5	3×10^{-10}	1×10^{-11}

* Final concentration = 10 ul of each standard is added to the assay tube, except for the NSB which is 30 ul.

Table 4-2. Weak Positive and Test Chemical Study Design

Sample[a]	Repetitions (Tubes)	Description	Concentration (M)[b]
Concentration 1	3	Complete assay with Test Compound added	1×10^{-3}
Concentration 2	3		1×10^{-4}
Concentration 3	3		1×10^{-5}
Concentration 4	3		1×10^{-6}
Concentration 5	3		1×10^{-7}
Concentration 6	3		1×10^{-8}
Concentration 7	3		1×10^{-9}
Concentration 8	3		1×10^{-10}
Vehicle only	3	Complete assay with Vehicle only	0

[a] Sampling setup is the same for each test compound and the concurrent weak positive dexamethasone.

[b] Final concentration = 10 μL of each Initial Concentration of test compound is added to the assay tube along with 300 μL of ventral prostate cytosol.

5.0 DATA MODELING AND ANALYSIS

5.1 Introduction

The AR binding assay is a member of the class of "radioligand binding assays." In these assays known concentrations of a radiolabeled "ligand" (a soluble molecule that binds to a receptor) is exposed to a receptor and the extent of binding is assessed based on the extent of radiation (dpm) emitted by the receptor ligand complex.

Much of the material in the sections that follow is discussed in greater detail in Motulsky and Christopoulos (2005) Section H, "Fitting radioligand and enzyme kinetics data."

5.2 Saturation Binding Assay

The discussion in this section is restricted to single site binding models.

5.2.1 Law of Mass Action and Receptor Binding

Reversible binding between a ligand and receptor can expressed schematically as

$$\mathbf{Receptor} + \mathbf{Ligand} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \mathbf{Receptor \bullet Ligand}$$

k_{on} and k_{off} represent the reaction rates of the binding and dissociation reactions respectively. K_d is defined as

$$K_d \equiv k_{off}/k_{on}$$

At equilibrium,

$$\text{Fraction of receptor bound} = \frac{[\text{Ligand}]}{[\text{Ligand}] + K_d}$$

This equation corresponds to a rectangular hyperbola relating fraction of receptor bound to concentration of free ligand. It starts at 0 when [Ligand] = 0 and has an upper asymptote of 1 as [Ligand] $\rightarrow \infty$.

5.2.2 Analyzing Saturation Radioligand Data

Two kinds of binding occur with the radioligand. These are referred to as specific binding and nonspecific binding respectively. Specific binding refers to binding of the radioligand to the ligand binding domain of the receptor. The binding referred to in the equations in the above section on law of mass action is specific binding. Nonspecific binding refers to binding of the radioligand to sites other than the ligand binding domain. The greater the concentration of free radioligand the greater the concentration of nonspecific binding will be.

The relationship between free radioligand concentration and concentration of radioligand bound with the receptor pertains to specific binding. The nonspecific binding is treated like background and is adjusted for by subtracting the average of the dpm's of the three nonspecific binding tubes corresponding to each total ligand concentration from the each of the total binding dpm's for that concentration.

The degree of nonspecific binding is determined by including special test specimens that contain the same concentrations of radioligand as the principal test specimens but also a sufficiently large concentration of unlabeled substance that will bind with all the receptors and leave no receptors remaining to bind with the radioligand. Any radiation that is emitted from the bound complex must then necessarily correspond to nonspecific binding of the radioligand.

There are several ways to estimate the extent of nonspecific binding. The most common way is to run multiple nonspecific binding tubes corresponding to each concentration of hot radioligand. In the tests run under the EDSP three parallel tubes are run for each total concentration of radioligand. Each concentration of radioligand is also run in triplicate. For each total concentration (controlled by the tester) of radioligand the averages of the radioactive decay (dpm) among the three total binding tubes and among the three total added tubes are determined. The total free radioligand is determined by subtracting the average total bound dpm from the average total added dpm. The specific bound decay is determined by subtracting the average nonspecific binding decay from each of the total bound decay determinations.

The specific bound ligand concentration is related to the total free ligand concentration by the nonlinear regression relation model

$$Y = \frac{B_{max} X}{X + K_d} + \varepsilon$$

In this relation X is the average free radioligand concentration (average total added minus average total bound) (nanomolar) among the three tubes corresponding to the same total added concentration and Y is the concentration of radioligand bound to the receptor (specific bound) (fmolar/mg protein). B_{max} is the maximum concentration bound as the concentration of free radioligand goes to ∞. K_d is the equilibrium dissociation constant discussed above and corresponds to the radioligand concentration at which half the receptor binding locations are filled. ε represents the random variation about the model and is often modeled as independently distributed with mean 0 and constant variance σ^2.

The model is fitted by nonlinear regression analysis. This results in parameter estimates and associated standard errors and confidence intervals and an estimate of residual variation.

Competitive binding fits can be compared across time within laboratories and across laboratories based on their values of B_{max}, K_d, error variance σ^2, and associated standard errors.

5.3 Competitive Binding Assay

5.3.1 Objective

The objective of the competitive binding assay is to assess the extent of inhibitory potential of environmental chemicals on AR binding.

5.3.2 Organization of Assay

The competitive binding assay involves a fixed concentration of radioligand (e.g. radiolabeled R1881) and graded concentrations of unlabeled competitor compound. As the concentration of unlabeled compound increases it binds with increasing numbers of binding sites, thereby leaving fewer receptor binding sites available to bind with the radioligand. This results in decreasing radiation emission as the concentration of the unlabeled compound increases, thereby resulting in a decreasing concentration response relation.

A single run of the assay includes specimens of various types.

- Complete nonbinder with AR receptors (e.g. ethanol, EtOH)). These specimens result in the maximal amount of radiation emission. Three tubes are tested at the beginning of the run and three tubes at the end of the run, to determine if there was any variation in conditions across the run.
- Nonspecific binding tubes (NSB). These tubes include a sufficiently high concentration of an unlabeled binder (e.g. unlabeled R1881) that all the receptor sites are taken up. Any emission of radiation is then due to nonspecific binding, which serves as a background to be corrected for. Three tubes are tested at the beginning of the run and three tubes at the end of the run, to determine if there was any variation in conditions across the run.
- Graded concentrations of an unlabeled standard compound (e.g. unlabeled R1881) that result in a standard concentration response curve. Three replicate tubes are run per concentration.
- Graded concentrations of a positive control (e.g. dexamethasone) that result in a positive control concentration response curve. Three replicate tubes are run per concentration.
- Graded concentrations of one or more test compounds that result in concentration response curves. Three replicate tubes are run per concentration.

The basic responses are measured in disintegrations per minute (dpm). The dpm in each tube are converted to "percent (specific) bound" by subtracting the average dpm in the six NSB tubes, dividing by the average dpm in the six EtOH tubes, and multiplying by 100. The average percent bound among the six NSB tubes is necessarily 0% and the average percent bound among the six EtOH tubes is necessarily 100%.

5.3.3 Concentration Response Relation

If the radioligand and the inhibitor both bind reversibly to the same (single) binding site, then (specific) binding at equilibrium follows a four parameter relation between percent bound (Y) and logarithm of inhibitor concentration (X). By convention logarithms to the base 10 are used in the receptor binding literature. The concentration response relation is described by a sigmoid curve, sometimes referred to as the "Hill equation" (1910). It is represented as:

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

The parameters in the equation represent the following quantities:

- B is the bottom plateau, i.e. the least expected percent bound
- T is the top plateau, i.e. the greatest expected percent bound
- β is the "hill slope," i.e. the steepness with which the curve declines. Since the curve declines with increasing X, β is necessarily negative.
- $Log_{10}EC_{50}$ is the logarithmic concentration at which E(Y) = (B + T)/2%
- ε is 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, σ^2).

For an ideal concentration response, B = 0, T = 100, and β = -1. In that case $\log_{10}EC_{50}$ is the logarithmic concentration at which E(Y) = 50%.

In general, the ideal values of the parameters do not hold. In that case $\log_{10}EC_{50}$ does not have a readily interpretable physical correspondence. Furthermore (B + T)/2 will differ for different chemicals and so the $\log_{10}EC_{50}$'s are not directly comparable between the test chemicals and the standard.

An alternative parameterization of the four parameter curve has parameters that are more physically interpretable. Namely

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

In this form of the equation T, B, and β are as above. $\log_{10}IC_{50}$ is the logarithmic concentration at which E(Y) = 50%. Thus $\log_{10}IC_{50}$ always corresponds to the same percentile of the concentration response and so can be directly compared between the test compounds and the standard.

The ratio of the IC_{50} concentrations between the test compound and the standard is referred to as the "relative binding affinity", RBA. Namely

$$RBA \equiv \frac{IC_{50,\,STD}}{IC_{50,\,TEST}}$$

5.3.4 Statistical Analysis

For each test chemical three or more test runs are carried out, structured as discussed above. Three replicate tubes of each graded concentration of unlabeled standard, weak positive control, and each test chemical are run as well as six replicate tubes of the EtOH non-binders and six replicate NSB tubes (three at the beginning of the run and three at the end).

For each test run the four parameter concentration response models are fitted to the concentration response data for each chemical by nonlinear regression 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.

Nonlinear regression analysis can be carried out using PRISM Version 4 or 5 (Motulsky 2003, 2007) software or general purpose statistical systems such as SAS (2003). The question of whether to use weighted or non-weighted least squares is still under discussion at EPA. An interim recommendation is to carry out non-weighted fits for the AR assay.

For each test chemical multiple runs are 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%
- $Log_{10}RBA$, ($\log_{10}(IC_{50,std}/ IC_{50,test})$)

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 RBA for each chemical are

[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).

estimated as 10 to the power $\log_{10}IC_{50}$ and 10 to the power $\log_{10}RBA$ respectively. Similarly for the lower and upper confidence bounds.

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 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.

6.0 REFERENCE AND TEST CHEMICALS

As part of the validation of the Androgen Receptor Binding Assay, test chemicals were selected by EPA on the basis of data in the scientific literature. Chemicals were chosen to represent a wide range of chemical classes and potential AR binding. Three validation steps were carried out: Preliminary Interlaboratory Studies (Table 6-1), Interlaboratory Validation Studies (Table 6-2), and Supplemental Validation Studies (Table 6-3). The tables contain a test chemical's descriptors and basic chemical class information. Additionally, the tables include a preliminary AR binding potential evaluation from ICCVAM. Some of the material in this chapter was adapted from the unpublished results of the prior EDSP Work Assignments 2-22 and 4-11, as well as work performed on the separate EPA contract 68-W-99-033 (Work Assignment 2-19).

Table 6-1. EPA AR Validation Reference Chemicals—Preliminary Interlaboratory Studies

Reference Chemical	Supplier	Mfr. Purity (%)	Chemical ID	CAS No.	Molecular Formula	Molecular Weight (g/mol)	Class	ICCVAM Comment[a]
4-Tert-octylphenol	Sigma-Aldrich	98.3	M004850	140-66-9	$C_{14}H_{22}O$	206.33	Environmental pollutant, ER agonist	ER agonist
Methoxychlor	Sigma	95.2	M004867	72-43-5	$C_{16}H_{15}Cl_3O_2$	345.65	Insecticide, estrogenic and antiandrogenic effects. Non-binder	weak ER agonist; AR antagonist
Progesterone	Sigma	100	M004868	57-83-0	$C_{21}H_{30}O_2$	314.47	Progestational hormone, binds to AR with moderate potency	AR agonist
Dexamethasone	Biomol Intl.	99.5	M004869	50-02-2	$C_{22}H_{29}FO_5$	392.43	Glucocorticoid, known weak AR binder	weak ER and AR agonist
Spironolactone	Sigma-Aldrich	99	M004876	52-01-7	$C_{24}H_{32}O_4S$	416.57	Diuretic, antagonist of aldosterone, known AR binder	AR agonist and antagonist
Atrazine	Chem Service	98	M004877	1912-24-9	$C_8H_{14}ClN_5$	215.69	Herbicide, known to be negative in the AR binding assay	Binds weakly to AR and ER
Di-(2-ethylhexyl)phthalate	Supelco	99.9	M004878	117-81-7	$C_{24}H_{38}O_4$	390.56	Plasticizer for resins & elastomers, an antiandrogen that does not operate through the AR pathway. Nonbinder.	
Procymidone	Chem Service	99	M004879	32809-16-8	$C_{13}H_{11}Cl_2NO_2$	284.14	Pesticide/herbicide. Antiandrogen. Weak binder	AR antagonist
Linuron	Chem Service	99	M004880	330-55-2	$C_9H_{10}Cl_2N_2O_2$	249.1	Pre-and post-emergence herbicide, antiandrogen and known AR binder	weak AR agonist and antagonist
Cyproterone acetate	Sigma-Aldrich	99.7	M004881	427-51-0	$C_{24}H_{29}ClO_4$	416.94	Anti-androgen, known AR binder	AR agonist and antagonist
17β-estradiol	Sigma-Aldrich	99	M004890	50-28-2	$C_{18}H_{24}O_2$	272.36	Estrogenic hormone known to bind to the AR at high concentrations	strong ER agonist; AR agonist and antagonist
3-(3,5-Dichlorophenyl)-5-ethenyl-5-methyl-2-4-oxazolidinedione	Chem Service	99	M004891	50471-44-8	$C_{12}H_9Cl_2NO_3$	286.11	Pesticide, an antiandrogen but not an AR binder.	AR antagonist

Reference Chemical	Supplier	Mfr. Purity (%)	Chemical ID	CAS No.	Molecular Formula	Molecular Weight (g/mol)	Class	ICCVAM Comment[a]
2,2-Bis(4-chlorophenyl)-1,1-dichloro-ethylene (pp'-DDE)	Aldrich	99.4	M004892	72-55-9	$C_{14}H_8Cl_4$	320.04	Insecticide, antiandrogen and AR binder	weak AR agonist and antagonist
6a-Methyl-17a-hydroxyprogesterone acetate	Aldrich	99.2	M004901	520-85-4	$C_{24}H_{37}O_4$	344.49	Contraceptive ingredient known to bind to the AR	
Methyltrienolone (R1881)	Perkin Elmer	99	M004902	965-93-5	$C_{19}H_{24}O_2$	284.37	Synthetic non-aromatizable androgen, strong binder to androgen receptor	
Testosterone	Sigma	>99	M004904	58-22-0	$C_{19}H_{28}O_2$	288.43	Natural androgenic hormone, precursor to dihydrotestosterone, convertible estradiol	strong AR agonist

a. ICCVAM, 2006. In some cases, this is a speculative evaluation.

Table 6-2. EPA AR Validation Reference Chemicals—Interlaboratory Validation Studies

Reference Chemical	Supplier	Mfr. Purity (%)	Chemical ID	CAS No.	Molecular Formula	Molecular Weight (g/mol)	Basis for Selection	ICCVAM Note[a]
Dihydrotestosterone	Sigma	99	CR42400	521-18-6	C19H30O2	290.44	Metabolite of testosterone, strong androgen receptor binder (30X testosterone)	weak ER agonist; strong AR agonist
Testosterone	Sigma-Aldrich	99	CR42401	58-22-0	$C_{19}H_{28}O_2$	288.43	Androgenic hormone, precursor to dihydrotestosterone, convertible estradiol	strong AR agonist
17β-estradiol	Sigma-Aldrich	100	CR42402	50-28-2	$C_{18}H_{24}O_2$	272.36	Estrogenic hormone	strong ER agonist; AR agonist and antagonist
6a-methyl-17a-hydroxyprogesterone acetate (MPA)	Aldrich	99.2	CR42403	71-58-9	$C_{24}H_{34}O_4$	386.53	Progesterone is known to bind to AR	weak AR agonist
Linuron	Chem Service	99.5	CR42404	330-55-2	$C_9H_{10}Cl_2N_2O_2$	249.1	Pre-and post-emergence herbicide	
2,2-Bis(4-chlorophenyl)-1,1-dichloro-ethylene (pp'-DDE)	Sigma-Aldrich	98.6	CR42405	72-55-9	$C_{14}H_8Cl_4$	320.04	Antiandrogen and AR antagonist	weak AR agonist and antagonist
Cyproterone acetate	Sigma-Aldrich	99.7	CR42406	427-51-0	$C_{24}H_{29}ClO_4$	416.94	AR antagonist	AR agonist and antagonist
Spironolactone	Sigma-Aldrich	100.9	CR42407	52-01-7	$C_{24}H_{32}O_4S$	416.57	Diuretic, antagonist of aldosterone	AR agonist and antagonist
Bis(2-ethylhexyl)phthalate (DEPH)	Supelco	99.9	CR42408	117-81-7	$C_{24}H_{38}O_4$	390.56	Plasticizer for resins & elastomers	
Atrazine	Chem Service	98	CR42409	1912-24-9	$C_8H_{14}ClN_5$	215.69	Herbicide	Binds weakly to AR and ER

a. ICCVAM, 2006. In some cases, this is a speculative evaluation.

Table 6-3. EPA AR Validation Reference Chemicals—Supplemental Validation Studies

Reference Chemical	Supplier	Mfr. Purity (%)	Chemical ID	CAS No.	Molecular Formula	Molecular Weight (g/mol)	Basis for Selection	ICCVAM Note[a]
Trenbolone	Sigma	98	CR42340	10161-33-8	$C_{18}H_{22}O_2$	270.37	Agricultural steroid to increase muscle growth, androgen receptor binder	binds strongly to the AR
Bicalutamide	Chemos	99.7	CR42341	90357-06-5	$C_{18}H_{14}F_4N_2O_4S$	430.37	Non-steroidal anti-androgen	
Mifepristone	Sigma	99.7	CR42342	84371-65-3	$C_{29}H_{35}NO_2$	429.6	Synthetic steroid, abortifacient	AR agonist and antagonist
Nilutamide	Sigma	100	CR42343	63612-50-0	$C_{12}H_{10}F_3N_3O_4$	317.22	On ICCVAM list as a moderate binder	
17□-Ethynyl estradiol	Sigma	99	CR42344	57-63-6	$C_{20}H_{24}O_2$	296.4	Synthetic derivative of estradiol, contraceptive extended bio-half-life	strong ER agonist
Hydroxyflutamide	Toronto Research Chemicals	>98	CR42345	52806-53-8	$C_{11}H_{11}F_3N_2O_4$	292.21	Anti-androgen, block conversion of testosterone to dihydrotestosterone	
Fluoxymestrone	Sigma	>99	CR42346	76-43-7	$C_{20}H_{29}FO_3$	336.44	Synthetic androgen	
Estrone	Aldrich	99.9	CR42347	53-16-7	$C_{18}H_{22}O_2$	270.37	ER agonist	strong ER agonist; AR agonist
Flutamide	Sigma	100	CR42348	13311-84-7	$C_{11}H_{11}F_3N_2O_3$	276.21	AR antagonist	AR antagonist
Diethylstilbestrol	Sigma	100	CR42349	56-53-1	$C_{18}H_{20}O_2$	268.36	ER agonist	ER agonist
o,p'DDT	Supelco	98	CR42350	789-02-6	$C_{14}H_9Cl_5$	354.49	Parent compound of antiadrogen	
Kepone	Supelco	99.9	CR42351	143-50-0	$C_{10}H_2Cl_{10}O$	490.68	Insecticide, carcinogenic	
Bisphenol A	Sigma	99.9	CR42352	80-05-7	$C_{15}H_{16}O_2$	228.29	ER agonist, primary monomer in polycarbonate plastic and epoxy resins	weak ER agonist
Fluoranthene	Sigma	98.9	CR42353	206-44-0	$C_{16}H_{10}$	202.26	PAH	
Ketoconazole	Fisher	99.2	CR42354	65277-42-1	$C_{26}H_{28}Cl_{12}N_4O_4$	531.4	Synthetic antifungal drug	weak AR agonist

Reference Chemical	Supplier	Mfr. Purity (%)	Chemical ID	CAS No.	Molecular Formula	Molecular Weight (g/mol)	Basis for Selection	ICCVAM Note[a]
4-Nonylphenol	Acros Organics	>98.5	CR42355	104-40-5	$C_{15}H_{24}O$	220.39	Industrial surfactants, pesticide, air pollutant	ER agonist and antagonist; AR antagonist
Phenobarbital	Sigma	>99	CR42357	57-30-7	$C_{12}H_{12}N_2O_3$	232.24	Barbiturate	enhances thyroid hormone excretion
Phorbol 12-Myristate 13-Acetate	Alexis Biochemical	>/-98	CR42358	16561-29-8	$C_{36}H_{56}O_8$	616.83	Tumor promoter	
2,4,5-Trichlorophenoxyacetic acid	Sigma	99.3	CR42359	93-76-5	$C_8H_5Cl_3O_3$	255.49	Synthetic auxin, herbicide	weak ER agonist
Bisphenol B	Aldrich	not provided	CR42360	77-40-7	$C_{16}H_{18}O_2$	242.32	ICCVAM list and weak ER agonist	ER agonist
Genistein	Sigma	99.4	CR42361	446-72-0	$C_{15}H_{10}O_5$	270.24	Isoflavone, antioxidant, act like estrogen	
Butylbenzyl phthalate	Chem Service	98.3	CR42362	85-68-7	$C_{19}H_{20}O_4$	312.37	Plasticiser (polyvinyl chloride	ER agonist
Kaempferol	TCI	97.7	CR42363	520-18-3	$C_{15}H_{10}O_6$	286.23	Natural flavonoid	weak ER agonist
Norethynodrel	Sigma	>99	CR42365	68-23-5	$C_{20}H_{26}O_2$	298.42	Progestin, contraceptive	Binds to ER
Finasteride	LKT	98.9	CR42367	98319-26-7	$C_{23}H_{36}N_2O_2$	372.55	Anti-androgen, block conversion of testosterone to dihydrotestosterone	5α-reductase inhibitor
17α-Estradiol	Sigma	min 99	CR42368	57-91-0	$C_{18}H_{24}O_2$	272.39	Steroid	ER agonist
Econazole	Sigma	>99	CR42369	27220-47-9	$C_{18}H_{15}Cl_3N_2O$	381.68	Antifungal agent	
Methyltrienolone (R1881) STD Curve Substrate	Perkin Elmer	99		965-93-5	$C_{19}H_{24}O_2$	284.37	Synthetic non-aromatizable androgen, strong binder to androgen receptor	

Reference Chemical	Supplier	Mfr. Purity (%)	Chemical ID	CAS No.	Molecular Formula	Molecular Weight (g/mol)	Basis for Selection	ICCVAM Note[a]
Dexamethasone Weak Positive	Fluka	99.5		50-02-2	$C_{22}H_{29}FO_5$	392.43	Glucocorticoid, known competitive binder	weak ER and AR agonist

a. ICCVAM, 2006. In some cases, this is a speculative evaluation.

7.0 ASSAY DEVELOPMENT AND OPTIMIZATION IN THE LEAD LABORATORY

The objective of the assay development and optimization experiments was to identify the optimal factors and conditions for the assay. This section generally follows the sequence of tasks to (1) confirm the performance of the AR assay, (2) evaluate the performance of the competitive assay with 3 unknown chemicals and (3) perform a series of competitive assays with 16 unknown chemical samples to expand the database for determining the effectiveness of the assay.
Note that all data in Chapter 7 were analyzed using the 2 parameter model as briefly presented in Chapter 5 with the top and bottom set to 100 and 0. Since the assay development and optimization involve changing experimental designs and were not intended as a rigorous interlaboratory validation, this data was not examined using the final model (presented in Chapter 5) as used in Chapters 8-10. Some of the material in this chapter was adapted from the unpublished results of the prior EDSP Work Assignment 2-22.

7.1 Confirmation of Standard Curves and Assay Performance

The confirmation of standard curves and assay performance involved demonstrating the correct performance of the AR binding assay protocol by conducting saturation and competitive assays using the R1881. Each technician involved with running the unknown chemicals performed the both the saturation and competitive assays in duplicate (simultaneous runs). The results were plotted on Scatchard plots for confirmation of assay performance.

7.1.1 Confirmation of Saturation Assay Performance

To demonstrate the correct performance of the AR binding assay protocol with the saturation binding assay using R1881, two technicians (designated "J" and "L") performed the assay in duplicate. The assay was performed as described in Chapter 4.

The resulting specific binding data (Figure 7-1) was fit using the one-site binding model (i.e., Y=Bmax*X/(Kd+X)). The regression of the observed values against the expected values was linear (all of the R^2 values for the two technician and days were greater than 0.97) with the duplicate analyses normally distributed about the expected line. Total binding and non-specific binding showed the expected linear trend with increasing [^{3}H] R1881 concentration, although two nominal outliers in the non-specific binding data were noted. Scatchard analyses were also linear as expected (Figure 7-2). To examine the interday and technician variability, an ANOVA analysis of the saturation data was conducted (Table 7-1). No statistically significant differences were found between technicians or day of assay. Bmax and Kd values were also consistent between days and technicians.

	288J	289L	290J	291L
One site binding (hyperbola)				
Best-fit values				
BMAX	13955	13465	14290	13663
KD	0.8982	0.7681	0.9698	0.8121
Std. Error				
BMAX	396.5	224.6	283.5	185.5
KD	0.07997	0.04215	0.05891	0.03564
95% Confidence Intervals				
BMAX	13105 to 14806	12983 to 13946	13682 to 14898	13265 to 14061
KD	0.7266 to 1.070	0.6777 to 0.8586	0.8434 to 1.096	0.7357 to 0.8886

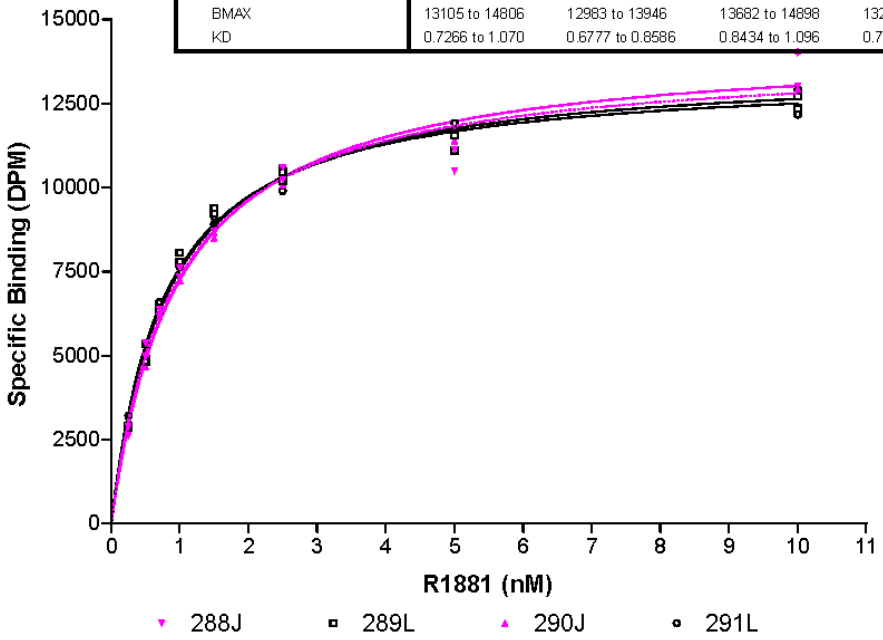


Figure 7-1. Specific binding data from the saturation assay. The 'J' and 'L' designations signify different technicians. The two runs per technician were conducted on different days.

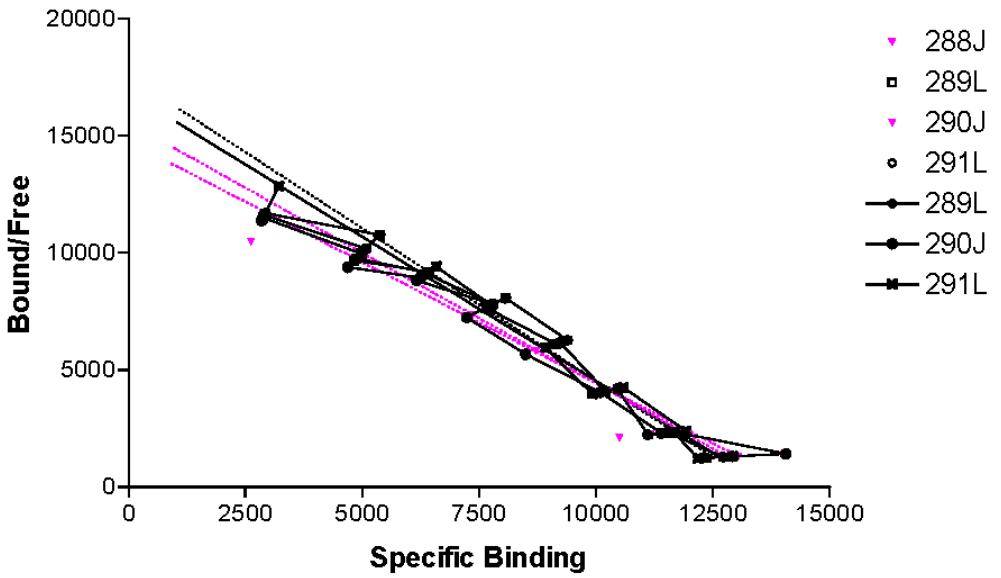


Figure 7-2. Scatchard analysis of saturation binding data. The 'J' and 'L' designations signify different technicians. The two runs per technician were conducted on different days.

Table 7-1. ANOVA analysis of competitive binding assay for R1881

CV versus day, technician (averaging over assay)

Factor	Type	Levels	Values
day	fixed	2	1 2
technician	fixed	2	1 2

Analysis of Variance for CV

Source	DF	SS	MS	F	P	
day	1	0.001130	0.001130	0.83	0.366	Not Significant
technician	1	0.000156	0.000156	0.11	0.736	Not Significant
day*technician	1	0.000483	0.000483	0.35	0.554	Not Significant
Error	92	0.125903	0.001369			
Total	95	0.127673				

In Section 4.4 a number of questions were raised regarding the evaluation of data from AR saturation assays. These questions can now be answered as follows:

Q1. As increasing concentrations of [3H]-R1881 were used, does the specific binding curve reach a plateau? Maximum specific binding must be reached, indicating saturation of AR with ligand.

A1. The saturation binding curve does reach a plateau, as noted in Figure 7-1

Q2. Does the data produce a linear Scatchard plot (a plot of bound/free ligand as a function of specific binding)?

A2. As noted in Figure 7-2, the Scatchard plot is linear.

Q3. Is the Kd within an acceptable range?

A3. The Kd is above the range noted by ICCVAM and falls between 0.8121 and 0.9698 nM. The Kd was consistently higher than the range noted in the ICCVAM Background Review Document throughout the validation program. All other parameters were consistent with expectations.

Q4. Is non-specific binding excessive? The value for non-specific binding should be less than 50% of the total binding.

A4. The non-specific binding for the assay optimization tasks ranged from 8.1 to 10.0%, well within the criteria.

7.1.2 Confirmation of Competitive Assay Performance

Following the saturation assay, a competitive binding assay was performed to demonstrate proficiency of the research staff using the reference chemical (R1881). Each technician prepared their own standard curve reagents and performed the assays twice on two separate days. The assay was performed as described in Chapter 4. The data are identified based on run number and technician (designated 'J' and 'L'). For the two assay runs, each technician used the same set of standard curve reagents, which they had prepared.

A composite of all standard curves for the four runs is presented in Figure 7-3. The IC_{50} values (Table 7-2) ranged from 1.19 $x10^{-9}$ M to 1.32 x 10^{-9} M. The difference observed between the averages of the two technicians was 0.066 $x10^{-9}$M and represents a difference of 5.17%. It is noted that the amount bound for the IC_{50}s were slightly higher than the anticipated target of $1x10^{-9}$ M, which is based on literature values; however, it is slightly below the sum of the molar concentration of [3H] R881 and the Kd—1.86 X 10^{-9} M—which is a reasonableness test for the IC_{50} recommended by ICCVAM (2003a).

Additionally, an assessment of the binding data was conducted to ensure that ligand depletion was not excessive. The percent bound ranged from 8.70 to 9.05% (Table 7-3) indicating that there was not a ligand depletion concern.

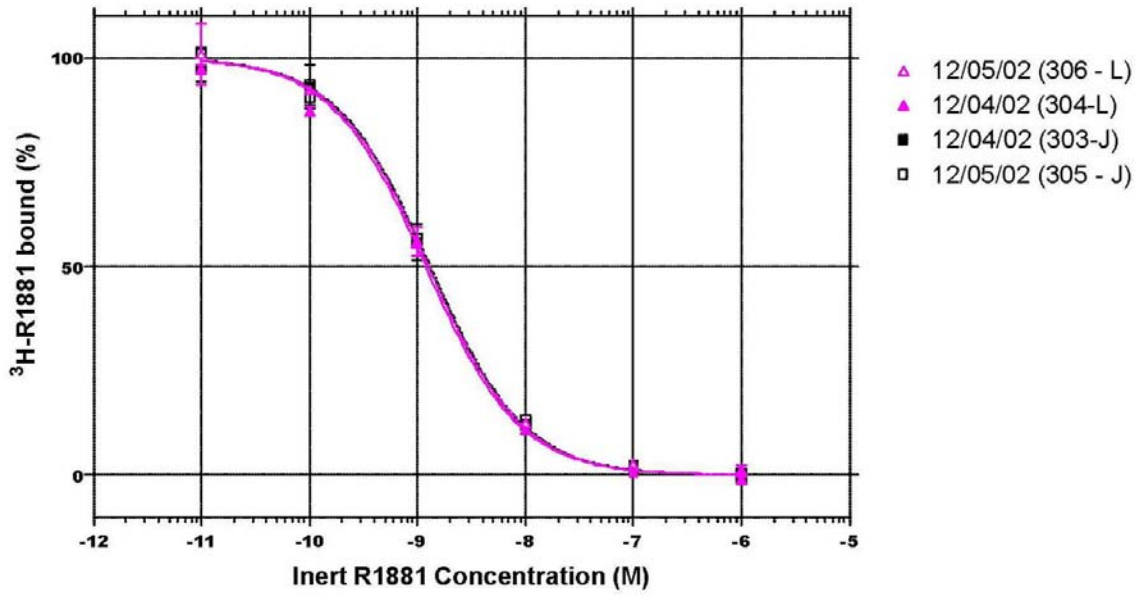


Figure 7-3. Standard curves for competitive assay using R1881.

Table 7-2. Fitted parameters for R1881 competitive assay

	IC50 (M)	
	Technician J	**Technician L**
Run 1	1.29E-09	1.19E-09
Run 2	1.32E-09	1.28E-09
Average	**1.30E-09**	**1.23E-09**
Standard Deviation	2.12E-11	5.87E-11

Grand Average	1.27E-09
Standard Deviation of Grand Average	5.60E-11

Table 7-3. Ligand depletion - 10% Rule Data

Run	*Adjusted DPMs 100% Tubes*	*DPMs Hot Tubes*	*% Bound*
303-J	4114.6	47638	
	4172.9	48569	
	4079.3	46840	
	4301.9	48625	
Average	4167.2	47918	8.70
SD	97.8	849.3	
304-L	4204.8	47419	
	3935.1	45468	
	4105.0	47130	
	4158.0	43853	
Average	4100.7	45967.5	8.92
SD	117.7	1651.1	
305-J	4312.7	47637	
	4282.6	48005	
	4272.7	49050	
	4213.8	48854	
Average	4270.5	48386.5	8.83
SD	41.4	674.8	
306-L	4442.9	48153	
	4330.1	47767	
	4087.3	46847	
	4267.8	46469	
Average	4282.0	47309	9.05
SD	148.7	783.4	

7.2 Evaluate the Performance of the Competitive Assay with 3 Unknown Chemicals

This section details a modification of the AR assay protocol tested using three chemicals with known binding affinities. The modification was made to examine the levels of non-specific binding due to processing of samples from assay incubation tubes on day "2." Originally, the appropriate amount of HAP (500 µl of 60 % slurry) was introduced directly into the assay incubation tubes (designated as Protocol A; prepared under the separate EPA Contract 68-W-99-033, Work Assignment 2-19). In the revised protocol [designated as Protocol B (Battelle, 2002)], 100 µl of reaction mixture is removed from each of the incubation tubes and added to pre-labeled tubes containing HAP. The revised protocol is presented in Appendix A.

The three chemicals tested in this experiment, 4-androstene-3, 17-dione (M004831), 5α-dihydrotestosterone (M004833), and corticosterone (M004837), were identified only by bar code labels. The assay activities were performed by two technicians. Each technician prepared their

own standard curve and test chemical dilutions and performed two assays on separate days using each protocol. For the assay runs, each technician used the same set of standard curve and unknown chemical reagents, which they had prepared.

The data are identified based on run number and technician (Figures 7-4 through 7-6). The IC_{50}s ranged from 1.14 $x10^{-9}$ to 1.47 x 10^{-9} M for Protocol A and from 0.89 x 10^{-9} to 1.16 $x10^{-9}$ M for Protocol B (Table 7-4). The coefficient of variation of the combined data for the two technical assistants for the two assay systems was 11.0% for Protocol A and 12.1% for Protocol B. The RBAs for all three tested chemicals were similar between protocols, indicating that the protocols produce similar results.

An assessment of the binding data to meet the required 10% Rule relative to ligand depletion showed the percent bound ranged from 5.9 % to 7.11% for Protocol A and from 8.63 to 9.72% for Protocol B. This data indicates that ligand depletion was not a concern.

The IC_{50}s and RBAs for the three "weak" binders are presented in Table 7-4. In general only slight differences were observed for the "weak" binders between the two assay protocols. Using Protocol A yielded slightly higher IC_{50}s and RBAs relative to data obtained using Protocol B. However, the standard deviations are smaller using Protocol B, indicating less systematic error in the procedure. Based on this, the modified protocol B was accepted as the standard protocol for all further testing.

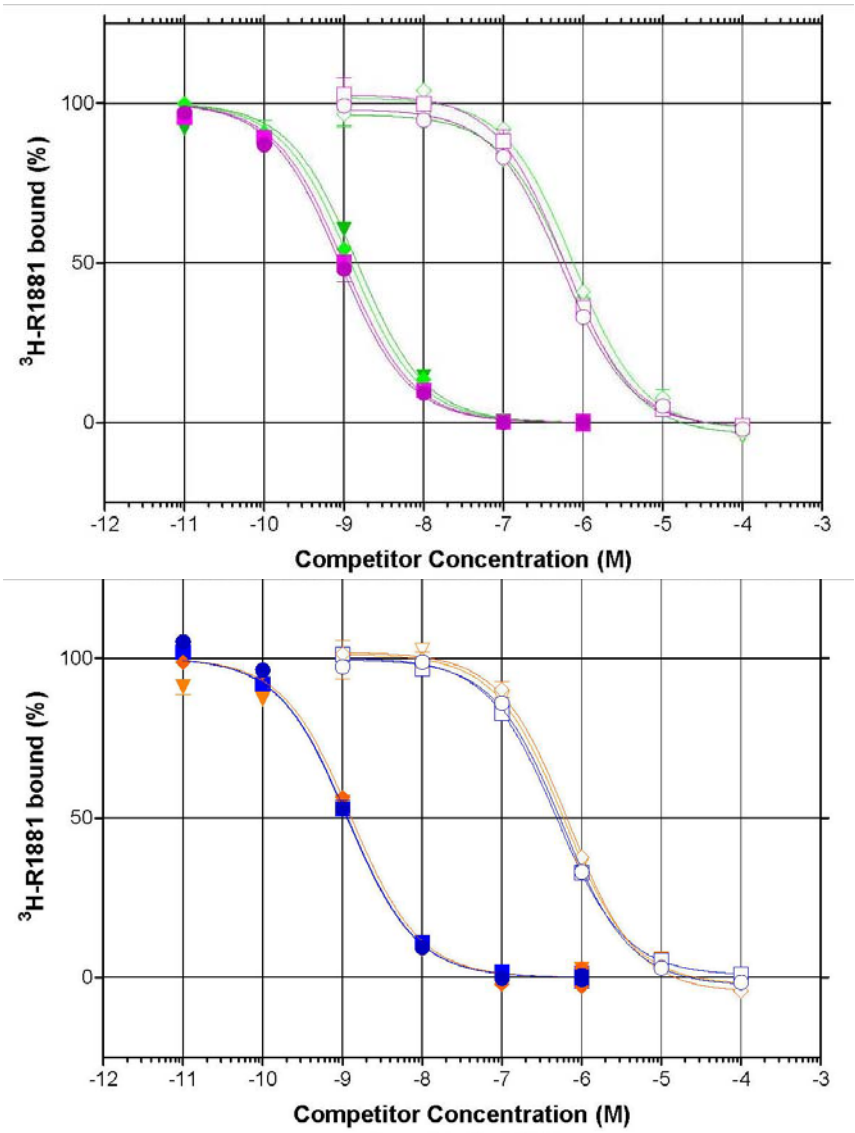


Figure 7-4. Comparison of protocols for 4-androstene-3, 17-dione (M004831) for technician 'J' (top panel) and 'L' (bottom panel). Standard curves are included on both panels (solid lines). Green and orange curves designate protocol A, magenta and blue for protocol B.

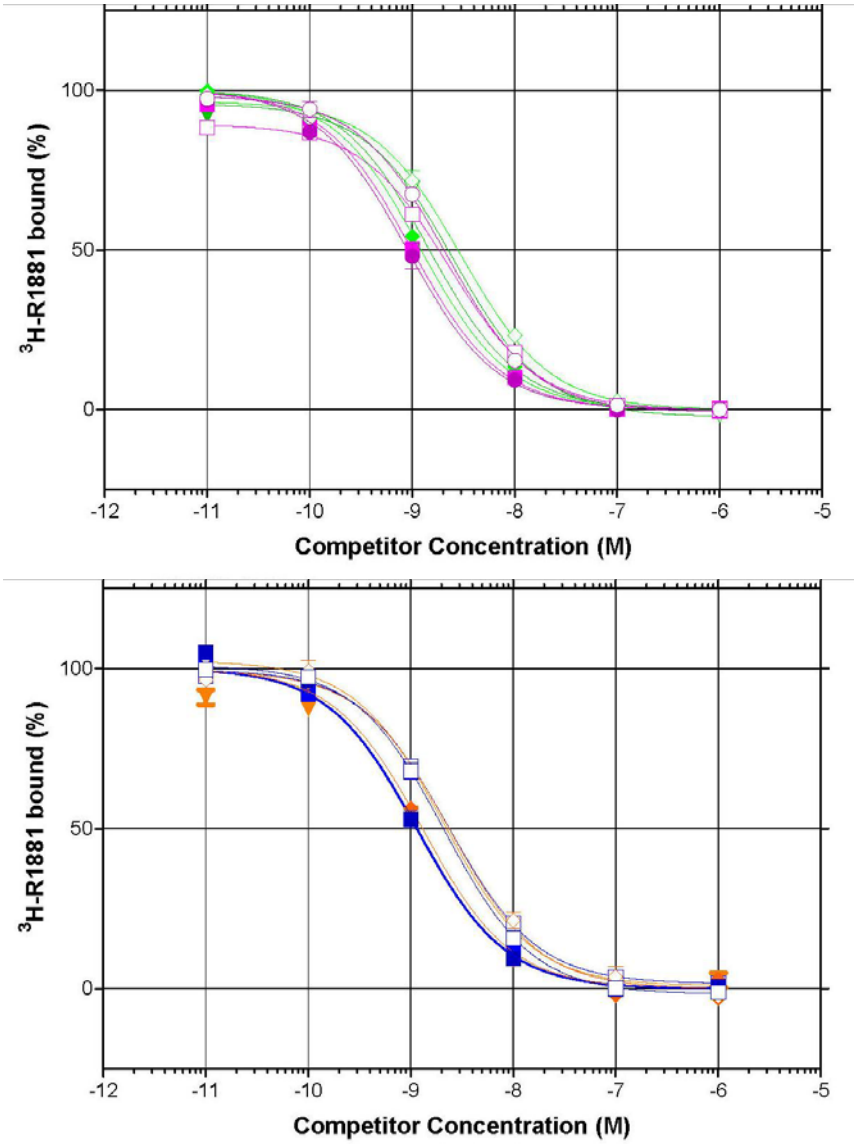


Figure 7-5. Comparison of protocols for 5α-dihydrotestosterone (M004833) for technician 'J' (top panel) and 'L' (bottom panel). Standard curves are included on both panels (solid lines). Green and orange curves designate protocol A, magenta and blue for protocol B.

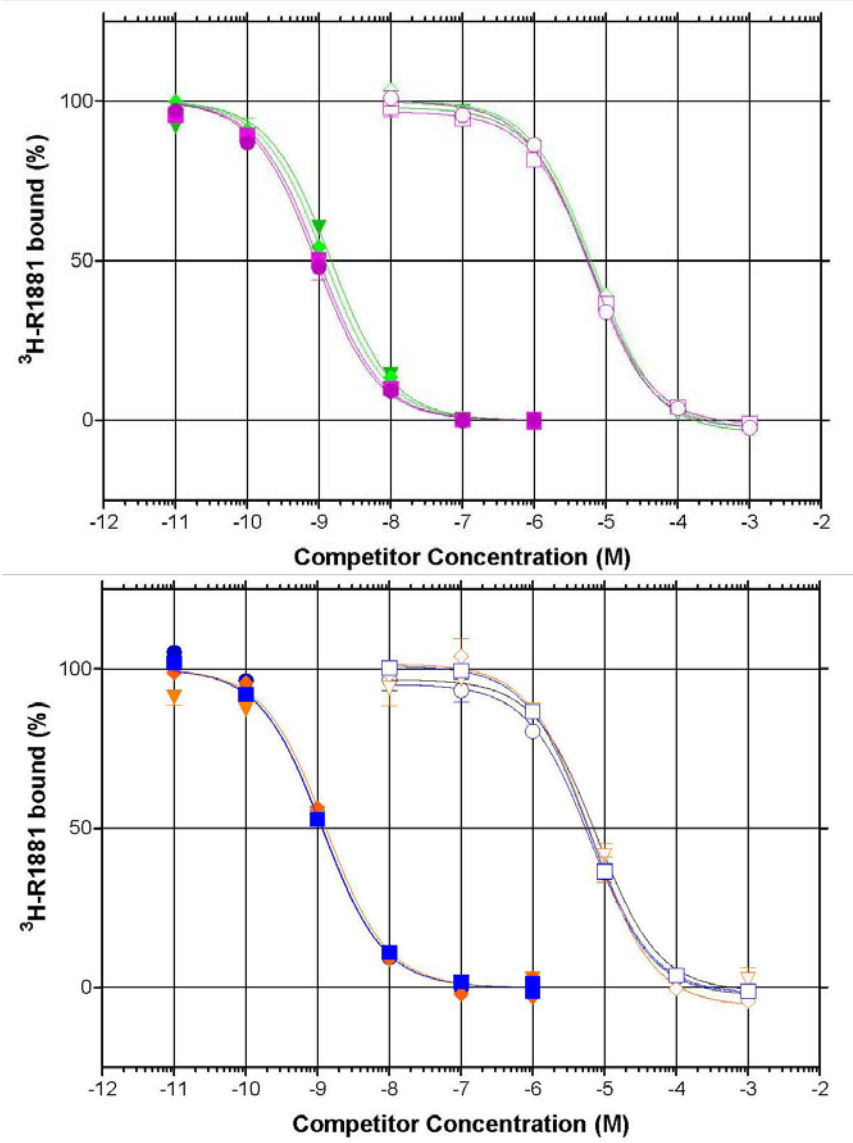


Figure 7-6. Comparison of protocols for corticosterone (M004837) for technician 'J' (top panel) and 'L' (bottom panel). Standard curves are included on both panels (solid lines). Green and orange curves designate protocol A, magenta and blue for protocol B.

Table 7-4. Comparison of IC50s and RBAs for "Weak" Binders

Protocol 'A'	IC_{50} (M)				RBA (%)		
	R1881 Standard	4-androstene-3, 17-dione (M004831)	5α-dihydro testosterone (M004833)	corticosterone (M004837)	4-androstene-3, 17-dione (M004831)	5α-dihydro testosterone (M004833)	corticosterone (M004837)
341-J	1.29E-09	7.60E-07	2.98E-09	6.55E-06	0.160	40.92	0.019
343-J	1.14E-09	6.73E-07	2.54E-09	6.49E-06	0.219	57.89	0.023
340-L	1.22E-09	6.86E-07	2.38E-09	6.51E-06	0.189	54.32	0.020
342-L	1.47E-09	5.99E-07	2.09E-09	7.69E-06	0.190	54.65	0.015
Average	1.28E-09	6.80E-07	2.50E-09	6.81E-06	0.19	51.95	0.019
STD	1.40E-10	6.60E-08	3.70E-10	5.89E-07	0.02	7.53	0.003
Protocol 'B'	IC50 (nM)				RBA		
332-J	1.00E-09	5.75E-07	2.29E-09	6.16E-06	0.173	43.48	0.016
334-J	8.90E-10	5.41E-07	2.13E-09	5.76E-06	0.165	41.91	0.016
335-L	1.14E-09	4.76E-07	2.31E-09	5.99E-06	0.239	49.29	0.019
337-L	1.16E-09	5.59E-07	2.10E-09	6.28E-06	0.209	55.37	0.019
Average	1.05E-09	5.38E-07	2.21E-09	6.05E-06	0.20	47.51	0.018
STD	1.30E-10	4.34E-08	1.10E-10	2.28E-07	0.03	6.12	0.002

7.3 Expand the Database of Tested Compounds by Performing Competitive Assays with 16 Unknown Chemical Samples

This section details the final validation step prior to inter-laboratory validation. The AR assay, as modified in the previous section, was tested with 16 unknown chemical samples. Sixteen chemicals were tested in the lead laboratory as coded unknowns (see Chapter 6, Table 6-3). Each chemical was run at least in duplicate and standards were run during each assay run. The data were again analyzed using the two parameter model with the top and bottoms set to 100 and 0 respectively.

The result summaries can be found Tables 7-5 and 7-6. The data are identified based on run number and technician. Graphic representation of the data (standard curves and competitive binding response curves for chemicals) is presented Figures 7-7 and 7-8.

The standard curve was run a total of 7 times during this task (Table 7-5, Figure 7-7). The average IC_{50} was $9.42x10^{-10} \pm 1.05x10^{-10}$ M, for a coefficient of variation of 11.2%. The

individual runs are evenly distributed about the mean and are consistent with previously reported IC_{50}s.

For several of the test compounds (procymidone - M004879, 17β-estradiol - M004890, and 3-(3,5-dichlorophenyl)-5-ethenyl-5-methyl-2-4-oxazolidinedione - M004891) the highest concentration samples (10^{-3} M) were not included in the data analysis due to the formation of precipitate in the sample tube, an indication of a solubility limit. Based on achieving a minimum % bound of 50%, there were 14 binders and 2 non-binders. The non-binders were atrazine (M004877) and di-(2-ethylhexyl)phthalate (M004878).

For the binding chemicals, the logIC_{50} values ranged from -8.89 to -3.08 log M with RBA of means from 72.111 to 0.001%. The R^2 values for the individual curve fits were above 0.9 for all except the two weakest binders (Methoxychlor—M004867 and Vinclozolin—M004891) where the highest experimental concentrations did not achieve full displacement of the radiolabel. Additionally, the run to run variability was quite small with the logIC_{50} standard deviations less than 10% of the IC_{50} values.

Table 7-5. Standard Curves—Nonlinear Parameter Estimates

Run Identification	*IC50 (Log M)*	*IC50 (M)*	*Std Error IC50 (Log M)*	*R²*
8/19/2003 (405-J)	-9.10	7.97E-10	0.0118	0.9994
8/20/2003 (406-L)	-9.04	9.15E-10	0.0691	0.9772
8/20/2003 (407-J)	-9.06	8.73E-10	0.0245	0.9971
8/25/2003 (408-J)	-9.01	9.78E-10	0.0231	0.9977
8/28/2003 (411-J)	-9.00	9.95E-10	0.0171	0.9987
8/28/2003 (412-L)	-9.04	9.10E-10	0.0267	0.9969
9/17/2003 (424-J)	-8.95	1.13E-09	0.0485	0.9909
Average	-9.03	9.42E-10	0.0315	0.9940
SD	0.05	1.05E-10	0.0202	0.0079
Coefficient of Variation		**11.20%**		

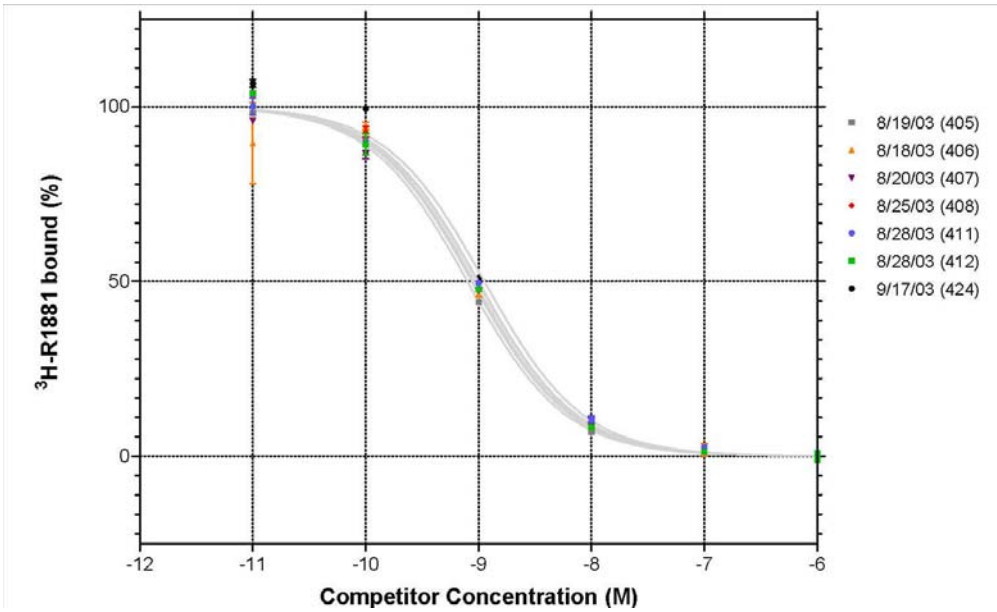


Figure 7-7. Standard Curves for Competitive Assay

Table 7-6. Summary for 16 Unknown Chemicals

	Parameter Estimates				*Relative Binding Affinity*			
Run Identification	***IC50 (nM)***	***IC50 (Log M)***	***Std Error IC50 (Log M)***	***R²***	***R-1881 IC50 (nM)***	***Unknown IC50 (nM)***	***RBA***	***RBA of means***
4-Tert-octylphenol (M004850)								
8/20/2003 (406-L)	5.37E-05	-4.27	0.061	0.964	0.91	53828.0	0.002%	
8/20/2003 (407-J)	4.27E-05	-4.37	0.047	0.975	0.87	42629.0	0.002%	
Mean	4.79E-05	-4.32	0.054	0.970	0.89	48228.5	0.002%	**0.002%**
SD	7.81E-06	0.07	0.010	0.008	0.03	7918.9	0.000%	
Methoxychlor (M004867)								
8/20/2003 (406-L)	7.76E-04	-3.11	0.145	0.631	0.91	782800.0	0.000%	
9/17/2003 (424-J)	8.71E-04	-3.06	0.151	0.654	1.13	880200.0	0.000%	
Mean	8.32E-04	-3.08	0.148	0.642	1.02	831500.0	0.000%	**0.000%**
SD	6.70E-05	0.04	0.005	0.016	0.15	68872.2	0.000%	
Progesterone (M004868)								
8/20/2003 (406-L)	3.98E-07	-6.40	0.041	0.994	0.91	395.6	0.231%	
8/20/2003 (407-J)	3.72E-07	-6.43	0.034	0.997	0.87	367.5	0.237%	
Mean	3.80E-07	-6.42	0.037	0.995	0.89	381.5	0.234%	**0.234%**
SD	1.88E-08	0.02	0.005	0.002	0.03	19.9	0.004%	
Dexamethasone (M004869)								
8/20/2003 (407-J)	2.82E-05	-4.55	0.098	0.950	0.87	28154.0	0.003%	
8/28/2003 (411-J)	5.37E-05	-4.27	0.029	0.996	0.99	54034.0	0.002%	
9/17/2003 (424-J)	6.31E-05	-4.20	0.060	0.983	1.13	63381.0	0.002%	
Mean	4.57E-05	-4.34	0.062	0.976	1.00	48523.0	0.002%	**0.002%**
SD	1.81E-05	0.19	0.035	0.024	0.13	18248.7	0.001%	
Spironolactone (M004876)								
8/20/2003 (406-L)	2.24E-07	-6.65	0.042	0.993	0.91	224.0	0.408%	
8/28/2003 (411-J)	1.78E-07	-6.75	0.023	0.998	0.99	178.5	0.557%	
Mean	2.00E-07	-6.70	0.033	0.996	0.95	201.3	0.483%	**0.477%**
SD	3.26E-08	0.07	0.013	0.003	0.06	32.2	0.105%	
Procymidone (M004879) *								
8/19/2003 (405-J)	6.31E-05	-4.20	0.027	0.992	0.80	62885.0	0.001%	
8/25/2003 (408-J)	7.24E-05	-4.14	0.065	0.932	0.98	71610.0	0.001%	
9/17/2003 (424-J)	6.03E-05	-4.22	0.067	0.958	1.13	60882.0	0.002%	
Mean	6.46E-05	-4.19	0.053	0.961	0.97	65125.7	0.001%	**0.001%**
SD	6.38E-06	0.04	0.023	0.030	0.17	5704.2	0.000%	
Linuron (M004880)								
8/25/2003 (408-J)	6.92E-05	-4.16	0.028	0.996	0.98	69739.0	0.001%	
8/28/2003 (411-J)	9.12E-05	-4.04	0.018	0.998	0.99	90865.0	0.001%	

	Parameter Estimates				Relative Binding Affinity			
Run Identification	*IC50 (nM)*	*IC50 (Log M)*	*Std Error IC50 (Log M)*	*R^2*	*R-1881 IC50 (nM)*	*Unknown IC50 (nM)*	*RBA*	*RBA of means*
Mean	7.94E-05	-4.10	0.023	0.997	0.99	80302.0	0.001%	**0.001%**
SD	1.56E-05	0.08	0.007	0.002	0.01	14938.3	0.000%	
Cyproterone acetate (M004881)								
8/20/2003 (407-J)	1.35E-07	-6.87	0.032	0.995	0.87	134.9	0.647%	
8/28/2003 (412-L)	1.05E-07	-6.98	0.031	0.996	0.91	104.4	0.871%	
Mean	1.17E-07	-6.93	0.032	0.996	0.89	119.7	0.759%	**0.751%**
SD	2.13E-08	0.08	0.000	0.000	0.03	21.5	0.158%	
17β-estradiol (M004890) *								
8/25/2003 (408-J)	3.24E-07	-6.49	0.031	0.996	0.98	322.5	0.303%	
8/28/2003 (412-L)	2.45E-07	-6.61	0.045	0.992	0.91	245.5	0.371%	
Mean	2.82E-07	-6.55	0.038	0.994	0.94	284.0	0.337%	**0.335%**
SD	5.52E-08	0.08	0.010	0.003	0.05	54.5	0.048%	
3-(3,5-Dichlorophenyl)-5-ethenyl-5-methyl-2-4-oxazolidinedione (Vinclozolin—M004891) *								
8/19/2003 (405-J)	8.13E-05	-4.09	0.106	0.800	0.80	81122.0	0.001%	
8/25/2003 (408-J)	7.76E-05	-4.11	0.071	0.907	0.98	78376.0	0.001%	
9/17/2003 (424-J)	1.41E-04	-3.85	0.099	0.820	1.13	142200.0	0.001%	
Mean	9.77E-05	-4.01	0.092	0.842	0.97	100566.0	0.001%	**0.001%**
SD	3.57E-05	0.15	0.019	0.057	0.17	36082.2	0.000%	
2,2-Bis(4-chlorophenyl)-1,1-dichloro-ethylene (pp'-DDE) (M004892)								
8/25/2003 (408-J)	7.24E-06	-5.14	0.079	0.966	0.98	7214.9	0.014%	
8/28/2003 (412-L)	1.05E-05	-4.98	0.110	0.935	0.91	10427.0	0.009%	
Mean	8.71E-06	-5.06	0.095	0.951	0.94	8821.0	0.011%	**0.011%**
SD	2.28E-06	0.11	0.022	0.022	0.05	2271.3	0.003%	
6a-Methyl-17a-hydroxyprogesterone acetate (M004901)								
8/25/2003 (408-J)	3.63E-08	-7.44	0.043	0.991	0.98	36.6	2.670%	
8/28/2003 (411-J)	2.88E-08	-7.54	0.028	0.997	0.99	28.7	3.468%	
8/28/2003 (412-L)	2.75E-08	-7.56	0.046	0.992	0.91	27.8	3.271%	
Mean	3.09E-08	-7.51	0.039	0.993	0.96	31.0	3.136%	**3.116%**
SD	4.73E-09	0.07	0.010	0.003	0.05	4.9	0.416%	
Methyltrienolone (R1881) (M004902)								
8/20/2003 (407-J)	1.23E-09	-8.91	0.011	0.998	0.87	1.2	70.31%	
8/28/2003 (411-J)	1.38E-09	-8.86	0.020	0.998	0.99	1.4	72.23%	
8/28/2003 (412-L)	1.23E-09	-8.91	0.029	0.997	0.91	1.2	73.81%	
Mean	1.29E-09	-8.89	0.020	0.998	0.93	1.3	72.12%	**72.11%**
SD	8.67E-11	0.03	0.009	0.001	0.06	0.1	1.750%	
Testosterone (M004904)								

	Parameter Estimates				Relative Binding Affinity			
Run Identification	***IC50 (nM)***	***IC50 (Log M)***	***Std Error IC50 (Log M)***	***R^2***	***R-1881 IC50 (nM)***	***Unknown IC50 (nM)***	***RBA***	***RBA of means***
8/20/2003 (407-J)	7.41E-09	-8.13	0.029	0.994	0.87	7.5	11.69%	
8/28/2003 (411-J)	7.76E-09	-8.11	0.027	0.997	0.99	7.8	12.75%	
8/28/2003 (412-L)	8.91E-09	-8.05	0.040	0.994	0.91	9.0	10.16%	
Mean	8.13E-09	-8.09	0.032	0.995	0.93	8.1	11.53%	**11.49%**
SD	7.85E-10	0.04	0.007	0.002	0.06	0.8	1.303%	

* -3 concentrations excluded for precipitate

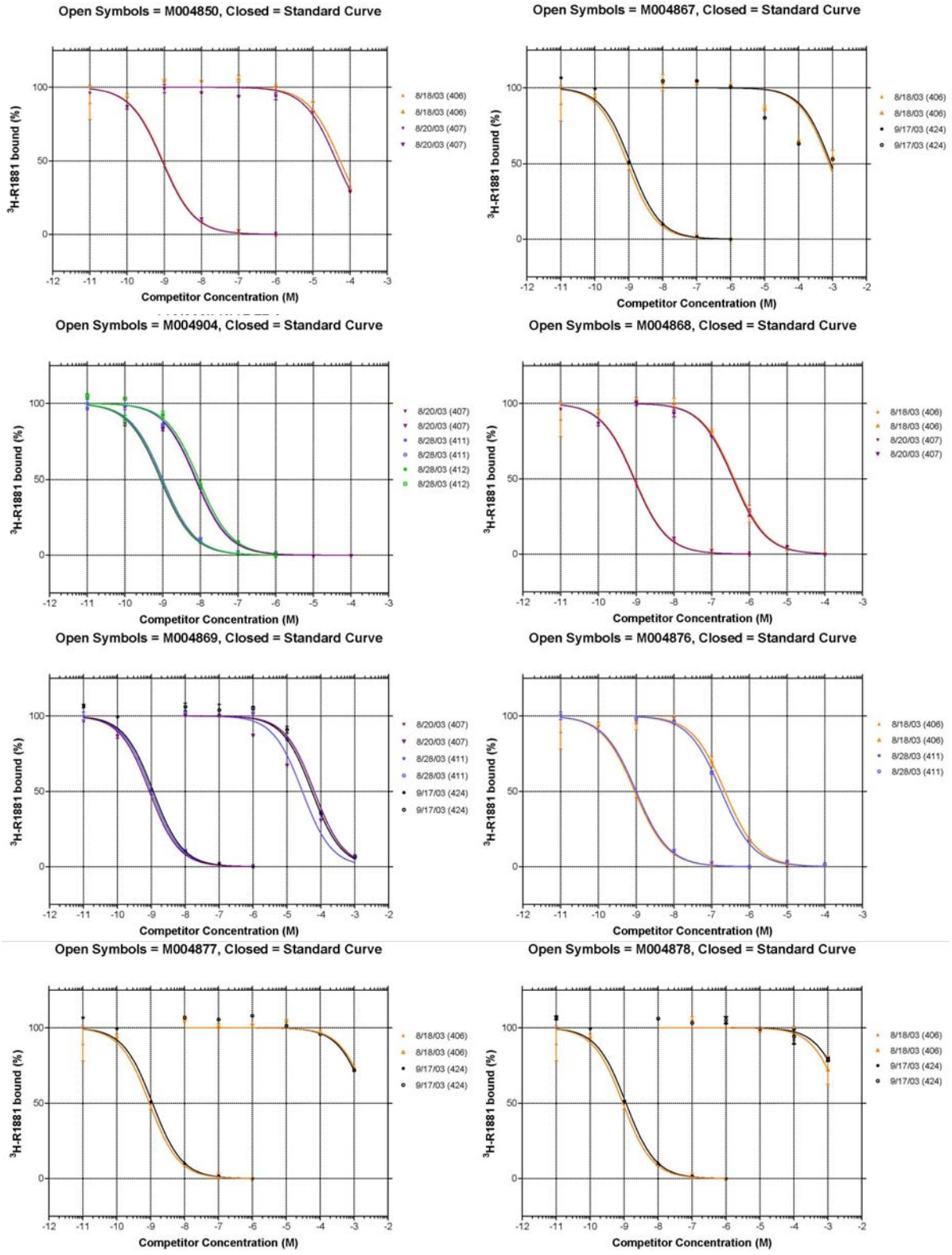


Figure 7-8. Test chemical results. Relevant standard curves, run during the same assay run as the test compounds are also shown. (See Table 7.6 above for chemical coding)

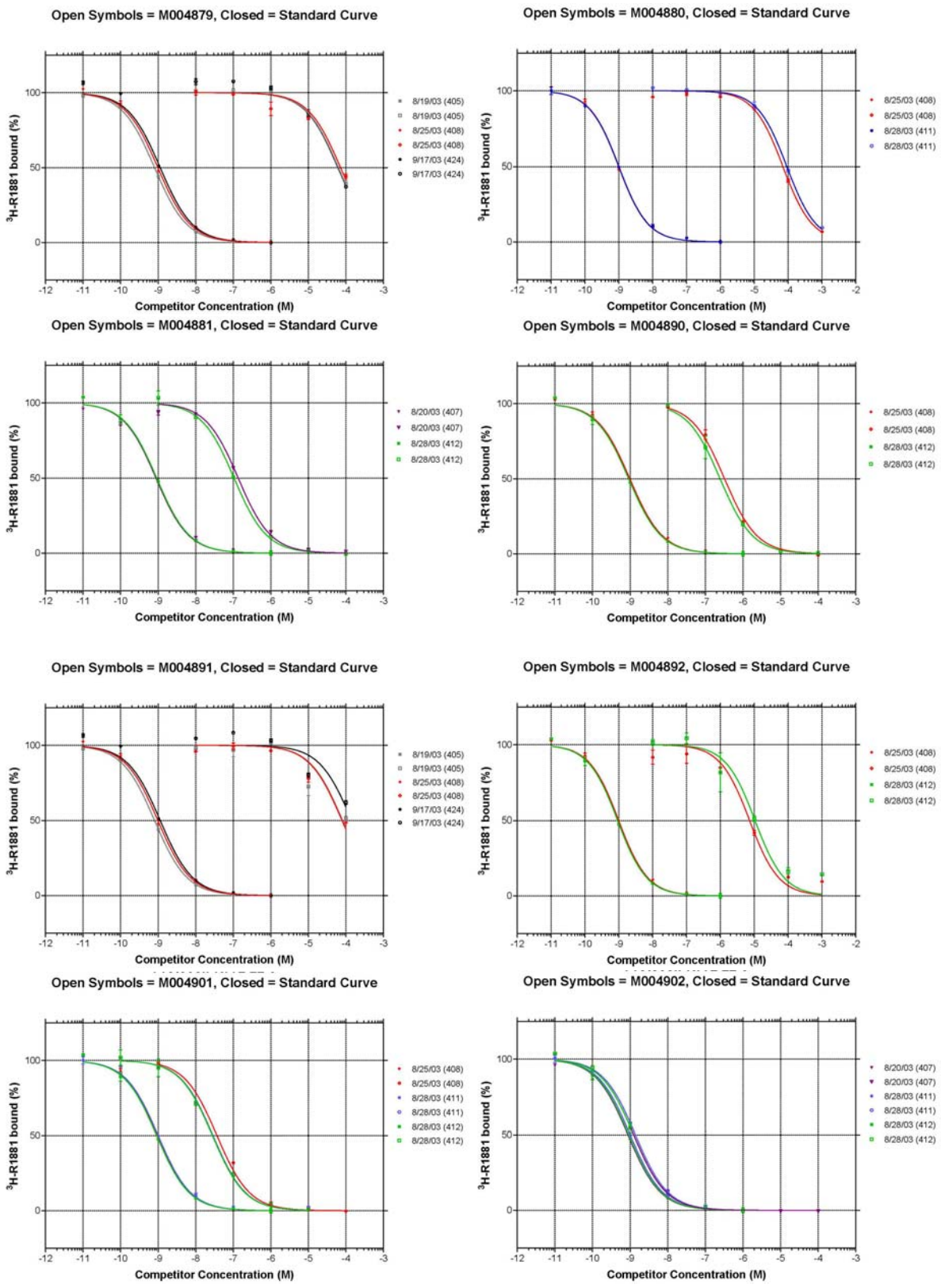


Figure 7-8. (continued)

8.0 PRELIMINARY INTERLABORATORY STUDIES

The objective for this task was to evaluate the intra- and interlaboratory variability of results among five independent laboratories when using Battelle-supplied R1881 from a common-lot, Battelle-supplied "weak binder" dexamethasone from a common lot, and a common lot of [^{3}H]-R1881. Stock concentrations of R1881 and dexamethasone were supplied by Battelle, Sequim Chemical Repository to the independent laboratories. To conduct this task, participating laboratories were supplied with a detailed protocol for androgen receptor (AR) saturation and competitive binding assays. This validation was conducted in two stages, first using the Battelle-supplied "standard" cytosol preparation, then using cytosol prepared by individual laboratories. Some of the material in this chapter was adapted from the results of the prior EDSP Work Assignment 4-11 (USEPA, 2006b,c).

Five laboratories participated in the first part of the validation with the standard cytosol, with all five performing acceptable saturation and competitive assays. The participating laboratories are shown in Table 8-1.

Table 8-1. Participating Laboratories

Lab ID	Laboratory Name	Abbreviation
LAB A	Illinois Institute Technologies Research Institute	IITRI
LAB B	Southern Research Institute	SRI
LAB C	In Vitro Technologies, Inc.	InVitro
LAB D	ABC Laboratories	ABC
LAB E	Battelle Richland	PNL

For the second part of the preliminary validation, all five laboratories attempted to prepare cytosol for the assay. Laboratories B, C, and E successfully prepared the cytosol and performed acceptable saturation and competitive assays. Laboratory D was not able to obtain cytosol with appropriate activity and was limited to several saturation binding assays which did not demonstrate appropriate binding activity. Therefore no competitive binding assays were conducted. Also during the preliminary validation, Lab A exhausted their funds and was requested to send their cytosol preparation to Lab D for later use. Lab A did not prepare or submit a report for this validation exercise.

The mean and coefficient of variation (CV) within laboratory results was evaluated to ensure that each laboratory was using the rat uterine cytosol preparations correctly and could reliably measure the relevant descriptors. In addition, the variability in the competitive binding assay was calculated from the measurements of log IC_{50} for R1881 and the weak binder (dexamethasone) and the relative binding affinity (RBA) for the weak binder. The goodness-of-fit (R^2 values ranging from 0 to 1) to the appropriate nonlinear binding equations were calculated. Finally, the sources of variability for the observed differences in laboratory results were examined.

Intra-laboratory variability of the resulting measurements was defined as the CV (standard deviation/mean x 100%) between the three separate assay repeats (indicated by the date of the run). Inter-laboratory variability was defined as the CV between the mean laboratory statistics

(average of the three runs). Other sources of variability associated with the estimation process of these statistics include the non-specific binding goodness-of-fit to a simple linear model for both the saturation and competitive binding assays and the variability in the activity of the radioactive labels.

Observations were removed from data analysis by the submitting laboratory based on their determination of outliers and level of saturation. Observations were removed from the intra- and inter laboratory comparison to allow convergence of the nonlinear one-site binding or competitive binding equations. The criteria used for model convergence and an appropriate measurement of the assay parameters were an R^2 value between 0 and 1, and a within replicate CV of less than 30%. Several laboratories did not calculate statistics. It is assumed that observations with absolute differences greater than 3 times the median value of the three within run replicates would have been removed when convergence of the nonlinear model was not obtained. Outliers that were not removed by the submitting laboratory and did not affect model convergence were indicated, and the statistical analysis was conducted with and without them.

8.1 Assay Performance with Standard Cytosol

Saturation binding Assay: Each of the five participating laboratories conducted three independent replicate saturation assays with three replicate runs of each concentration. The data were fit using the one-site binding model (Figure 8-1). The goodness-of-fit to the one-site binding equation ranged from 0.57 to 1.00 with a mean value of 0.96 for the 15 runs (Table 8-1). The range of B_{max} (fmole/100 μg) values was 6.67 to 15.6 with a mean value of 11.0. The range of K_d (nM) values was 0.685 to 1.57 with a mean value of 0.978. The intra-laboratory CVs for B_{max} ranged from 3.4% to 27% with a mean of 11% and for K_d ranged from 3.0% to 22% with a mean of 10.0%. Note that one data point from lab C was not removed by the reporting laboratory but is obviously in error (see note to Figure 8-1).

The inter-laboratory variability of the two saturation binding measurements was 16% and 25% for B_{max} and K_d, respectively (Table 8-2). When the single outlier from run C2-2/24/05 was removed these CVs became 20% and 24% respectively. The variability in these measurements was fairly large and can be explained by the variability in the fitted one-site binding curves which resulted from each laboratory's interpretation and reproduction of the saturation assay protocol (Figure 8-2).

Table 8-2. Intra-Laboratory variability of the statistics associated with the saturation assay. Values in parentheses were achieved by removing an outlier not removed by the submitting laboratory.

Statistic	Assay	Lab A	Lab B	Lab C	Lab D	Lab E	Overall
B_{max} (fmole/100 μg)	1	11.58	10.45	13.81	10.52	12.76	
	2	8.96	12.03	(10.03) 14.44	8.59	12.09	
	3	6.67	11.08	15.61	9.18	12.01	
Mean		9.07	11.19	(13.15) 14.62	9.43	12.28	(11.02) **11.32**
CV		27.1%	7.1%	(21.7%) 6.3%	10.5%	3.4%	(16.0%) **20.0%**
K_d (nM)	1	1.444	0.918	1.009	0.721	0.907	
	2	1.570	0.989	(0.481) 0.894	0.685	0.844	
	3	1.003	0.890	1.215	0.724	0.857	
Mean		1.339	0.933	(0.902) 1.039	0.710	0.869	(0.951) **0.978**
CV		22.3%	5.5%	(42.0%) 15.6%	3.0%	3.8%	(24.6%) **24.0%**
Goodness of Fit	1	0.99	0.88	0.90	0.98	0.99	
	2	0.90	0.98	(0.57) 0.95	0.95	1.00	
	3	0.92	0.97	0.99	0.96	1.00	
Mean		0.94	0.94	(0.82) 0.95	0.96	0.99	(0.93) **0.96**

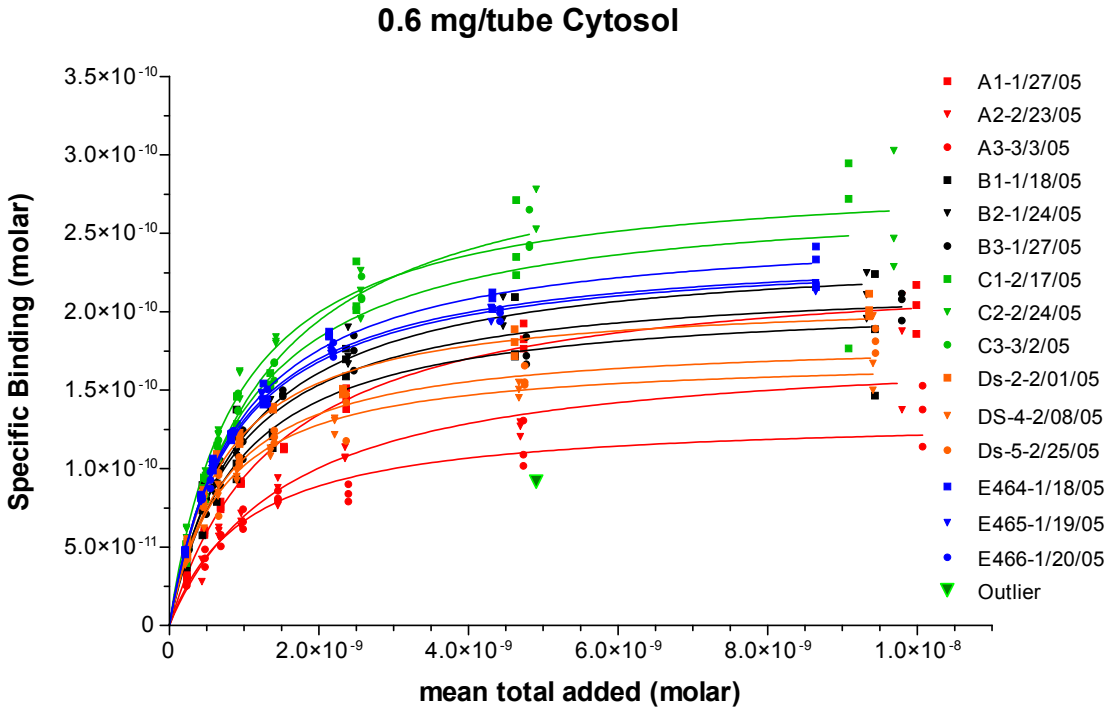


Figure 8-1. Inter-laboratory variability of one-site binding curves. The fitted curve for run C2-2/24/05 is without the designated outlier (highlighted at approximately $5x10^{-9}$ M).

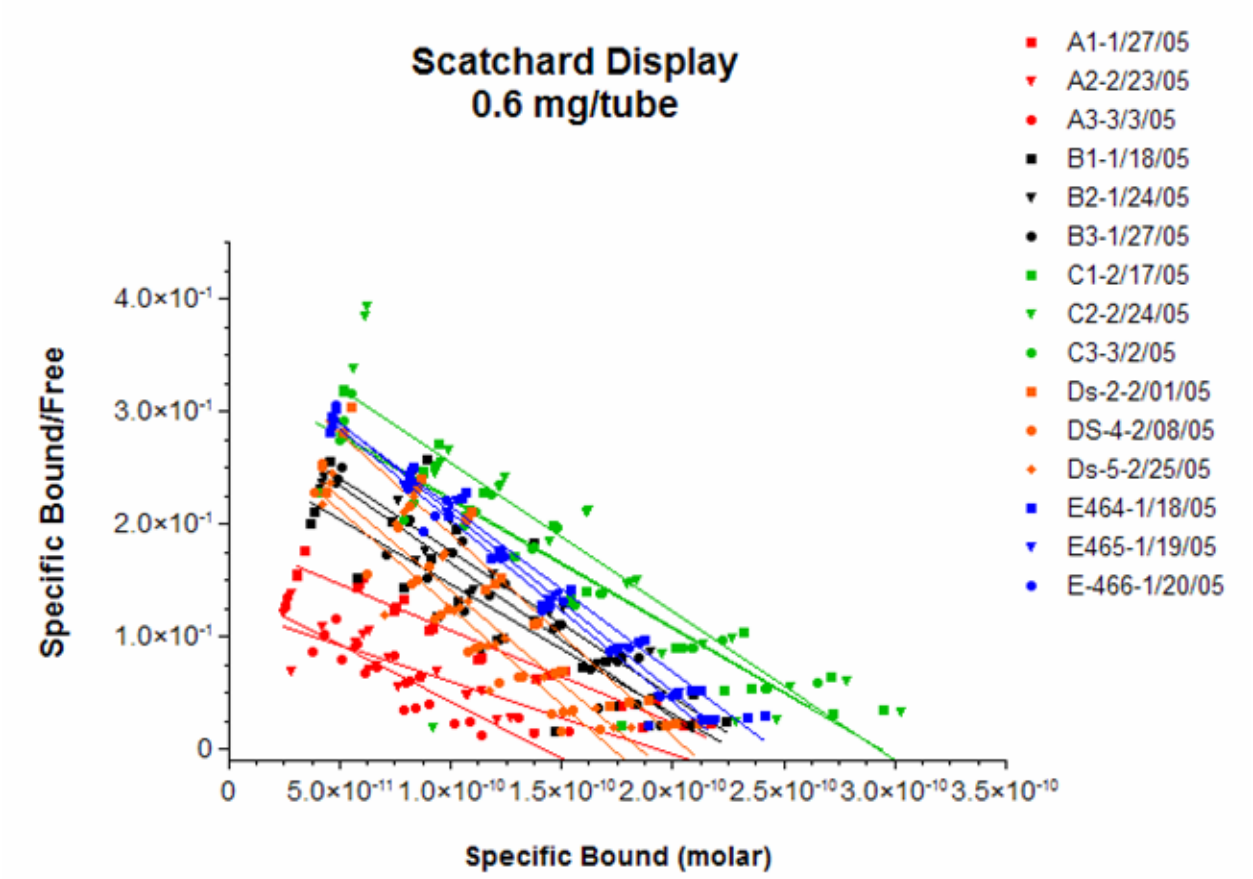


Figure 8-2. Inter-laboratory variability of Scatchard plot

Competitive Binding Assay: Each of the five participating laboratories were approved to conduct three independent replicate competitive binding assays with a standard and a weak positive control with three replicate runs for each concentration. No data other than those removed by the submitting laboratory were removed for statistical analysis.

The goodness-of-fit to the one-site competition equation for the standard ranged from 0.97 to 1.00 with a mean value of 0.99 for the 15 runs (Table 8-3, Figure 8-3). The goodness-of-fit for the weak positive control ranged from 0.82 to 1.00 with a mean value of 0.95. The range of IC_{50} values for the standard was 1.24E-09 to 2.27E-09 with a mean value of 1.61E-09. The range of IC_{50} values for the weak positive control was 2.33E-05 to 9.16E-05 with a mean value of 3.77E-05. The resulting RBAs ranged from 0.0017% to 0.0097% with a mean value of 0.0046%. The intra-laboratory CVs for RBA ranged from 2.9% to 92% with a mean of 14.7%. The highest RBA CV is from lab A which ran both the highest and lowest RBA runs. The inter-laboratory variability of the three competitive binding measurements was 13.1%, 16.7% and 14.7% for the standard and weak positive log IC_{50} values and RBA, respectively (Table 8-3).

Table 8-3. Intra-Laboratory variability of the statistics associated with the competitive assay

Statistic	Assay	Lab A	Lab B	Lab C	Lab D	Lab E	Overall
IC_{50} (M) Standard	1	2.265E-09	1.349E-09	1.416E-09	1.742E-09	1.977E-09	
	2	1.578E-09	1.549E-09	1.346E-09	1.459E-09	1.845E-09	
	3	1.242E-09	1.496E-09	1.291E-09	1.991E-09	1.837E-09	
Mean		1.643E-09	1.462E-09	1.350E-09	1.717E-09	1.885E-09	1.61E-09
CV		1.50%	0.35%	0.23%	0.77%	0.21%	13.1%
IC_{50} (M) Weak Positive	1	2.333E-05	3.508E-05	3.436E-05	2.944E-05	4.236E-05	
	2	9.162E-05	3.873E-05	3.664E-05	2.838E-05	4.159E-05	
	3	4.477E-05	3.673E-05	2.965E-05	3.373E-05	4.305E-05	
Mean		4.574E-05	3.681E-05	3.342E-05	3.043E-05	4.233E-05	3.77E-05
CV		6.85%	0.49%	1.05%	0.87%	0.17%	16.7%
RBA (%)	1	0.0097%	0.0038%	0.0041%	0.0059%	0.0047%	
	2	0.0017%	0.0040%	0.0037%	0.0052%	0.0044%	
	3	0.0028%	0.0041%	0.0044%	0.0059%	0.0043%	
Mean		0.0047%	0.0040%	0.0041%	0.0057%	0.0045%	0.0046%
CV		91.6%	2.9%	8.7%	7.8%	4.5%	14.7%
Goodness of Fit Standard	1	0.97	1.00	1.00	0.99	1.00	
	2	0.97	1.00	0.99	0.98	1.00	
	3	0.97	1.00	0.99	1.00	1.00	
Mean		0.97	1.00	0.99	0.99	1.00	0.99
Goodness of Fit Weak Positive	1	0.96	0.99	0.98	0.85	1.00	
	2	0.82	0.96	0.99	0.96	0.99	
	3	0.88	0.99	0.98	0.96	1.00	
Mean		0.89	0.98	0.98	0.92	1.00	0.95

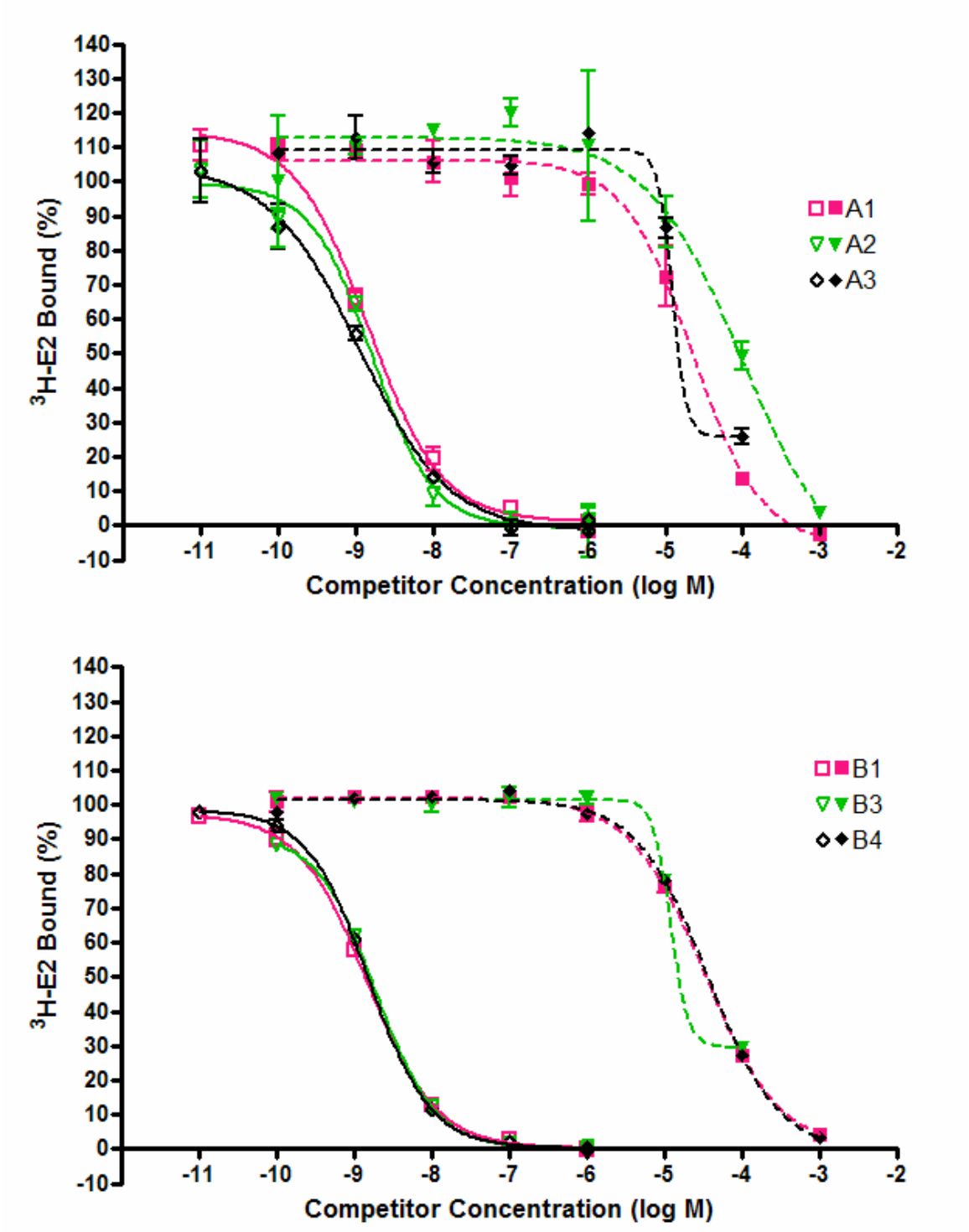


Figure 8-3. Competitive binding assay percent binding by laboratory A-E for weak positive dexamethasone (dashed lines, closed symbols). Standard Curves (solid lines, open symbols) are also shown.

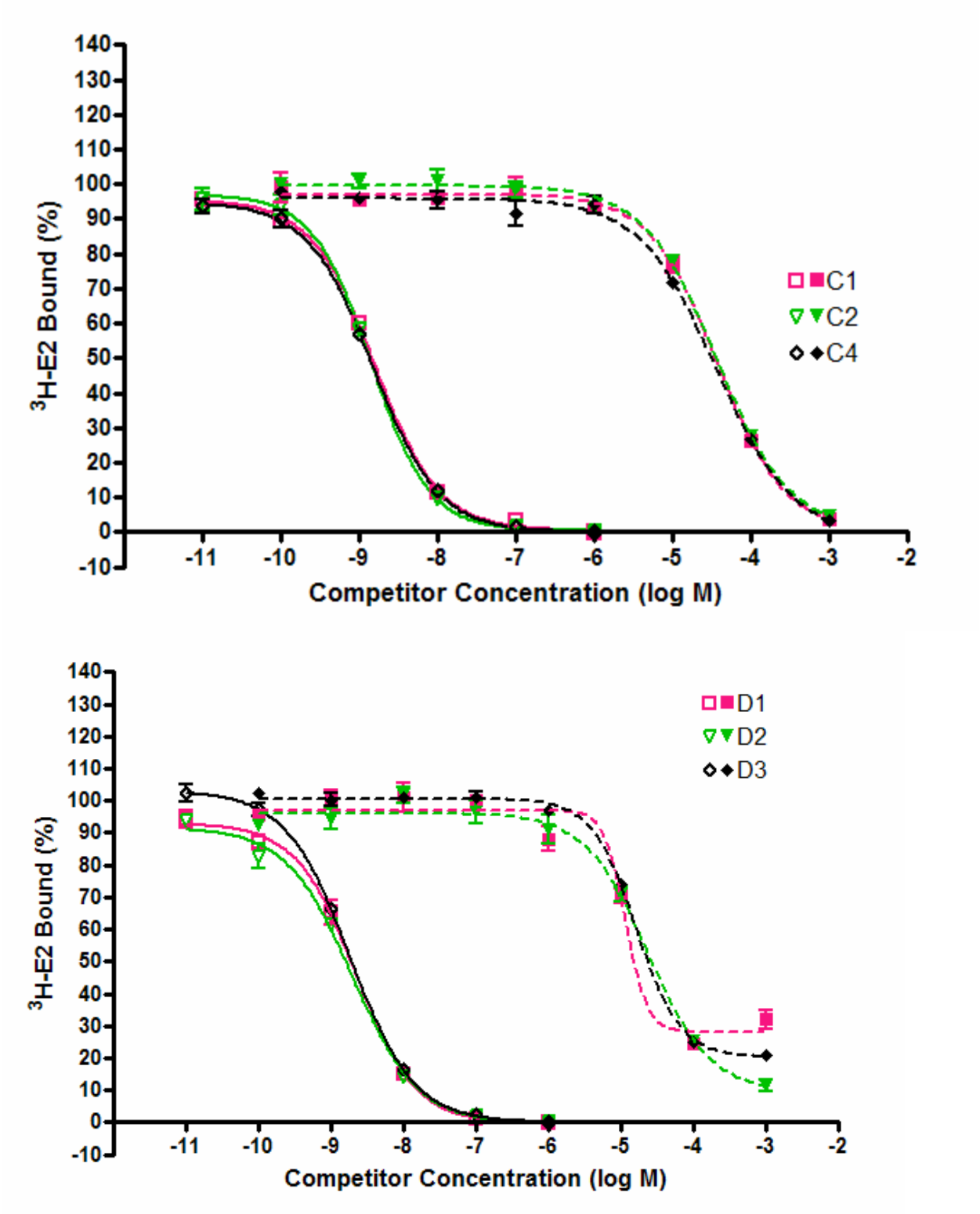


Figure 8-3. (continued)

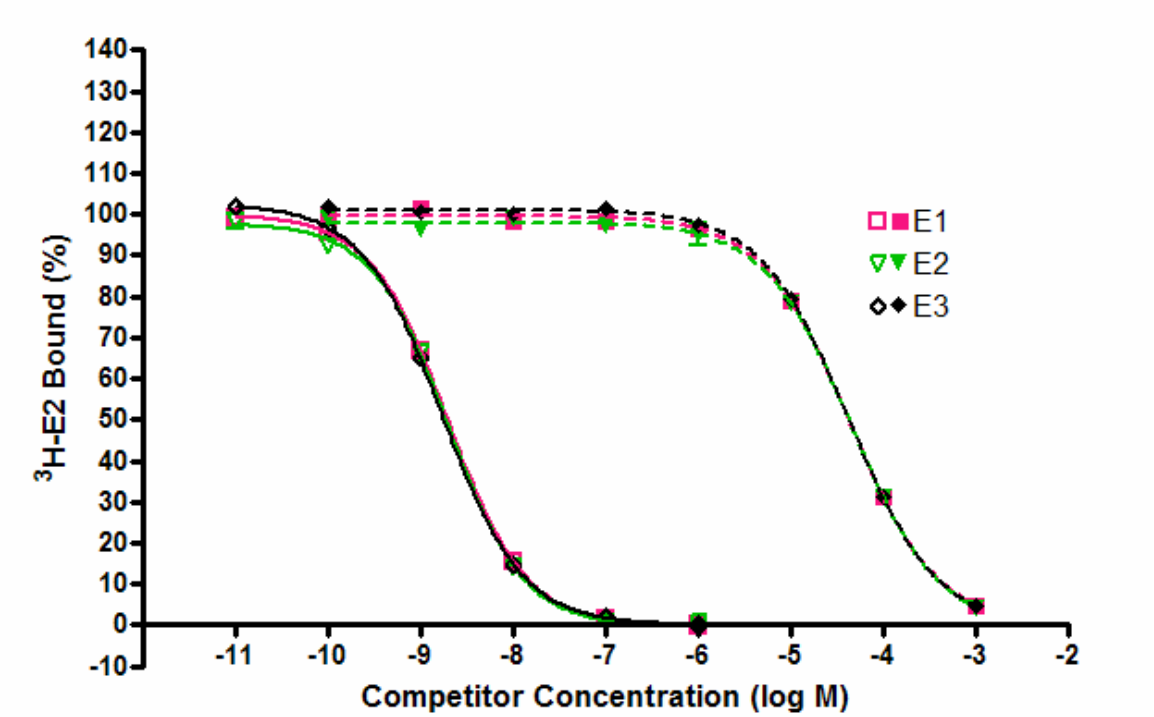


Figure 8-3. (continued)

8.2 Assay Performance with Laboratory Prepared Cytosol

8.2.1 Saturation Binding Assay

Each of the five participating laboratories conducted three independent runs of the saturation assay with triplicate tubes of each concentration. Two of the runs from Lab D did not converge and one run had a poor goodness-of-fit ($R^2 = 0.46$) and they were not used in the statistical analysis. The data from these runs, however, were included in the plots. The goodness-of-fit to the one-site binding equation ranged from 0.86 to 1.00 with a median value of 0.95 for the remaining 12 runs (Table 8-4). The range of B_{max} (fmole/100 µg) values was 4.81 to 16.8 with a median value of 9.25. The range of K_d (nM) values was 0.660 to 2.88 with a median value of 0.928. The intra-laboratory CVs for B_{max} ranged from 4.4% to 11% with a median of 6.9% and for K_d ranged from 2.9% to 24% with a median of 8.3%.

The inter-laboratory variability of the saturation binding measurements from the four laboratories was 53% and 61% for B_{max} and K_d, respectively (Table 8-4). The variability in these measurements was large and can be explained by the variability in the fitted one-site binding curves which resulted from each laboratory's interpretation and reproduction of the saturation assay protocol (Figure 8-4).

Table 8-4. Intra-Laboratory variability of the statistics associated with the saturation assay. The results from Lab D were not used in the statistical calculations.

Statistic	Assay	Lab A	Lab B	Lab C	Lab D[1]	Lab E	Overall
B_{max} (fmole/100 µg)	1	6.24	5.30	14.3		14.4	
	2	6.10	4.81	16.8		12.3	
	3	5.72	5.32	15.3	0.611	15.6	
Mean		6.02	5.14	15.4	0.61	14.1	10.2
CV		4.4%	5.6%	8.1%	NA	11.9%	53%
K_d (nM)	1	1.92	0.699	0.835		0.930	
	2	2.00	0.677	0.930		0.834	
	3	2.88	0.660	1.03	0.307	0.926	
Mean		2.27	0.68	0.93	0.31	0.90	1.19
CV		23.5%	2.9%	10.4%	NA	6.1%	61%
Goodness of Fit	1	0.95	0.94	0.98		0.97	
	2	0.96	0.92	0.97		0.86	
	3	0.95	0.88	0.98	0.46	1.00	
Mean		0.95	0.91	0.98	0.46	0.94	

[1] Model did not converge for data from assays 1 and 2 for Lab D

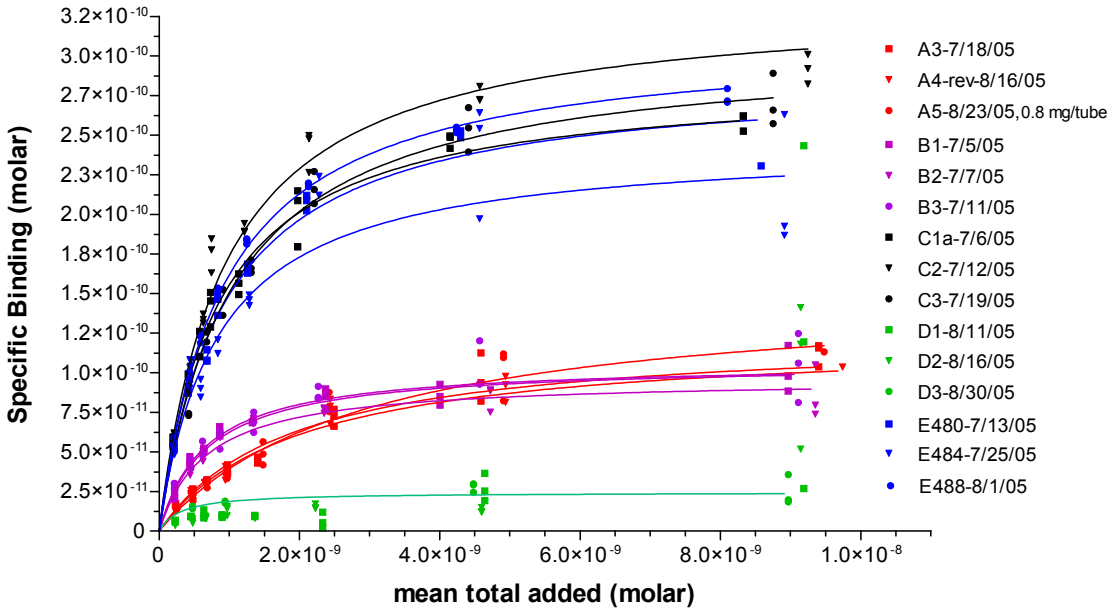


Figure 8-4. Inter-laboratory variability of saturation assay using one-site binding curves. The fitted curve for run C2-2/24/05 is without the designated outlier.

8.2.2 Competitive Binding Assay

Only three of the participating laboratories conducted three independent runs of the competitive binding assay with a standard and a weak positive control with triplicate tubes of each concentration.

The goodness-of-fit to the one-site competition equation for the standard ranged from 0.65 to 1.00 with a median value of 0.99 for the 9 runs (Table 8-5). One data point from Lab C Run 6-9/1/05 (see Figure 8-5 top panel) for the standard was considered an outlier and was removed from the analysis. Without the outlier the goodness-of-fit for Lab C Run 6-9/1/05 increased from 0.65 to 0.81. However, the percentage R1881 bound for this run was suppressed at the lower competitor concentrations (Figure 8-5 bottom panel). If the top of the curve is not constrained to 100% bound, then the goodness of fit becomes 0.99. The goodness-of-fit for the weak positive control ranged from 0.11 to 1.00 with a median value of 0.99. The low goodness-of-fit was associated with the percent bound being suppressed for Lab C Run 6-9/1/05 (Figure 8-5). Again, if the top of the curve is not constrained to 100% bound, then the goodness of fit becomes 1.00.

When the above changes were made for Lab C Run 6-9/1/05, the range of log IC_{50} values for the standard ranged from -9.158 to -8.710 with a mean value of -8.854. The range of log IC_{50} values for the weak positive control was -4.311 to -4.114 with a mean value of -4.219. The resulting RBAs ranged from 0.0010% to 0.0033% with a mean value of 0.0024%. The intra-laboratory CVs for RBA ranged from 1% to 46% with a median of 5%. The variability in these measurements was can be inferred by the variability in the fitted one-site competitive curves (Figure 8-6). Figure 8-6 is not intended to show each individual run but rather the overall run to run variability.

Table 8-5. Intra-Laboratory variability of the statistics associated with the competitive assay with and without an identified outlier and modeling constraints shown in parentheses

Statistic	Assay	Lab B	Lab C	Lab E	Overall
IC50 Standard	1	-8.879	-9.053	-8.830	
	2	-8.866	-8.710	-8.810	
	3	-8.754	-9.158 (-8.873)	-8.785	
Mean		-8.830	-8.928 (-8.857)	-8.810	-8.854 (-8.833)
CV		17%	57% (38%)	5%	14% (5%)
IC50 Weak Positive	1	-4.242	-4.239	-4.311	
	2	-4.234	-4.114	-4.298	
	3	-4.127	-4.156 (-4.044)	-4.308	
Mean		-4.198	-4.166 (-4.125)	-4.305	-4.219 (-4.203)
CV		15%	14% (22%)	2%	16% (20%)
RBA	1	0.002%	0.002%	0.003%	
	2	0.002%	0.003%	0.003%	
	3	0.002%	0.001% (0.002%)	0.003%	
Mean		0.002%	0.002% (0.002%)	0.003%	0.002% (0.002%)
CV		1%	46% (32%)	5%	30% (27%)
Goodness of Fit Standard	1	0.99	0.98	1.00	
	2	1.00	0.99	1.00	
	3	0.99	0.65 (0.99)	1.00	
Mean		99%	87% (99%)	100%	
Goodness of Fit Weak Positive	1	0.99	0.99	0.98	
	2	0.98	0.99	1.00	
	3	0.97	0.11 (0.94)	0.99	
Mean		98%	70% (97%)	99%	

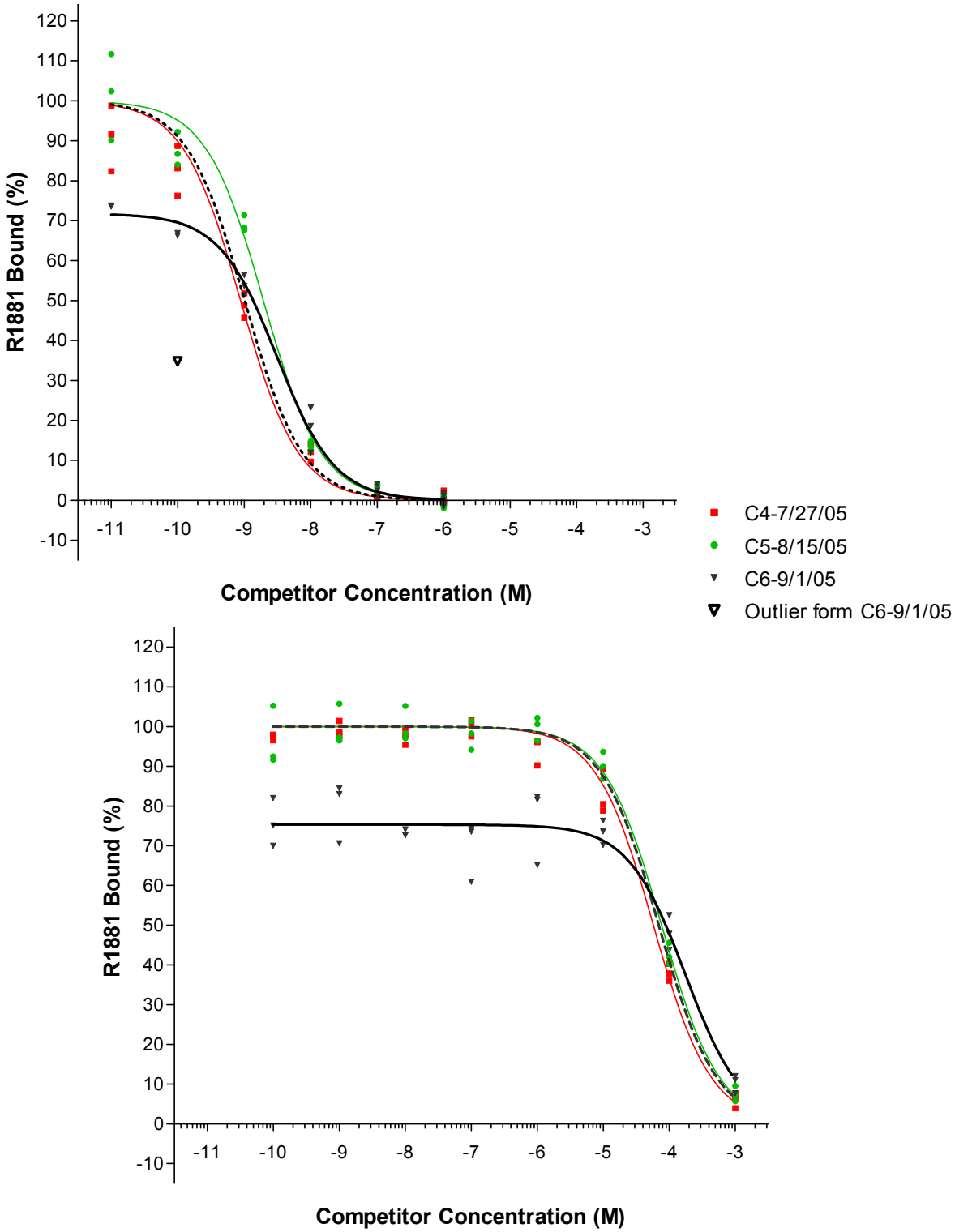


Figure 8-5. Lab C one-site competitive curves for the standard R1881 (top panel) and weak positive (bottom panel) with (dashed line) and without (solid line) the top parameter constrained to 100%

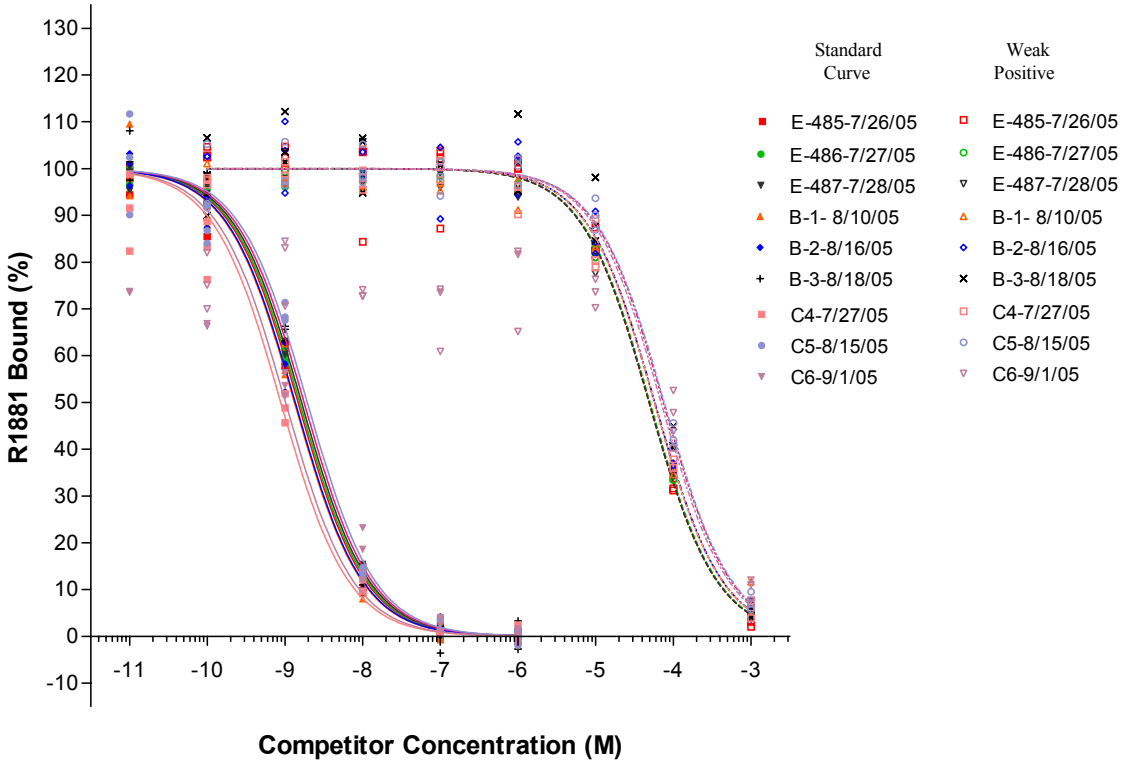


Figure 8-6. Inter-laboratory variability of one-site competitive curves using all data and top and bottom model constraints

The data were then re-fitted using the 4 parameter model (Table 8-6, Figure 8-7). No data other than those removed by the submitting laboratory were removed for statistical analysis. The goodness-of-fit for the weak positive control were all greater than 0.875 with a median value of 0.979. The range of $\log_{10}IC_{50}$ (log M) values for the weak positive control was -4.3056 to -4.036 with a mean value of -4.210.

Table 8-7 shows the parameter estimates across laboratories with the associated 95% confidence intervals. Labs B, C, and E all produced similar aggregate log IC_{50}s for the R1881 and dexamethasone with top and bottom estimates close to 100 and 0 respectively.

Table 8-6 Intra-Laboratory variability of the statistics associated with the competitive binding assay with dexamethasone (weak positive) using the 4 parameter model.

	Best-fit values				Goodness of Fit
	$\log_{10}IC_{50}$ (log M) ±SE	B	T	β slope	R^2
Lab B					
1	-4.245±0.036	2.2	98.1	-0.976	0.991
2	-4.239±0.048	0.9	100.6	-1.027	0.983
3	-4.132±0.052	-0.3	102.2	-1.043	0.978
Lab C					
1	-4.230±0.029	-1.9	98.3	-0.899	0.994
2	-4.116±0.036	3.1	98.9	-1.167	0.987
3	-4.036±0.066	7.1	74.8	-1.501	0.941
Lab E					
1	-4.305±0.050	-1.3	100.5	-1.041	0.982
2	-4.288±0.014	-1.1	98.2	-0.950	0.999
3	-4.298±0.014	-0.3	97.3	-0.943	0.999
Average of all labs					
	-4.210	0.9	96.6	-1.061	0.984

Table 8-7. Interlaboratory Parameter Estimates with 95% Confidence Intervals

Lab	$\log_{10}IC_{50}$	β slope	T	B
Standard Curve				
B	-8.848 (-8.938,-8.757)	-1.045 (-1.145,-0.944)	101.033 (99.099,102.968)	-0.350 (-1.664,0.963)
C	-8.900 (-9.148,-8.652)	-0.937 (-1.093,-0.781)	86.162 (62.518,109.805)	-0.225 (-2.467,2.017)
E	-8.813 (-8.832,-8.795)	-0.998 (-1.046,-0.950)	98.933 (97.933,99.932)	-0.108 (-0.813,0.596)
Weak Positive				
B	-4.231 (-4.281,-4.180)	-1.001 (-1.143,-0.858)	99.970 (96.974,102.966)	1.344 (-3.437,6.125)
C	-4.157 (-4.293,-4.021)	-0.946 (-1.084,-0.808)	90.749 (70.144,111.355)	0.606 (-4.131,5.343)
E	-4.307 (-4.327,-4.287)	-0.950 (-1.000,-0.900)	97.951 (97.150,98.752)	-0.737 (-2.598,1.123)

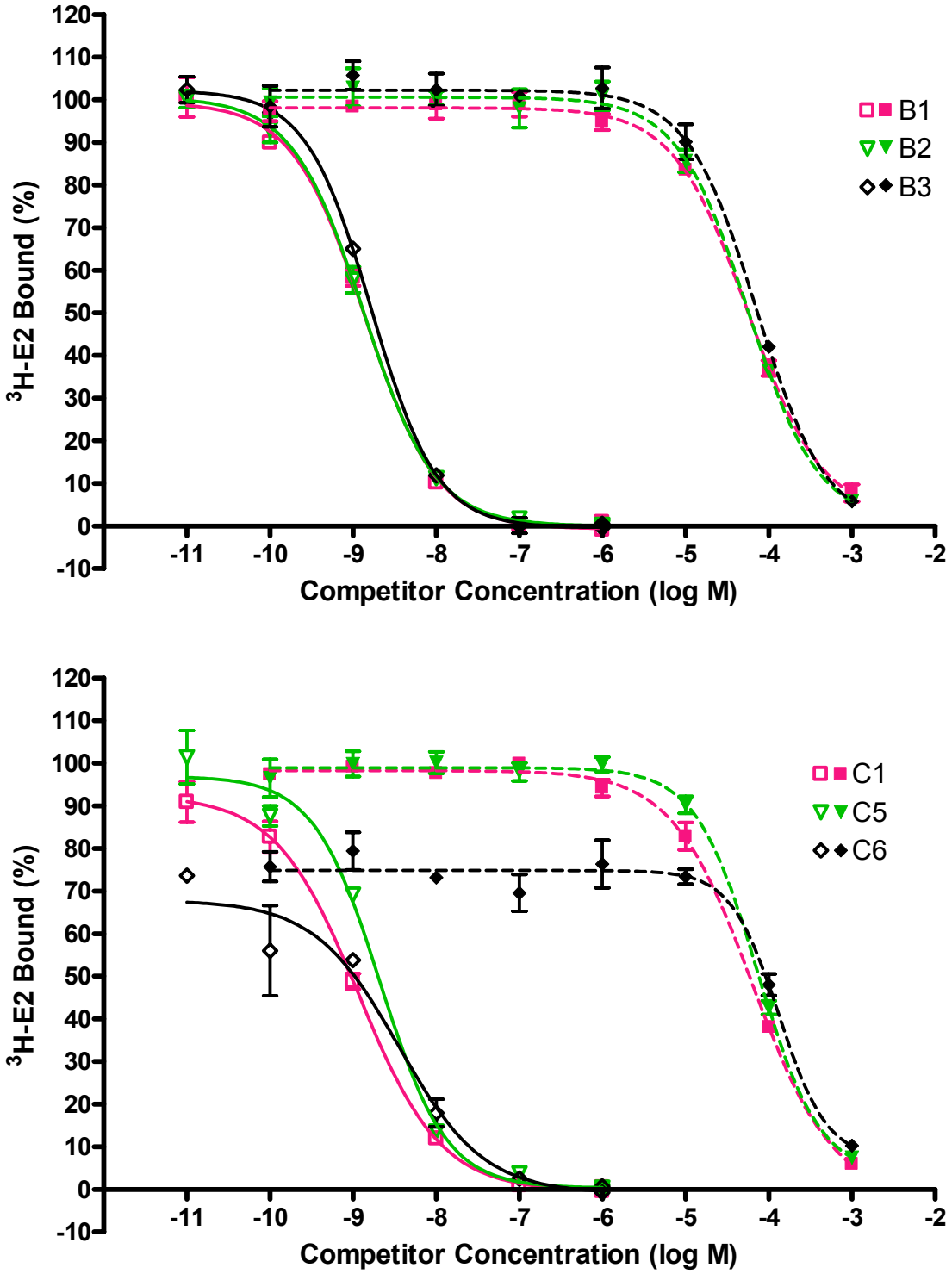


Figure 8-7. **Competitive binding assay percent binding by laboratory B (top), C (middle) and E (bottom) for weak positive dexamethasone (dashed lines, closed symbols). Standard Curves (solid lines, open symbols) are also shown.**

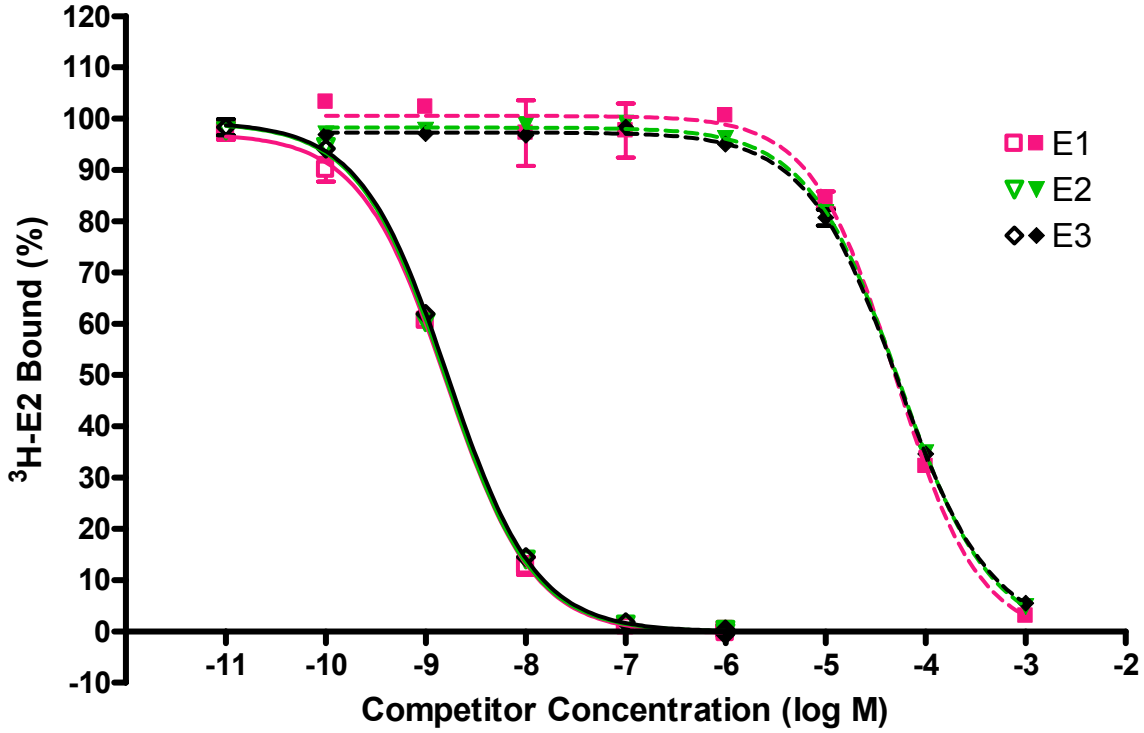


Figure 8-7. (continued)

9.0 INTERLABORATORY VALIDATION STUDIES

This chapter presents the results of the main interlaboratory validation study. Four laboratories tested 10 chemicals whose binding affinities were known. The objective of these studies was to establish the variability of results among independent laboratories when using cytosol prepared by each of the individual laboratories, Battelle-supplied R 1881 from a common-lot, Battelle-supplied "weak binder" dexamethasone from a common lot, and a common lot of ^{3}H-R1881 (all the same as in the preliminary validation studies) and a set of ten blinded test chemicals (Table 6-2, above). Stock concentrations of R1881, and dexamethasone and unknown test chemicals were supplied by Battelle, Sequim Chemical Repository to the independent laboratories. To conduct this task participating laboratories were supplied with a detailed protocol for androgen receptor (AR) saturation and competitive binding assays. Laboratory designations A-E are the same as in Chapter 8. Some of the material in this chapter was adapted from the results of the prior EDSP Work Assignment 4-11 (USEPA, 2006d).

Supporting data related to this work, Interlaboratory Analysis for the Standard Chemical, the Weak Positive Binder, and Test Chemicals, is presented in Appendix B.

Following preliminary validation (Chapter 8), laboratories B, C, and E had performed acceptable saturation and competitive assays and moved forward to the interlaboratory validation. Laboratory D was not able to obtain cytosol with appropriate activity and therefore could not complete the preliminary validation. Lab A exhausted their funds prior to completion of the preliminary validation and sent their cytosol preparation to Lab D for use in the interlaboratory validation. Lab A was dropped from the study and did not participate in this validation exercise.

9.1 Preliminary Analysis

9.1.1 Saturation Binding Assay

Since the cytosol preparations used in this validation were previously characterized in Labs B, C, and E, no additional saturation runs were conducted in these labs. Lab D ran a single run of the saturation assay with triplicate tubes of each concentration using cytosol prepared by Lab A (Figure 9-1). The fitted parameters for this run were Bmax (fmole/100 µg) 6.96, Kd (nM) 3.77, and Goodness of Fit 0.90.

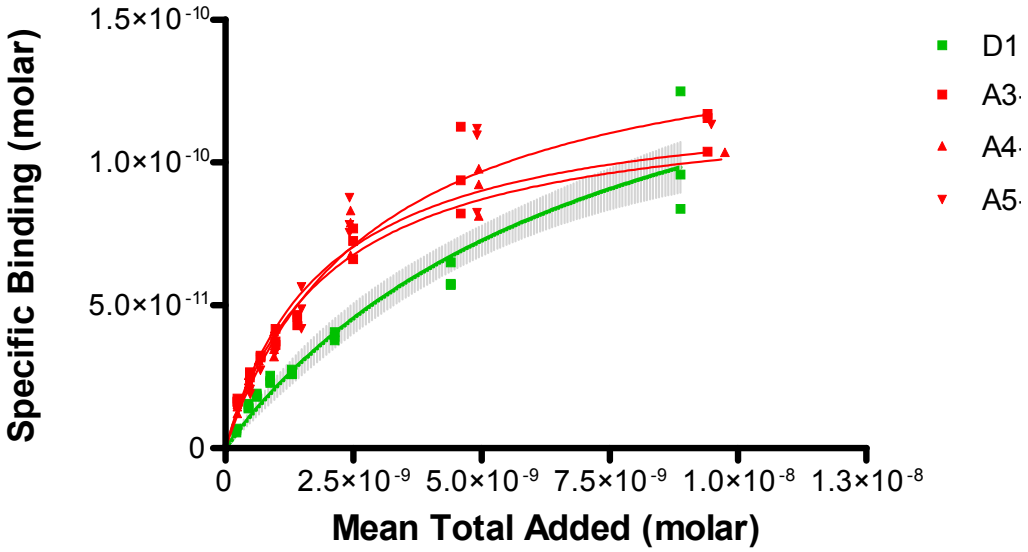


Figure 9-1 Comparison of saturation binding assay run in Lab D using Lab A prepared cytosol. The prior Lab A fits (red) are included as comparison. The gray area represents the 95% confidence bands about the fitted curve.

9.1.2 Competitive Binding Assay

Each participating laboratory conducted three independent runs of the competitive binding assay with a standard, a weak positive control, and ten test chemicals. Triplicate tubes of each concentration were performed during each run.

The goodness-of-fit to the one-site competition equation for the standard ranged from 0.30 to 1.00 with a median value of .99 for the 29 runs (Table 9-1). The low goodness-of-fit was associated with Lab C run C2-11/15/05. The percent bound was all less than 20% for this run (Figure 9-3). The goodness-of-fit for the weak positive control ranged from 0.02 to 0.99 with a median value of 0.96. Low goodness-of-fit was associated with several runs from Lab D (D5-11/29/05, D6-12/06/05, and D7-12/08/05 for which the percent bound was extremely variable for similar competitor concentrations (Figure 9-4). The range of log IC_{50} values for the standard was -8.947 to -8.184 with a mean value of -8.624. The range of log IC_{50} values for the weak positive control was -4.548 to -3.016 with a mean value of -4.183. The resulting RBAs ranged from 0.0004% to 0.0100% with a median value of 0.0039%. The intra-laboratory CV for RBA was 27% (Table 9-1). The variability in these measurements can be observed in the fitted one-site competitive curves for the standard and weak positive for all runs (Figures 9-2 to 9-5). Note that curves for Lab B and Lab E would generally overlap. Figures 9-2 to 9-5 show multiple runs and fits and are intended only to show general trends and comparison of fits, means and standard errors are shown in Figures 9-6 to 9-16.

Table 9-1. Intra-Laboratory variability of the statistics associated with the competitive assay for the standard and weak positive

Run	Standard Curve		Positive Control		
	log IC_{50}	R^2	log IC_{50}	R^2	RBA
Lab B					
B1-10/18/05	-8.807	0.99	-4.199	0.97	0.0025%
B2-10/20/05	-8.796	0.99	-4.229	0.97	0.0027%
B3-10/24/05	-8.790	0.99	-4.217	0.94	0.0027%
B4-10/26/05	-8.883	0.99	-4.292	0.96	0.0026%
B5-11/03/05	-8.785	0.99	-4.246	0.97	0.0029%
B6-11/07/05	-8.812	1.00	-4.237	0.99	0.0027%
B7-11/09/05	-8.883	0.99	-4.320	0.98	0.0027%
B8-11/14/05	-8.947	0.99	-4.247	0.96	0.0020%
Mean	-8.836	0.99	-4.246	0.97	0.0026%
CV	0.7%		0.9%		10%
Lab C					
C2-11/15/05	-8.346	0.30	-4.088	0.96	0.0055%
C3-11/28/05	-8.287	0.97	-3.963	0.96	0.0048%
C4-11/29/05	-8.184	0.85	-3.883	0.67	0.0050%
C5-11/16/05	-8.678	0.99	-4.159	0.98	0.0030%
C7-11/22/05	-8.544	0.99	-4.148	0.96	0.0040%
C8-12/19/05	-8.738	0.98	-4.159	0.95	0.0026%
Mean	-8.417	0.85	-4.053	0.92	0.0042%
CV	2.7%		2.9%		27%
Lab D					
D1-11/04/05	-8.298	0.95	-3.818	0.53	0.0033%
D2-11/08/05	-8.339	0.96	-4.339	0.88	0.0100%
D3-11/15/05	-8.456	0.95	-4.136	0.67	0.0048%
D4-11/22/05	-8.336	0.93	-4.209	0.75	0.0075%
D5-11/29/05	-8.530	0.96	-3.971	0.08	0.0028%
D6-12/06/05	-8.460	0.97	-3.016	0.02	0.0004%
D7-12/08/05	-8.352	0.89	-4.092	0.32	0.0055%
D8-12/08/05	-8.511	0.95	-4.209	0.84	0.0050%
D9-12/15/05	-8.676	0.97	-4.269	0.82	0.0039%
Mean	-8.426	0.95	-3.750	0.55	0.0048%
CV	1.4%		10.8%		58%
Lab E					
E517-11/08/05	-8.818	0.98	-4.530	0.96	0.0051%
E518-11/10/05	-8.752	0.99	-4.547	0.96	0.0062%
E519-11/17/05	-8.836	1.00	-4.363	0.99	0.0034%
E520-11/22/05	-8.752	1.00	-4.398	0.93	0.0044%
E522-12/08/05	-8.721	1.00	-4.548	0.94	0.0067%
E523-12/20/05	-8.772	1.00	-4.474	0.96	0.0050%
Mean	-8.775	0.99	-4.470	0.96	0.0051%
CV	0.5%		1.8%		24%
Lab E					
Mean	-8.572		-4.050		0.0042%
CV	2.6%		6.9%		27%

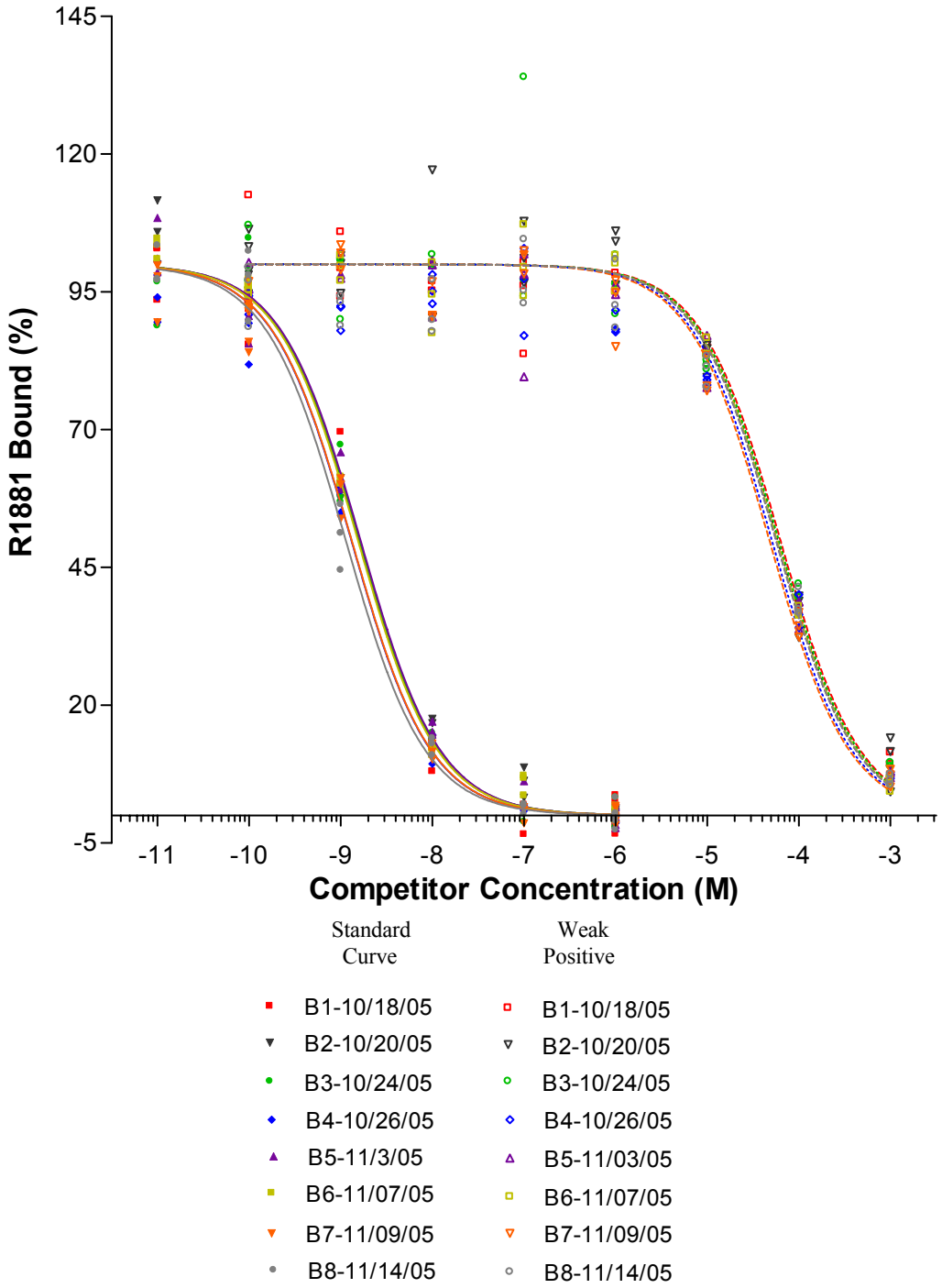


Figure 9-2. Two parameter model curves for the standard R1881 and weak positive for Lab B.

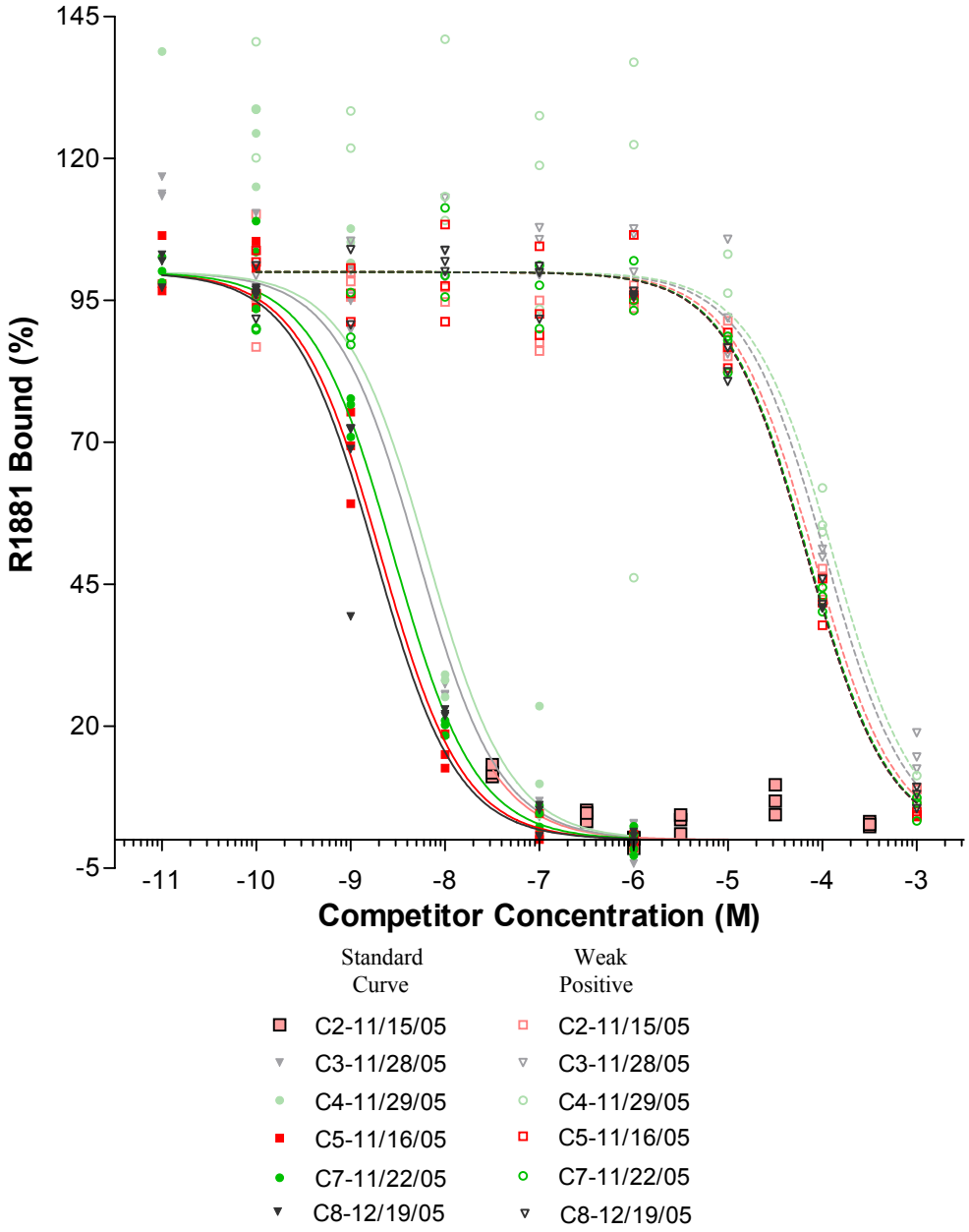


Figure 9-3. Two parameter model curves for the standard R1881 and weak positive for Lab C. Note run C2-11/15/05 had poor goodness-of-fit for the standard curve.

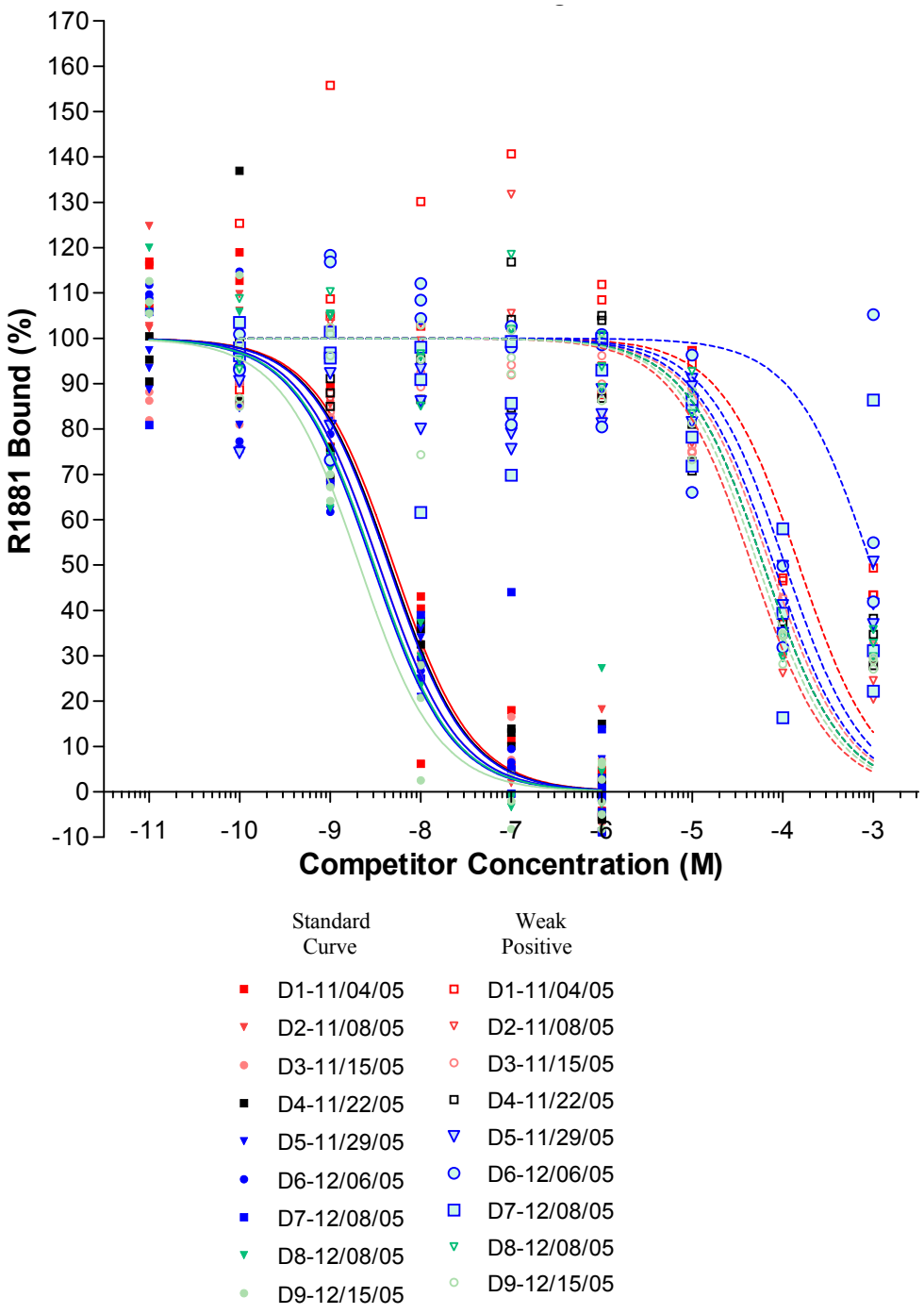


Figure 9-4. Two parameter model curves for the standard R1881 and weak positive for Lab D. Note runs D5, D6, and D7 had poor goodness-of-fit for the weak positive curves.

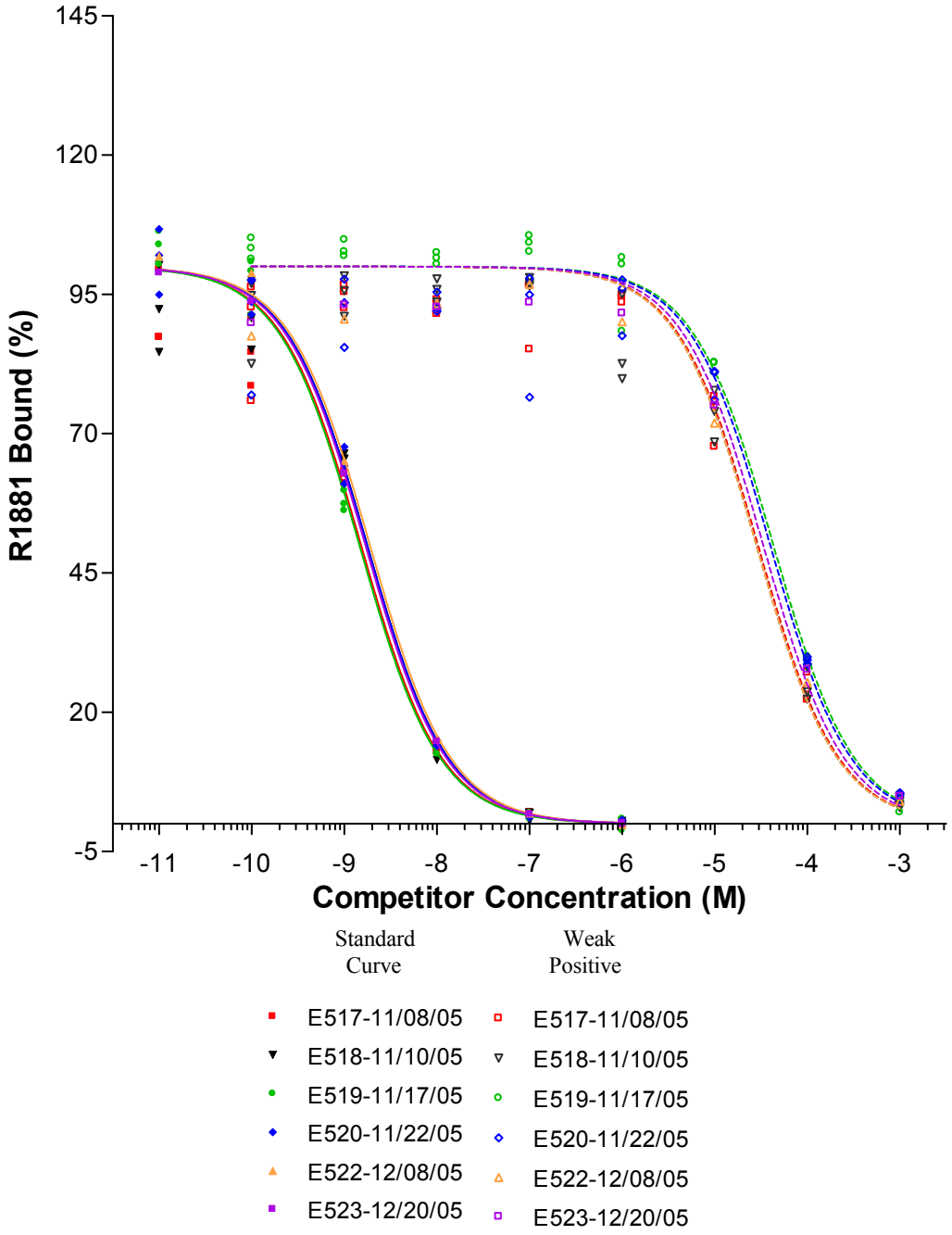


Figure 9-5. Two parameter model curves for the standard R1881 and weak positive for Lab E.

The goodness-of-fit to the one-site competitive equation for the ten test chemicals reflected the characteristics of the chemical being tested (Tables 9-2 to 9-6). Thus, the lack of convergence did not cause any data to be removed from the analysis. Most (80%) of the R^2 values were greater than 0.8. One run of CR42404, linuron, from Lab C and Lab D either did not converge or produced a poor fit to the curve (R^2 values less than or equal to 0.4), but the other two runs in both cases had R^2 values greater than 0.5. One run of CR42406, cyproterone acetate, from Lab D had a poor fit to the curve (R2 = 0.32), but again this was an anomaly. Chemicals CR42408, bis(2-ethylhexyl) phthalate (DEPH), and CR42409, atrazine, either did not converge or have poorly fitted curves for nearly all laboratories and runs. However, DEHP and atrazine were chosen to be negative chemicals and would not be expected to fit the model. The mean log IC_{50}, R^2, and RBA and intra-laboratory CVs for the test chemicals are also presented in each of the Tables 9-2 to 9-6. A detailed statistical analysis is presented following the data re-analysis with the 4 parameter model. Note that RBA errors appear large when the log IC_{50} of the test chemical is very close to the log IC_{50} of the R1881 standard and is not necessarily indicative of large experimental variability. Thus, the CVs are actually poor indicators of variability for this parameter.

Table 9-2. Intra-Laboratory Competitive Assay Results for Associated Runs of the Standard, Weak Positive, and Test Chemicals 5a-Androstan-17B-ol-3-one (4,5a-Dihydrotestosterone) and Testosterone

	Standard Curve		Positive Control			CR42400 5a-Androstan-17B-ol-3-one (4,5a-Dihydrotestosterone)			CR42401 Testosterone		
Run	log IC_{50}	R^2	log IC_{50}	R^2	RBA	log IC_{50}	R^2	RBA	log IC_{50}	R^2	RBA
B1-10/18/05	-8.81	0.99	-4.20	0.97	-4.61	-9.00	0.99	0.20	-8.11	0.97	-0.69
B4-10/26/05	-8.88	0.99	-4.29	0.96	-4.59	-8.94	1.00	0.06	-8.13	1.00	-0.75
B6-11/07/05	-8.81	1.00	-4.24	0.99	-4.58	-8.97	1.00	0.16	-8.08	1.00	-0.73
					Mean	-8.97	0.99	0.14	-8.11	0.99	-0.72
					CV	-0.3%		50.7%	-0.3%		-3.9%
C2-11/15/05	-8.35	0.30	-4.09	0.96	-4.26	-8.63	0.94	0.28	-7.71	0.98	-0.64
C3-11/28/05	-8.29	0.97	-3.96	0.96	-4.32	-8.54	0.87	0.26	-7.85	0.98	-0.43
C4-11/29/05	-8.18	0.85	-3.88	0.67	-4.30	-8.31	0.78	0.12	-7.51	0.94	-0.67
					Mean	-8.49	0.86	0.22	-7.69	0.97	-0.58
					CV	-2.0%		39.0%	-2.2%		-22.2%
D1-11/04/05	-8.30	0.95	-3.82	0.53	-4.48	-8.15	0.75	-0.15	-7.64	0.84	-0.66
D2-11/08/05	-8.34	0.96	-4.34	0.88	-4.00	-8.49	0.93	0.15	-7.68	0.96	-0.66
D3-11/15/05	-8.46	0.95	-4.14	0.67	-4.32	-8.31	0.98	-0.15	-7.69	0.99	-0.77
					Mean	-8.32	0.89	-0.05	-7.67	0.93	-0.70
					CV	-2.0%		-361.8%	-0.3%		-9.0%
E517-11/08/05	-8.82	0.98	-4.53	0.96	-4.29	-8.79	0.99	-0.02	-8.14	1.00	-0.68
E518-11/10/05	-8.75	0.99	-4.55	0.96	-4.21	-8.69	0.98	-0.06	-8.02	0.99	-0.73
E519-11/17/05	-8.84	1.00	-4.36	0.99	-4.47	-8.64	1.00	-0.19	-8.04	1.00	-0.80
					Mean	-8.71	0.99	-0.09	-8.07	1.00	-0.74
					CV	-0.9%		-93.3%	-0.8%		-8.4%

Table 9-3. Intra-Laboratory Competitive Assay Results for Associated Runs of the Standard, Weak Positive, and Test Chemicals 17β-Estradiol and 6α-Methyl-17α-hydroxyprogesterone acetate (MPA)

	Standard Curve		Positive Control			CR42402 17β-Estradiol			CR42403 6α-Methyl-17α-hydroxyprogesterone acetate (MPA)		
Run	log IC_{50}	R^2	log IC_{50}	R^2	RBA	log IC_{50}	R^2	RBA	log IC_{50}	R^2	RBA
B1-10/18/05	-8.81	0.99	-4.20	0.97	-4.61	-6.54	0.99	-2.27			
B2-10/20/05	-8.80	0.99	-4.23	0.97	-4.57				-7.64	0.98	-1.15
B4-10/26/05	-8.88	0.99	-4.29	0.96	-4.59	-6.62	0.99	-2.26	-7.78	0.99	-1.11
B7-11/09/05	-8.88	0.99	-4.32	0.98	-4.56	-6.72	1.00	-2.16	-7.84	0.99	-1.04
					Mean	-6.63	1.00	-2.23	-7.75	0.99	-1.10
					CV	-1.4%		-2.7%	-1.3%		-5.1%
C2-11/15/05	-8.35	0.30	-4.09	0.96	-4.26	-6.34	0.99	-2.00	-7.36	0.98	-0.99
C3-11/28/05	-8.29	0.97	-3.96	0.96	-4.32	-6.38	0.99	-1.90	-7.43	0.99	-0.85
C4-11/29/05	-8.18	0.85	-3.88	0.67	-4.30	-6.28	0.98	-1.91	-7.29	0.76	-0.89
					Mean	-6.34	0.99	-1.94	-7.36	0.91	-0.91
					CV	-0.8%		-2.9%	-0.9%		-7.5%
D1-11/04/05	-8.30	0.95	-3.82	0.53	-4.48	-6.82	0.66	-1.48			
D2-11/08/05	-8.34	0.96	-4.34	0.88	-4.00	-6.00	0.83	-2.34			
D3-11/15/05	-8.46	0.95	-4.14	0.67	-4.32	-5.50	0.70	-2.95			
D4-11/22/05	-8.34	0.93	-4.21	0.75	-4.13				-6.89	0.96	-1.45
D5-11/29/05	-8.53	0.96	-3.97	0.08	-4.56				-6.87	0.97	-1.66
D6-12/06/05	-8.46	0.97	-3.02	0.02	-5.44				-6.52	0.92	-1.94
					Mean	-6.10	0.73	-2.26	-6.76	0.95	-1.68
					CV	-10.9%		-32.7%	-3.1%		-14.7%
E517-11/08/05	-8.82	0.98	-4.53	0.96	-4.29	-6.52	1.00	-2.30			
E518-11/10/05	-8.75	0.99	-4.55	0.96	-4.21	-6.55	0.99	-2.21			
E519-11/17/05	-8.84	1.00	-4.36	0.99	-4.47	-6.38	0.99	-2.45			
E520-11/22/05	-8.75	1.00	-4.40	0.93	-4.35				-7.76	1.00	-0.99
E522-12/08/05	-8.72	1.00	-4.55	0.94	-4.17				-7.83	0.99	-0.89
E523-12/20/05	-8.77	1.00	-4.47	0.96	-4.30				-7.77	1.00	-1.00
					Mean	-6.48	0.99	-2.32	-7.79	0.99	-0.96
					CV	-1.3%		-5.4%	-0.5%		-6.0%

Table 9-4. Intra-Laboratory Competitive Assay Results for Associated Runs of the Standard, Weak Positive, and Test Chemicals Linuron and pp'-DDE

	Standard Curve		Positive Control			CR42404 Linuron			CR42405 1,1-Dichloro-2,2-bis(4-chlorophenyl) ethane (pp'-DDE)		
Run	log IC_{50}	R^2	log IC_{50}	R^2	RBA	log IC_{50}	R^2	RBA	log IC_{50}	R^2	RBA
B2-10/20/05	-8.80	0.99	-4.23	0.97	-4.57	-4.04	0.96	-4.76	-4.77	0.94	-4.03
B5-11/03/05	-8.79	0.99	-4.25	0.97	-4.54	-3.95	0.97	-4.83	-4.78	0.87	-4.00
B7-11/09/05	-8.88	0.99	-4.32	0.98	-4.56	-4.11	0.98	-4.77	-4.94	0.90	-3.94
					Mean	-4.03	0.97	-4.79	-4.83	0.91	-3.99
					CV	-1.9%		-0.8%	-2.0%		-1.1%
C2-11/15/05	-8.35	0.30	-4.09	0.96	-4.26	-3.89	0.97	-4.46			
C3-11/28/05	-8.29	0.97	-3.96	0.96	-4.32	-3.95	0.98	-4.33			
C4-11/29/05	-8.18	0.85	-3.88	0.67	-4.30	-4.30	0.15	-3.88			
C5-11/16/05	-8.68	0.99	-4.16	0.98	-4.52				-4.95	0.96	-3.72
C7-11/22/05	-8.54	0.99	-4.15	0.96	-4.40				-4.74	0.70	-3.81
C8-12/19/05	-8.74	0.98	-4.16	0.95	-4.58				-4.84	0.93	-3.89
					Mean	-4.05	0.70	-4.22	-4.84	0.86	-3.81
					CV	-5.5%		-7.2%	-2.3%		-2.2%
D4-11/22/05	-8.34	0.93	-4.21	0.75	-4.13	-4.01	0.78	-4.33	-5.27	0.95	-3.07
D5-11/29/05	-8.53	0.96	-3.97	0.08	-4.56	**Did not Converge**			-5.03	0.95	-3.50
D6-12/06/05	-8.46	0.97	-3.02	0.02	-5.44	-3.69	0.58	-4.77	-4.74	0.88	-3.72
					Mean	-3.85	0.68	-4.55	-5.02	0.93	-3.43
					CV	-5.8%		-6.9%	-5.3%		-9.6%
E517-11/08/05	-8.82	0.98	-4.53	0.96	-4.29	-4.13	0.97	-4.69	-4.81	0.99	-4.01
E518-11/10/05	-8.75	0.99	-4.55	0.96	-4.21	-4.02	0.94	-4.73	-4.81	0.97	-3.95
E519-11/17/05	-8.84	1.00	-4.36	0.99	-4.47	-3.99	0.87	-4.84	-4.74	0.99	-4.10
					Mean	-4.05	0.93	-4.75	-4.78	0.98	-4.02
					CV	-1.8%		-1.7%	-0.9%		-1.9%

Table 9-5. Intra-Laboratory Competitive Assay Results for Associated Runs of the Standard, Weak Positive, and Test Chemicals Cyproterone Acetate and Spironolactone

	Standard Curve		Positive Control			CR42406 Cyproterone Acetate			CR42407 Spironolactone		
Run	log IC_{50}	R^2	log IC_{50}	R^2	RBA	log IC_{50}	R^2	RBA	log IC_{50}	R^2	RBA
B3-10/24/05	-8.79	0.99	-4.22	0.94	-4.57	-6.84	1.00	-1.95	-6.55	0.99	-2.24
B5-11/03/05	-8.79	0.99	-4.25	0.97	-4.54	-6.79	0.99	-2.00	-6.51	0.99	-2.27
B8-11/14/05	-8.95	0.99	-4.25	0.96	-4.70	-6.88	0.99	-2.07	-6.50	0.98	-2.45
					Mean	-6.83	0.99	-2.01	-6.52	0.99	-2.32
					CV	-0.7%		-3.1%	-0.4%		-4.9%
C5-11/16/05	-8.68	0.99	-4.16	0.98	-4.52	-6.67	0.99	-2.00	-6.44	1.00	-2.23
C7-11/22/05	-8.54	0.99	-4.15	0.96	-4.40	-6.78	0.99	-1.77	-6.53	0.99	-2.01
C8-12/19/05	-8.74	0.98	-4.16	0.95	-4.58	-6.51	0.98	-2.23	-6.33	0.99	-2.41
					Mean	-6.65	0.99	-2.00	-6.44	0.99	-2.22
					CV	-2.0%		-11.6%	-1.6%		-9.0%
D7-12/08/05	-8.35	0.89	-4.09	0.32	-4.26	-6.27	0.95	-2.08	-6.08	0.79	-2.28
D8-12/08/05	-8.51	0.95	-4.21	0.84	-4.30	-6.14	0.95	-2.37	-6.09	0.87	-2.42
D9-12/15/05	-8.68	0.97	-4.27	0.82	-4.41	-6.31	0.32	-2.37	-6.07	0.87	-2.60
					Mean	-6.24	0.74	-2.27	-6.08	0.84	-2.43
					CV	-1.4%		-7.3%	-0.1%		-6.7%
E520-11/22/05	-8.75	1.00	-4.40	0.93	-4.35	-6.88	1.00	-1.88	-6.63	0.99	-2.12
E522-12/08/05	-8.72	1.00	-4.55	0.94	-4.17	-7.05	0.99	-1.68	-6.64	0.98	-2.08
E523-12/20/05	-8.77	1.00	-4.47	0.96	-4.30	-6.92	0.99	-1.85	-6.60	1.00	-2.17
					Mean	-6.95	0.99	-1.80	-6.62	0.99	-2.13
					CV	-1.3%		-6.1%	-0.3%		-2.1%

Table 9-6. Intra-Laboratory Competitive Assay Results for Associated Runs of the Standard, Weak Positive, and Test Chemicals Bis(2-ethylhexyl) phthalate and Atrazine

Run	Standard Curve		Positive Control			CR42408 Bis(2-ethylhexyl) phthalate (DEPH)			CR42409 Atrazine		
	log IC_{50}	R^2	log IC_{50}	R^2	RBA	log IC_{50}	R^2	RBA	log IC_{50}	R^2	RBA
B3-10/24/05	-8.79	0.99	-4.22	0.94	-4.57	**Did not Converge**			-2.05	0.33	-6.74
B6-11/07/05	-8.81	1.00	-4.24	0.99	-4.58	**Did not Converge**			-2.05	0.20	-6.76
B8-11/14/05	-8.95	0.99	-4.25	0.96	-4.70	**Did not Converge**			**Did not Converge**		
					Mean	**Did not Converge**			-2.05	0.27	-6.75
					CV	NA		NA	0.0%		-0.2%
C5-11/16/05	-8.68	0.99	-4.16	0.98	-4.52	-1.58	0.03	-7.10	-2.25	0.41	-6.42
C7-11/22/05	-8.54	0.99	-4.15	0.96	-4.40	**Did not Converge**			**Did not Converge**		
C8-12/19/05	-8.74	0.98	-4.16	0.95	-4.58	**Did not Converge**			**Did not Converge**		
					Mean	-1.58	0.03	-7.10	-2.25	0.41	-6.42
					CV	NA		NA	NA		NA
D7-12/08/05	-8.35	0.89	-4.09	0.32	-4.26	-2.80	0.27	-5.55	-2.91	0.51	-5.45
D8-12/08/05	-8.51	0.95	-4.21	0.84	-4.30	-2.28	0.04	-6.23	**Did not Converge**		
D9-12/15/05	-8.68	0.97	-4.27	0.82	-4.41	**Did not Converge**			**Did not Converge**		
					Mean	-2.54	0.16	-5.89	-2.91	0.51	-5.45
					CV	-14.7%		-8.2%	NA		NA
E520-11/22/05	-8.75	1.00	-4.40	0.93	-4.35	-2.11	0.52	-6.65	-2.33	0.44	-6.42
E522-12/08/05	-8.72	1.00	-4.55	0.94	-4.17	**Did not Converge**			7.90E-03	0.33	-2.10
E523-12/20/05	-8.77	1.00	-4.47	0.96	-4.30	-1.84	0.34	-6.94	-2.21	0.86	-6.56
					Mean	-1.97	0.43	-6.79	-2.21	0.54	-6.53
					CV	-9.7%		-3.0%	-5.2%		-1.6%

9.2 Data Analysis Using the Four Parameter Model

Following the preliminary analysis of the data, the four parameter AR competitive binding assay model was applied (see Chapter 5 for model description). The re-fits of the R1881 standard, the weak positive dexamethasone and the test chemicals are shown in Table 9-7. For the model parameters $\log_{10}IC_{50}$, and hill slope, and for the $\log_{10}RBA$ ($\log_{10}IC_{50,\ standard} - \log_{10}IC_{50,\ test}$), the parameter estimate and the associated within laboratory standard error and degrees of freedom are shown for each laboratory. Additionally, the data are shown graphically in Figures 9-6 to 9.16.

The majority of the fitted IC_{50}s are not greatly different between the one site model and the 2 and 4 parameter models; however, some of the model fits require additional discussion:

- Run 5 (Seq#6953) for Lab C (Figure 9-6b) shows very high specific binding at the lowest concentrations, with the Top fit of 140.3 and 133.3 for the standard and weak positive respectively. Additionally, the top values for test compounds run during this assay run are high (e.g. dihydrotestosterone in Figure 9-7). The log IC_{50}s are slightly higher for these runs.
- The standard curves and weak positive for Lab D (Figure 9-6c) demonstrate some of the limitations of the four parameter model. For dexamethasone run 120805, the log IC_{50} value (-4.648) is only slightly higher than the log IC_{50} for the whole lab D analysis (-4.340); however, the slope of this fit is -8.045 indicating that the quality of the curve is poor. The slope parameter should be -1 for ideal data, indicating that this run should be discarded. Similar slopes are noted for several of the Lab D weak positive runs in Figure 9-6c (e.g., runs 11042005, 11292005 and 121205).
- Many of the test runs converged and had better fits after excluding values at the highest test concentrations. In some cases the laboratories made notations that indicated solubility limitations (precipitation). Even in cases where solubility was not limiting, it is possible that such high chemical concentrations can cause physical or biological changes to the system which are not directly related to the binding activity of the AR.
- For the standard curve and for the weak positive binder Laboratories C and D display relatively large variation among studies for the log IC_{50}, the slope, and the log RBA parameters.
- DEHP and atrazine (Figures 9-15 and 9-16) were found to be non binders in Prism. The SAS model was able to obtain parameters for some of the runs but the 95% confidence intervals were unrealistically large and the estimates are considered unreliable (SAS, 2003).

Table 9-7. Four Parameter Fits for Inter-Laboratory Validation

Lab Code	$log_{10}IC_{50}$ (log M)			β slope			$log_{10}RBA$		
	Estimate (95% CI)	Degree of Freedom	Standard Error	Estimate (95% CI)	Degree of Freedom	Standard Error	Estimate (95% CI)	Degree of Freedom	Standard Error
Standard Curve – R1881									
B	-8.853(-8.892,-8.815)	6.40	0.016	-0.961(-1.043,-0.879)	15.05	0.039			
C	-8.539(-8.705,-8.373)	17.00	0.079	-1.041(-1.191,-0.891)	17.01	0.071			
D	-8.490(-8.580,-8.401)	17.00	0.042	-0.799(-0.907,-0.691)	17.00	0.051			
E	-8.784(-8.824,-8.743)	7.01	0.017	-1.000(-1.032,-0.968)	61.07	0.016			
Average	-8.775(-8.846,-8.704)	7.68	0.031	-0.999(-1.031,-0.966)	24.03	0.016			
Weak Positive - Dexamethasone									
B	-4.251(-4.288,-4.215)	107.26	0.018	-0.956(-1.052,-0.860)	113.02	0.048	-4.602(-4.652,-4.552)	30.83	0.024
C	-4.091(-4.140,-4.043)	36.36	0.024	-0.891(-1.247,-0.535)	7.13	0.151	-4.448(-4.619,-4.276)	20.16	0.082
D	-4.340(-4.465,-4.215)	33.05	0.062	-1.134(-1.469,-0.798)	29.82	0.164	-4.150(-4.300,-4.000)	49.97	0.075
E	-4.439(-4.493,-4.384)	8.15	0.024	-1.006(-1.085,-0.927)	82.98	0.040	-4.345(-4.407,-4.283)	14.33	0.029
Average	-4.352(-4.438,-4.266)	16.21	0.041	-0.943(-0.965,-0.920)	83.58	0.011	-4.386(-4.539,-4.233)	8.62	0.067
CR42400 Dihydrotestosterone									
B	-8.967(-8.990,-8.944)	34.157	0.011	-0.963(-1.041,-0.884)	28.777	0.038	0.114(0.071,0.156)	13.752	0.020
C	-8.564(-8.748,-8.381)	2.9606	0.057	-1.007(-1.099,-0.915)	49.555	0.046	0.025(-0.182,0.232)	15.253	0.097
D	-8.358(-8.630,-8.086)	2.2278	0.070	-0.698(-0.873,-0.524)	35.874	0.086	-0.132(-0.356,0.092)	4.1062	0.082
E	-8.688(-8.788,-8.588)	2.7029	0.029	-1.054(-1.117,-0.991)	34.751	0.031	-0.096(-0.185,-0.006)	4.6248	0.034
Average	-8.652(-8.958,-8.347)	4.0181	0.110	-0.952(-1.143,-0.762)	2.7193	0.056	-0.013(-0.148,0.121)	5.9245	0.055
CR42401 Testosterone									
B	-8.133(-8.175,-8.092)	4.4275	0.016	-0.945(-1.019,-0.872)	42.592	0.037	-0.720(-0.770,-0.670)	10.566	0.022
C	-7.776(-7.991,-7.560)	2.8088	0.065	-0.956(-1.122,-0.790)	3.7976	0.058	-0.764(-0.985,-0.542)	12.588	0.102
D	-7.722(-7.788,-7.656)	21.735	0.032	-0.824(-0.950,-0.698)	19.295	0.060	-0.769(-0.876,-0.661)	33.321	0.053
E	-8.087(-8.169,-8.005)	3.3701	0.027	-0.982(-1.016,-0.948)	19.567	0.016	-0.697(-0.775,-0.618)	6.0684	0.032
Average	-7.934(-8.188,-7.681)	4.0709	0.092	-0.954(-1.011,-0.897)	4.0511	0.021	-0.720(-0.755,-0.684)	24.063	0.017

Lab Code	$log_{10}IC_{50}$ (log M)			β slope			$log_{10}RBA$		
	Estimate (95% CI)	Degree of Freedom	Standard Error	Estimate (95% CI)	Degree of Freedom	Standard Error	Estimate (95% CI)	Degree of Freedom	Standard Error
CR42402 17β-estradiol									
B	-6.655(-6.773,-6.536)	3.4907	0.040	-1.072(-1.169,-0.975)	36.496	0.048	-2.199(-2.313,-2.084)	4.6258	0.043
C	-6.384(-6.424,-6.344)	40.375	0.020	-0.949(-1.126,-0.772)	2.3984	0.048	-2.155(-2.325,-1.986)	19.181	0.081
D	-6.212(-7.019,-5.404)	3.1338	0.260	-0.698(-0.932,-0.465)	32.799	0.115	-2.279(-3.075,-1.483)	3.302	0.263
E	-6.496(-6.614,-6.377)	2.9562	0.037	-1.015(-1.102,-0.927)	4.0903	0.032	-2.288(-2.398,-2.178)	4.2791	0.041
Average	-6.492(-6.647,-6.338)	5.899	0.063	-0.984(-1.177,-0.791)	1.709	0.038	-2.235(-2.315,-2.154)	4.377	0.030
CR42403 MPA									
B	-7.786(-7.892,-7.679)	3.0502	0.034	-0.868(-0.959,-0.777)	45.78	0.045	-1.068(-1.168,-0.968)	4.4745	0.037
C	-7.436(-7.498,-7.374)	20.227	0.030	-0.931(-1.037,-0.824)	20.001	0.051	-1.103(-1.277,-0.928)	21.811	0.084
D	-6.754(-6.935,-6.573)	3.6466	0.063	-0.983(-1.333,-0.634)	6.5953	0.146	-1.736(-1.913,-1.559)	7.4005	0.076
E	-7.776(-7.794,-7.759)	30.814	0.009	-0.925(-0.963,-0.886)	28.413	0.019	-1.007(-1.050,-0.965)	10.979	0.019
Average	-7.441(-8.022,-6.860)	3.9397	0.208	-0.919(-0.955,-0.882)	9.9917	0.016	-1.223(-1.631,-0.815)	3.9027	0.146
CR42404 Linuron									
B	-4.092(-4.190,-3.993)	3.4388	0.033	-1.043(-1.209,-0.877)	49.269	0.082	-4.762(-4.857,-4.667)	5.0741	0.037
C	-3.983(-4.042,-3.924)	20	0.028	-1.097(-1.375,-0.818)	20	0.133	-4.556(-4.729,-4.382)	21.358	0.084
D	-4.005(-4.254,-3.756)	18.077	0.118	-0.395(-1.453,0.663)	20.217	0.508	-4.485(-4.745,-4.225)	22.611	0.126
E	-4.065(-4.124,-4.006)	16	0.028	-0.620(-0.717,-0.524)	16	0.045	-4.719(-4.787,-4.651)	22.891	0.033
Average	-4.043(-4.097,-3.988)	14.269	0.025	-0.871(-1.138,-0.604)	13.511	0.124	-4.688(-4.822,-4.555)	2.7779	0.040
CR42405 pp'-DDE									
B	-4.868(-4.949,-4.787)	48.026	0.040	-0.719(-0.833,-0.605)	52.504	0.057	-3.985(-4.072,-3.898)	54.227	0.043
C	-4.998(-5.079,-4.916)	29.379	0.040	-1.216(-1.596,-0.835)	28.941	0.186	-3.542(-3.723,-3.360)	25.957	0.088
D	-5.086(-5.454,-4.718)	3.1235	0.118	-1.016(-1.204,-0.828)	43.691	0.093	-3.404(-3.754,-3.054)	3.9644	0.126
E	-4.786(-4.823,-4.749)	32.079	0.018	-0.835(-1.009,-0.662)	3.7825	0.061	-3.998(-4.050,-3.947)	24.925	0.025
Average	-4.905(-5.041,-4.768)	4.7491	0.052	-0.886(-1.089,-0.684)	4.35	0.075	-3.754(-4.117,-3.391)	3.8798	0.129
CR42406 Cyproterone acetate									

Lab Code	$log_{10}IC_{50}$ (log M)			β slope			$log_{10}RBA$		
	Estimate (95% CI)	Degree of Freedom	Standard Error	Estimate (95% CI)	Degree of Freedom	Standard Error	Estimate (95% CI)	Degree of Freedom	Standard Error
B	-6.843(-6.893,-6.794)	4.2608	0.018	-0.828(-0.975,-0.681)	3.5674	0.050	-2.010(-2.065,-1.955)	9.5699	0.024
C	-6.673(-6.842,-6.503)	2.657	0.049	-0.879(-0.963,-0.794)	48.492	0.042	-1.867(-2.063,-1.670)	16.597	0.093
D	-6.199(-6.280,-6.117)	39.314	0.040	-1.069(-1.330,-0.809)	36.002	0.128	-2.291(-2.409,-2.174)	45.534	0.058
E	-6.936(-7.075,-6.797)	2.0638	0.033	-0.861(-0.903,-0.819)	28.93	0.021	-1.848(-1.962,-1.734)	3.2283	0.037
Average	-6.664(-7.058,-6.270)	4.0002	0.142	-0.864(-0.899,-0.829)	47.731	0.017	-2.006(-2.250,-1.763)	3.9786	0.088
CR42407 Spironolactone									
B	-6.530(-6.609,-6.451)	3.3461	0.026	-0.925(-1.009,-0.841)	59.891	0.042	-2.324(-2.399,-2.248)	5.8961	0.031
C	-6.446(-6.578,-6.314)	2.9945	0.041	-0.994(-1.169,-0.819)	3.0143	0.055	-2.093(-2.279,-1.907)	19.304	0.089
D	-6.265(-6.375,-6.155)	29.4	0.054	-1.254(-1.537,-0.970)	33.028	0.139	-2.225(-2.363,-2.087)	46.358	0.069
E	-6.606(-6.629,-6.583)	42.075	0.011	-0.937(-0.982,-0.892)	41.49	0.022	-2.178(-2.222,-2.134)	14.087	0.020
Average	-6.470(-6.645,-6.296)	3.79	0.061	-0.946(-0.983,-0.909)	42.009	0.018	-2.222(-2.320,-2.124)	6.2334	0.040

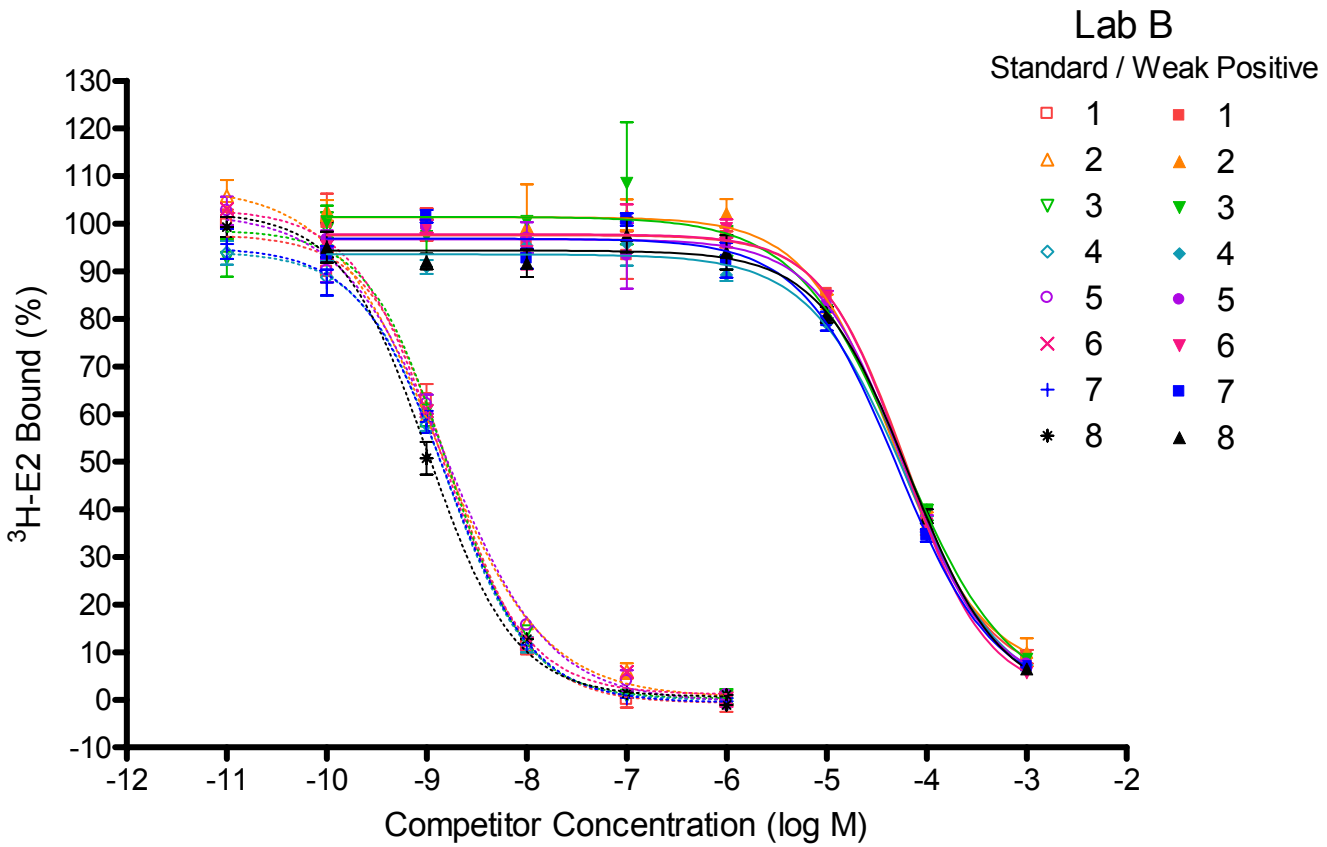


Figure 9-6a. Lab B - Weak Positive – Dexamethasone with corresponding standard curve runs in the 4-parameter model. Note that individual runs are represented as means and SEM. Standard curves are dotted lines and open symbols, test compound is solid lines and closed symbols.

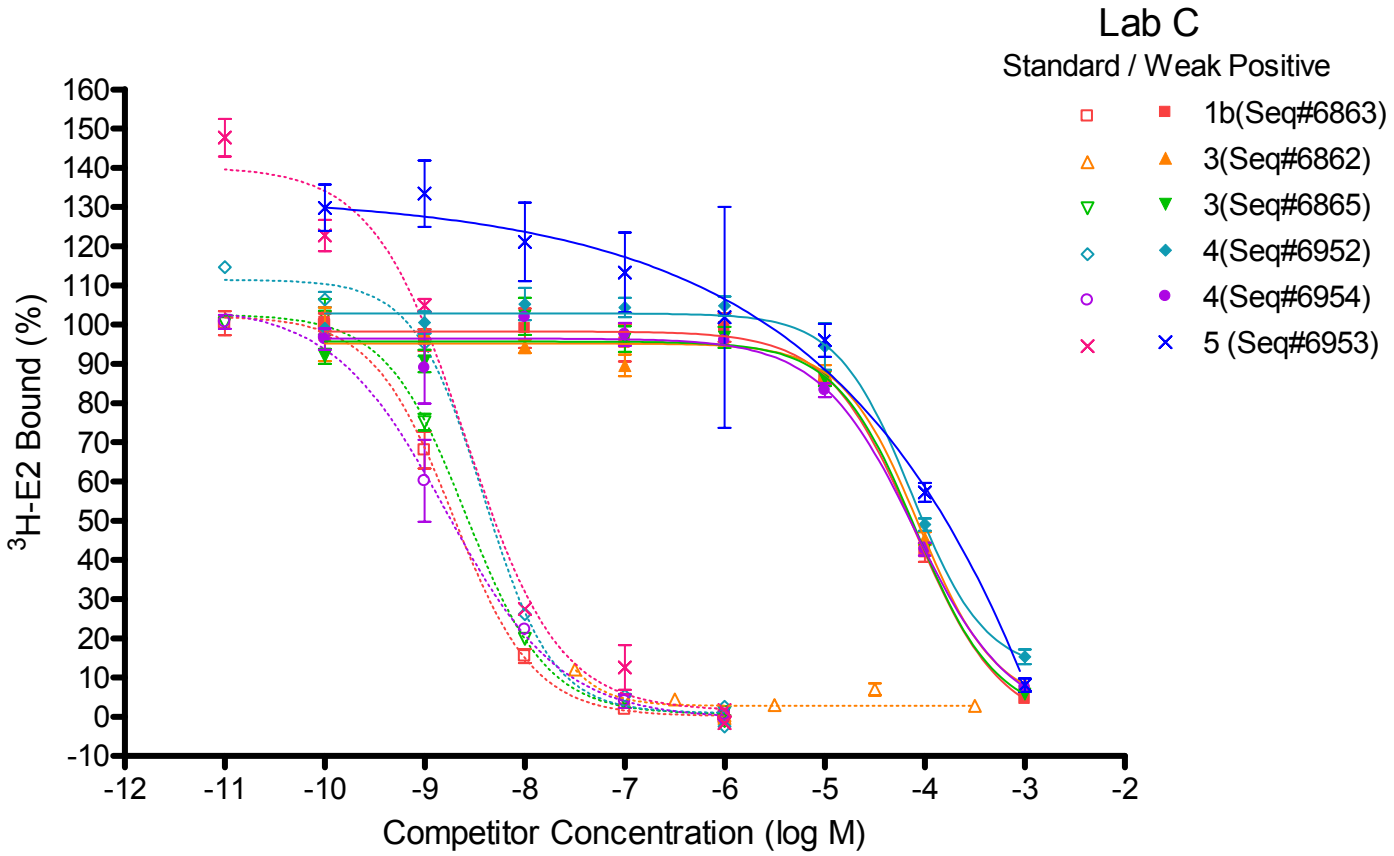


Figure 9-6b. Lab C - Weak Positive - Dexamethasone with corresponding standard curve runs in the 4-parameter model. Note that individual runs are represented as means and SEM. Standard curves are dotted lines and open symbols, test compound is solid lines and closed symbols.

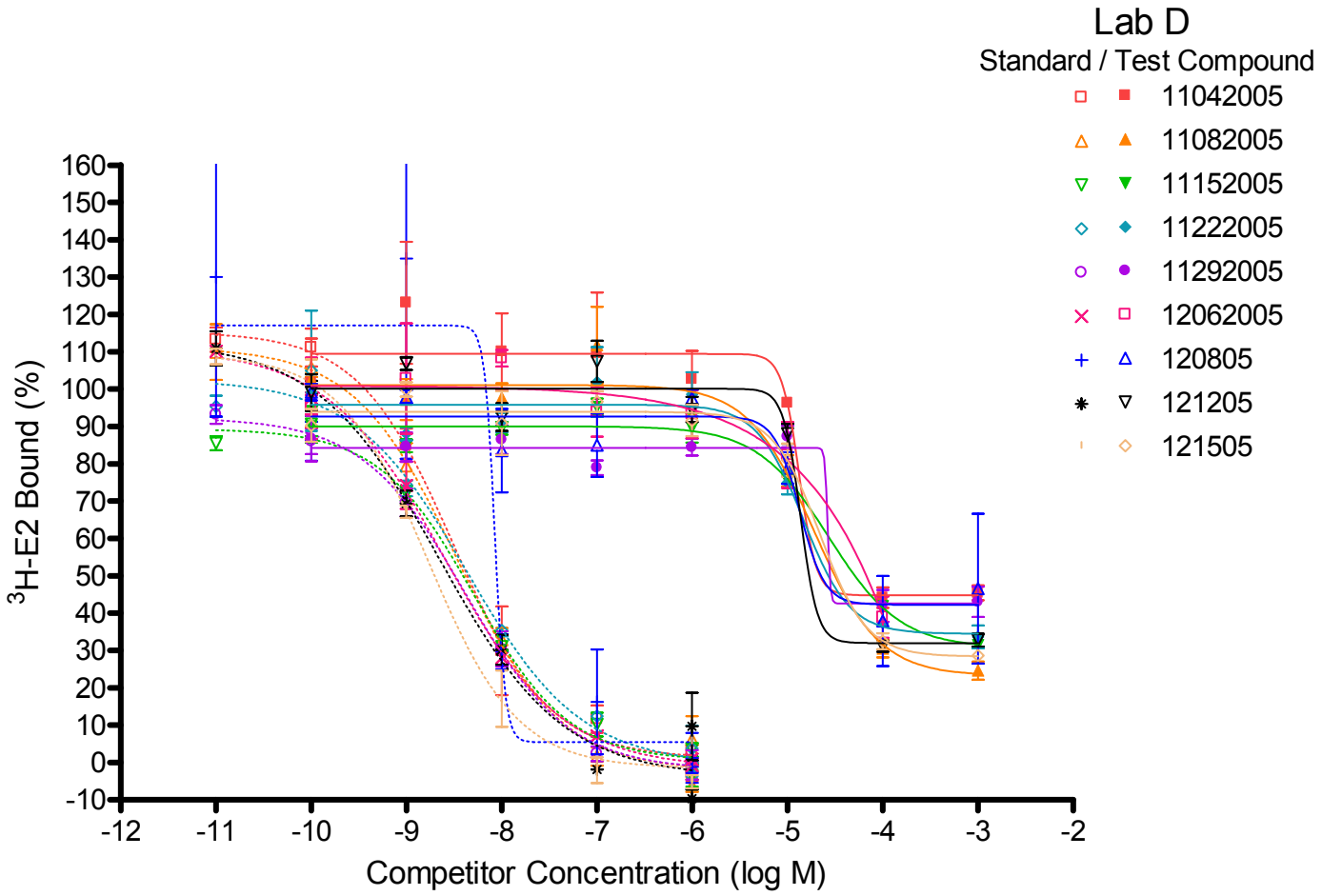


Figure 9-6c. **Lab D - Weak Positive - Dexamethasone with corresponding standard curve runs in the 4-parameter model. Note that individual runs are represented as means and SEM. Standard curves are dotted lines and open symbols, test compound is solid lines and closed symbols.**

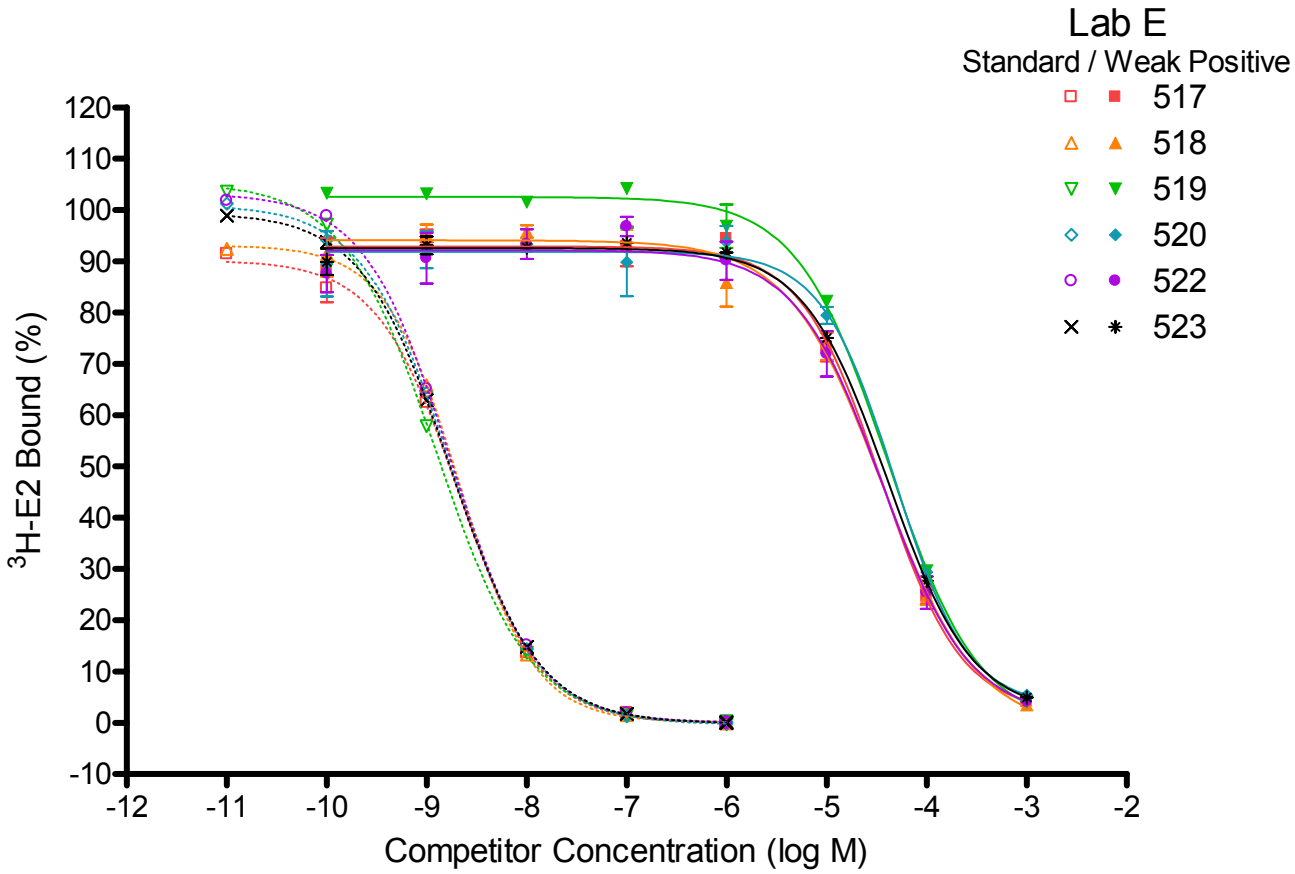


Figure 9-6d. **Lab E - Weak Positive - Dexamethasone with corresponding standard curve runs in the 4-parameter model. Note that individual runs are represented as means and SEM. Standard curves are dotted lines and open symbols, test compound is solid lines and closed symbols.**

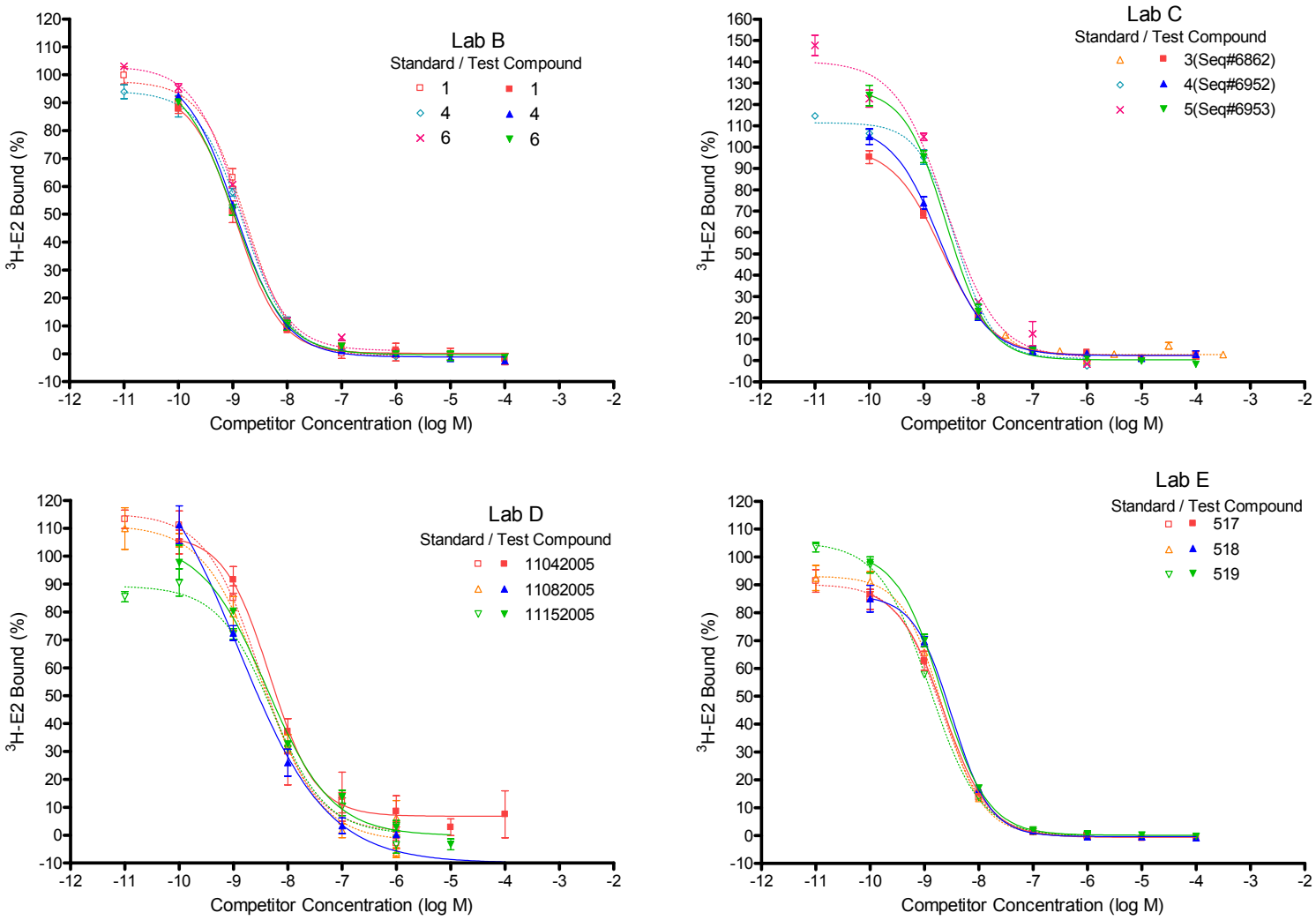


Figure 9-7. CR42400 Dihydrotestosterone with corresponding standard curve runs. Note that individual runs are represented as means and SEM. Standard curves are dotted lines and open symbols, test compound is solid lines and closed symbols.

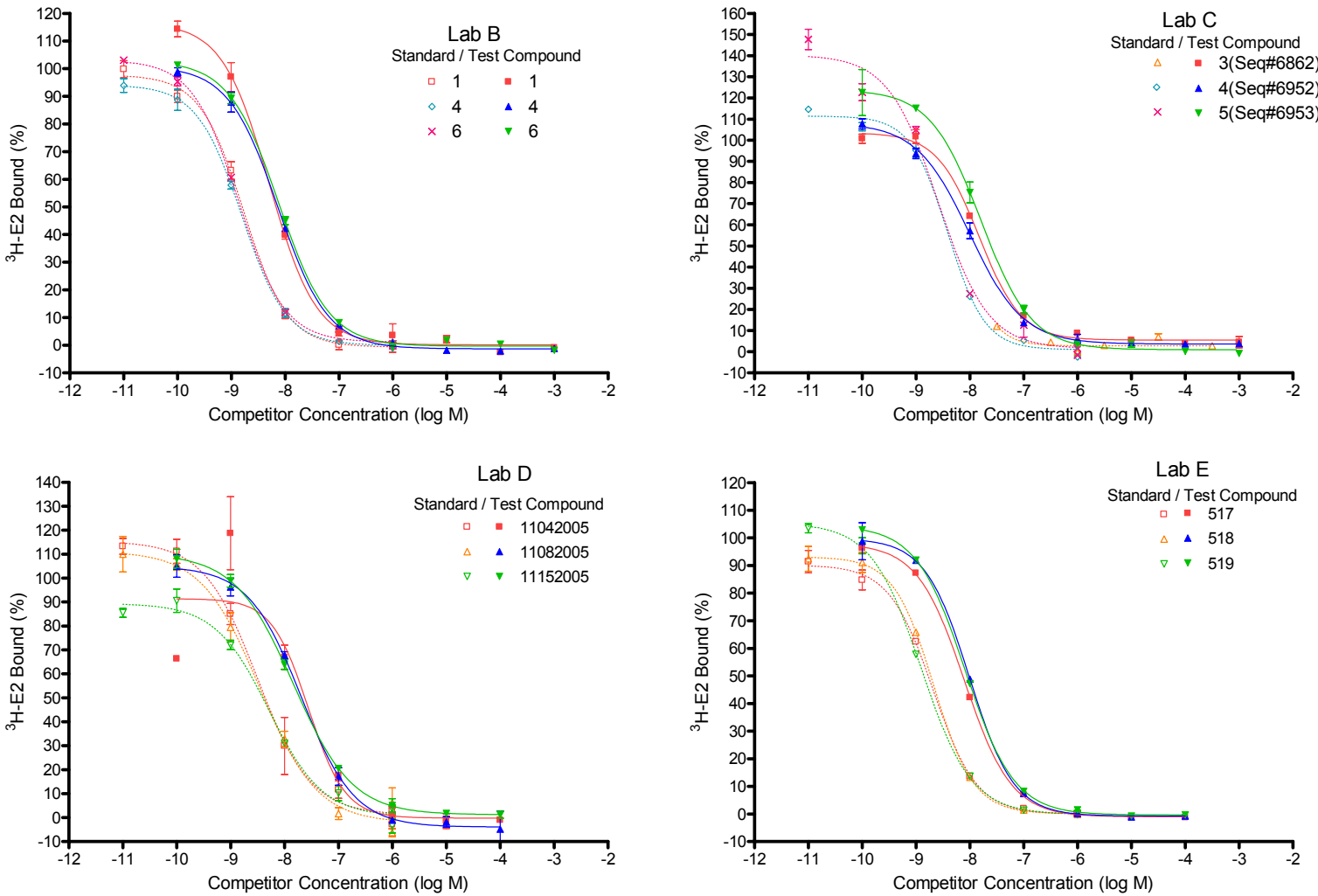


Figure 9-8. CR42401 Testosterone with corresponding standard curve runs. Note that individual runs are represented as means and SEM. Standard curves are dotted lines and open symbols, test compound is solid lines and closed symbols.

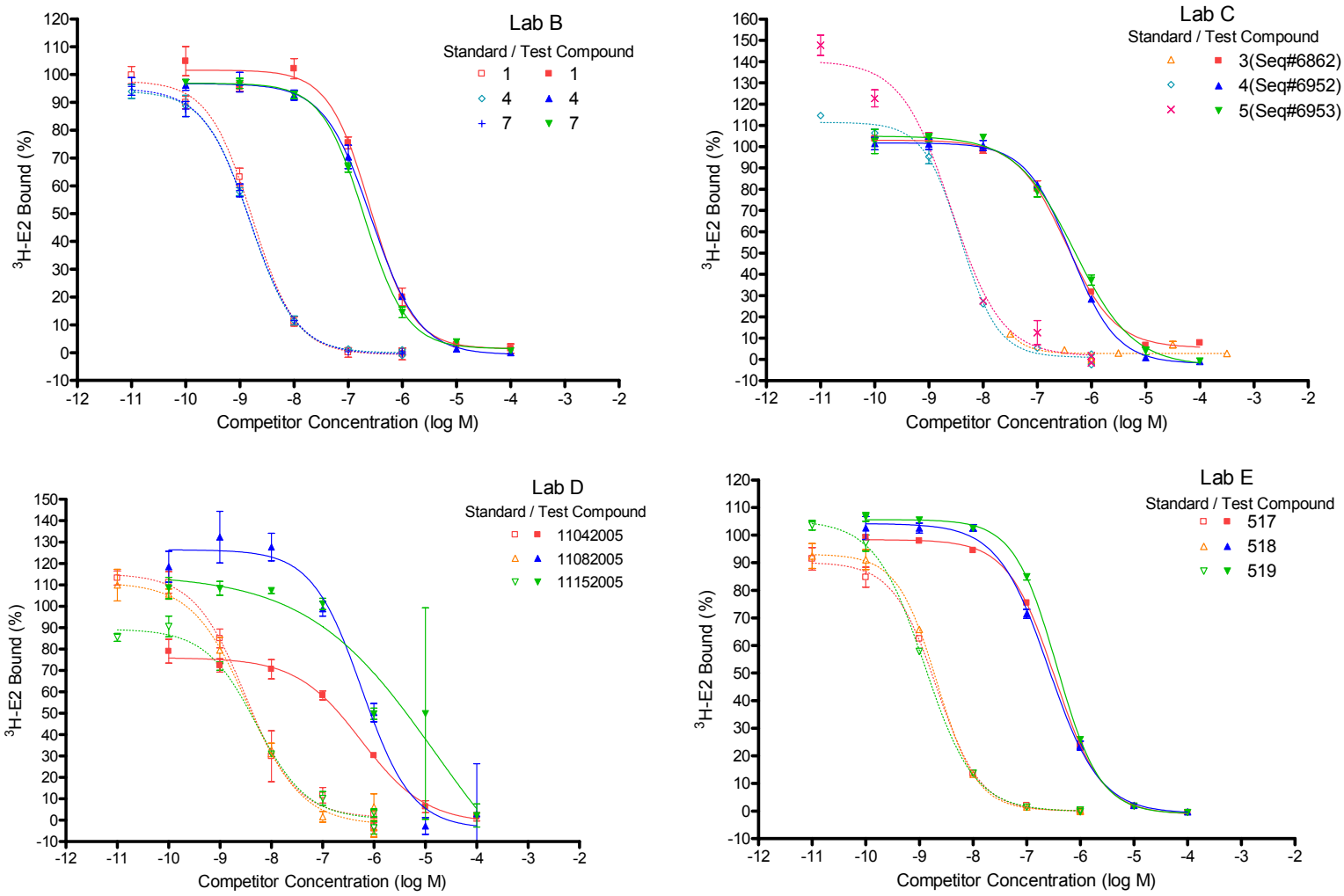


Figure 9-9 CR42402 17ß-estradiol with corresponding standard curve runs. Note that individual runs are represented as means and SEM. Standard curves are dotted lines and open symbols, test compound is solid lines and closed symbols.

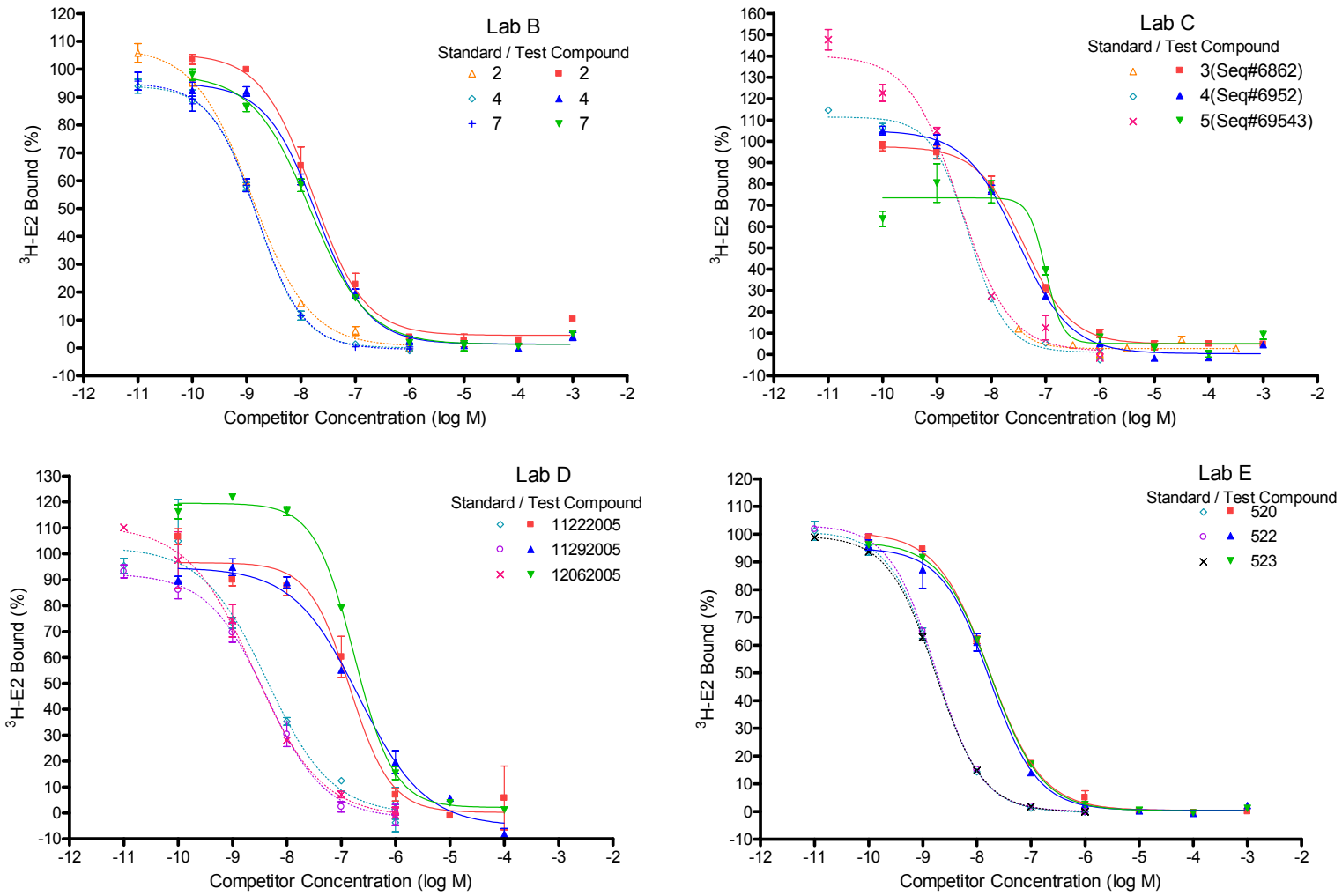


Figure 9.10 CR42403 MPA with corresponding standard curve runs. Note that individual runs are represented as means and SEM. Standard curves are dotted lines and open symbols, test compound is solid lines and closed symbols.

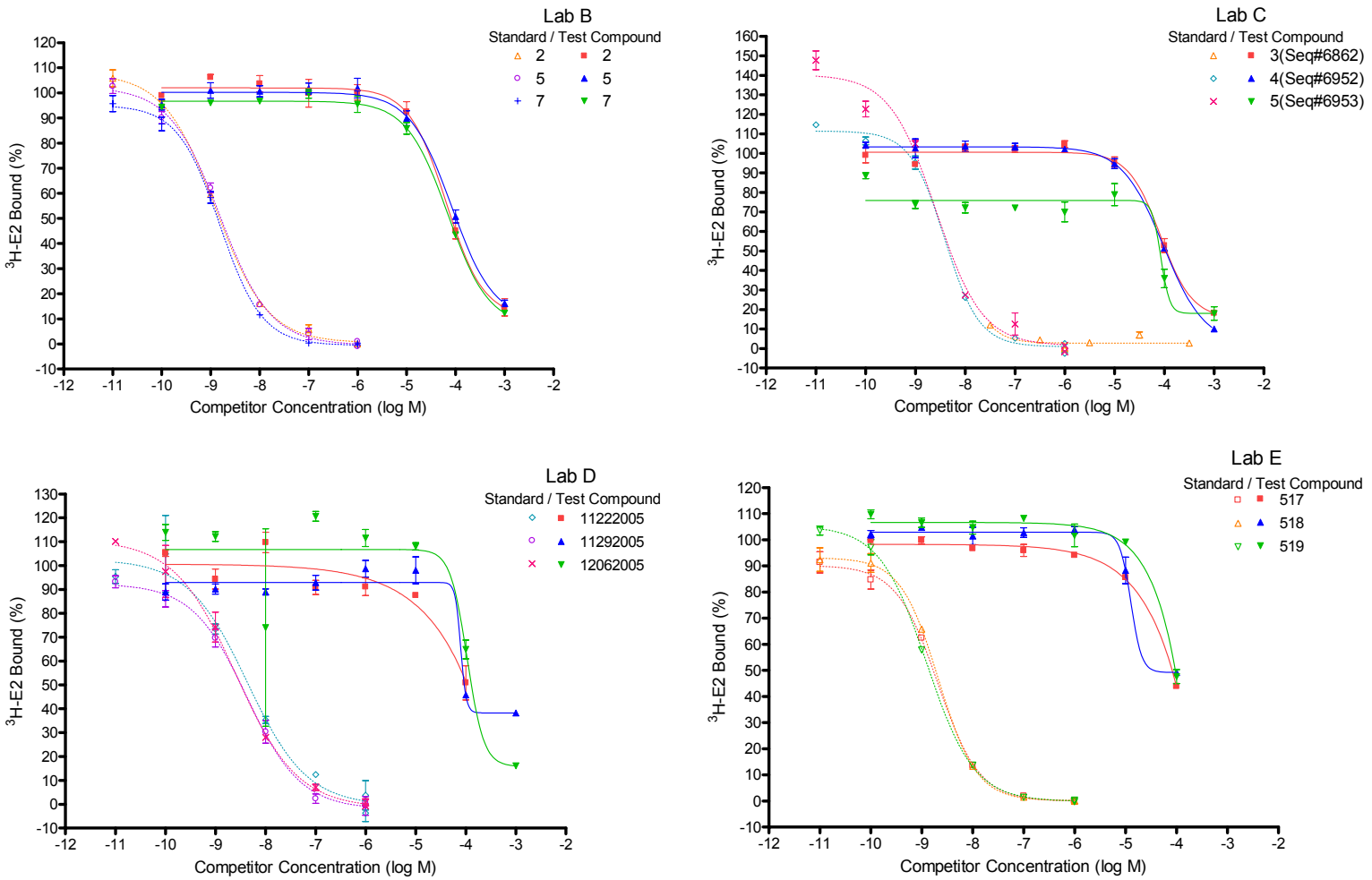


Figure 9-11. CR42404 Linuron with corresponding standard curve runs. Note that individual runs are represented as means and SEM. Standard curves are dotted lines and open symbols, test compound is solid lines and closed symbols.

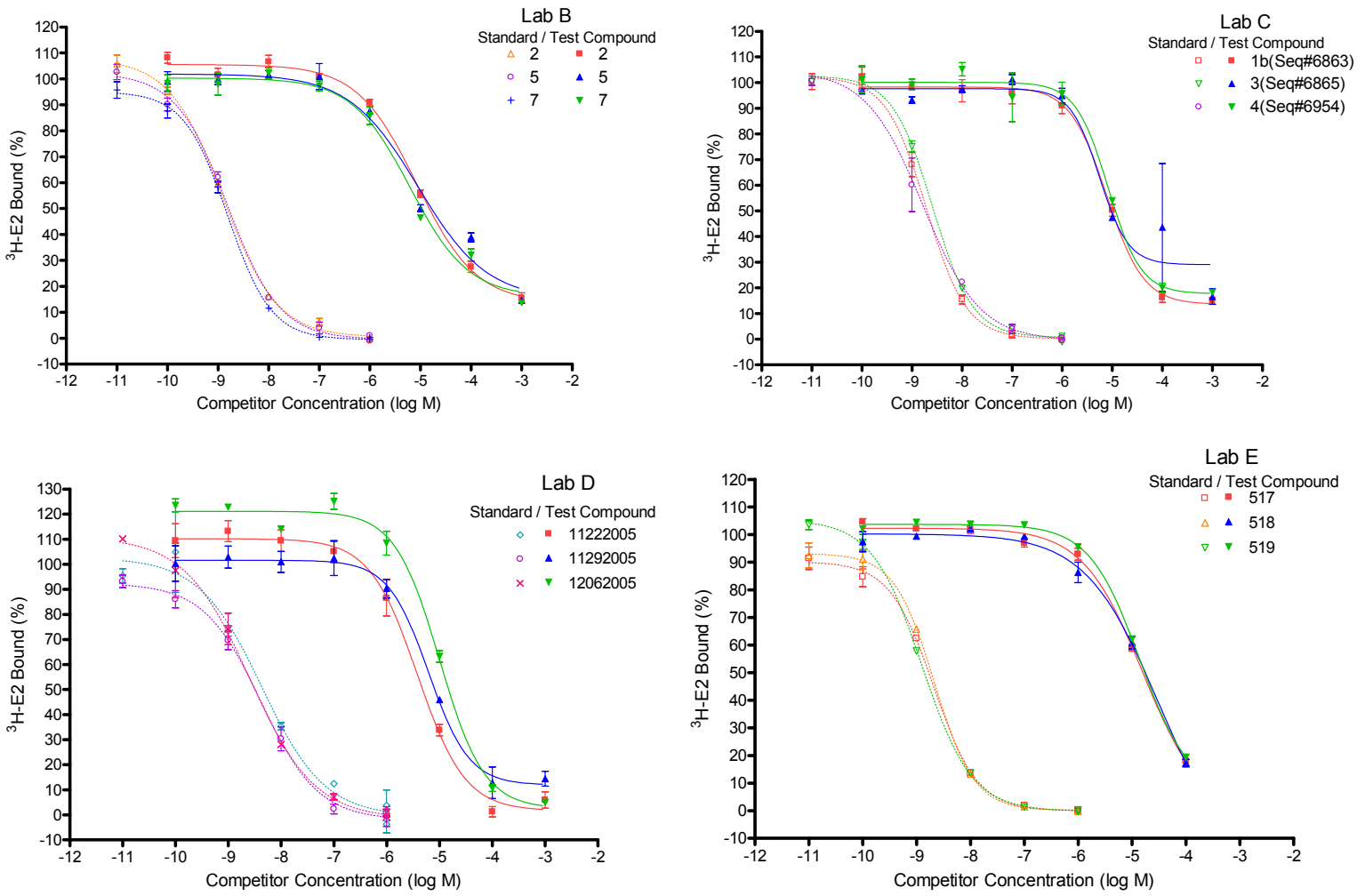


Figure 9-12. CR42405 pp'-DDE with corresponding standard curve runs. Note that individual runs are represented as means and SEM. Standard curves are dotted lines and open symbols, test compound is solid lines and closed symbols.

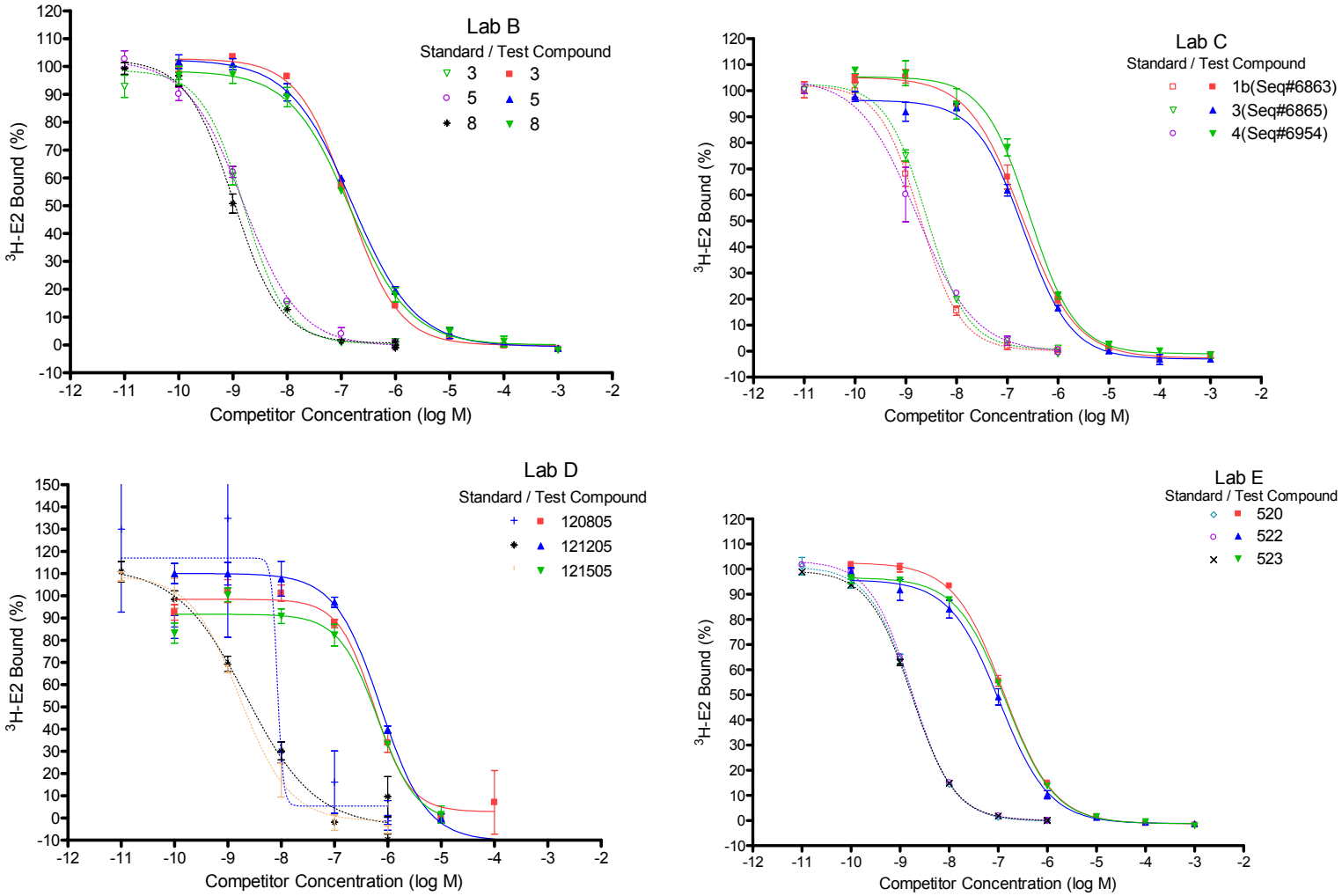


Figure 9-13. CR42406 Cyproterone acetate with corresponding standard curve runs. Note that individual runs are represented as means and SEM. Standard curves are dotted lines and open symbols, test compound is solid lines and closed symbols.

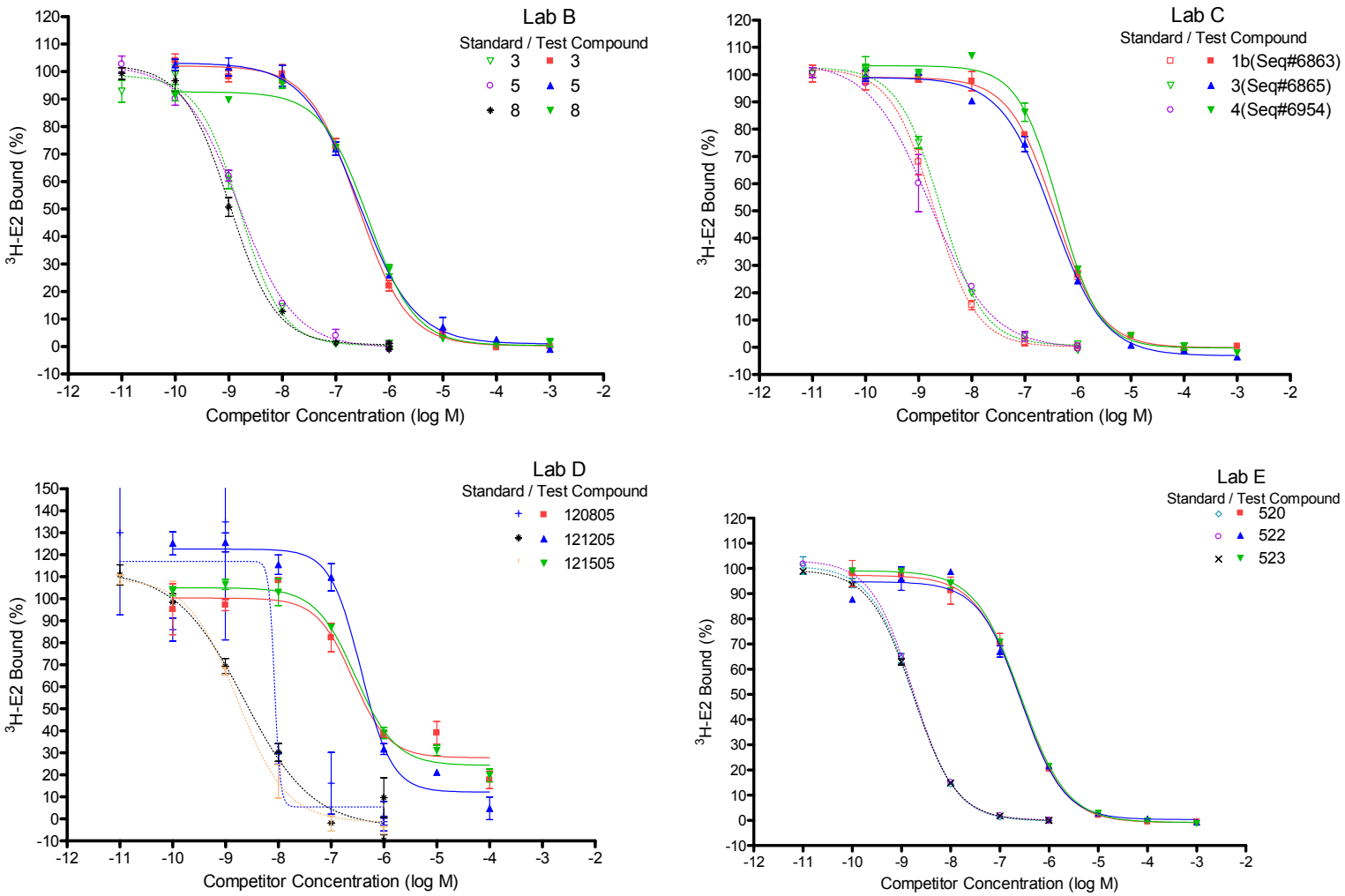


Figure 9-14. CR42407 Spironolactone with corresponding standard curve runs. Note that individual runs are represented as means and SEM. Standard curves are dotted lines and open symbols, test compound is solid lines and closed symbols.

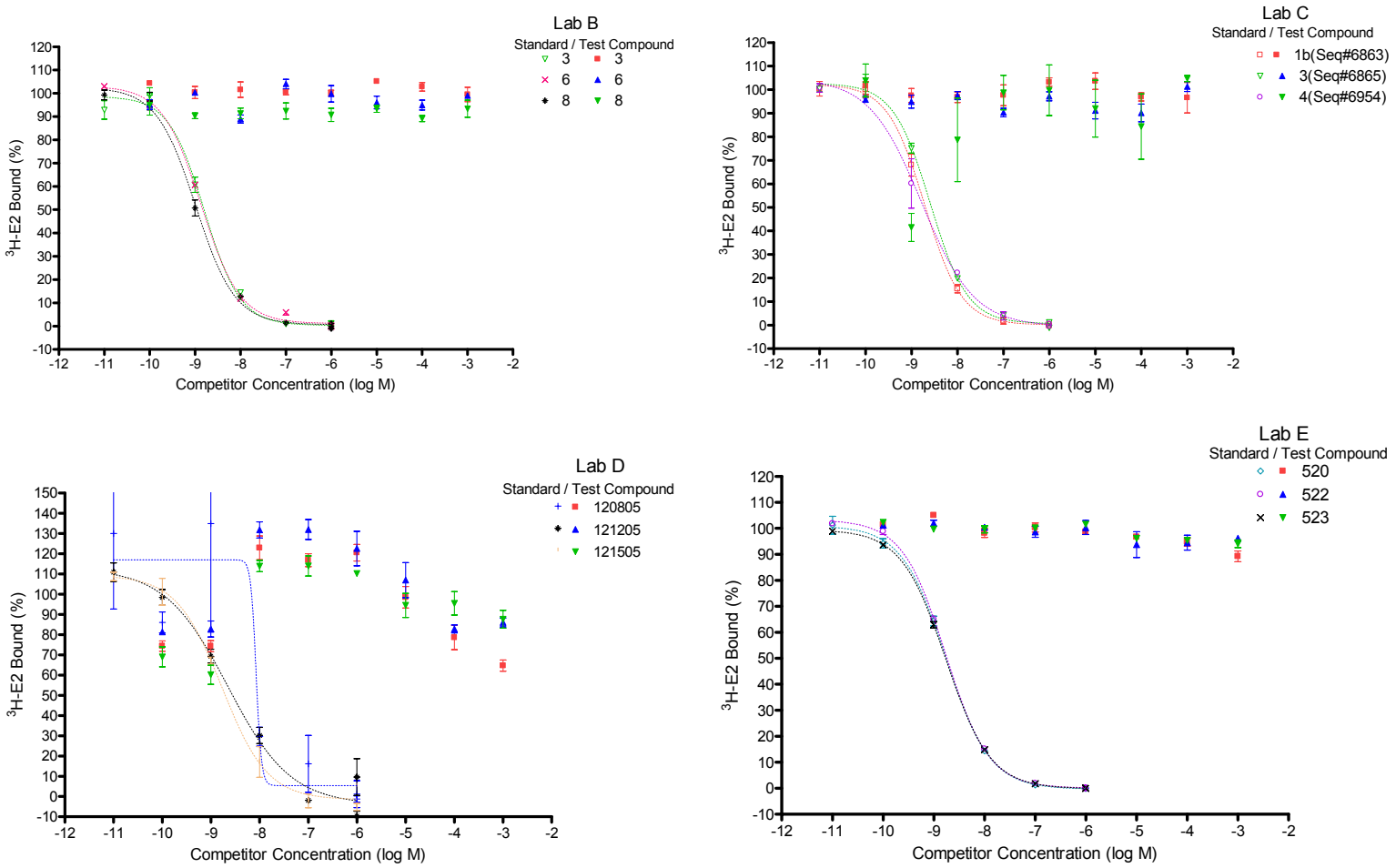


Figure 9-15. CR42408 DEHP with corresponding standard curve runs. Note that individual runs are represented as means and SEM. Standard curves are dotted lines and open symbols, test compound is solid lines and closed symbols.

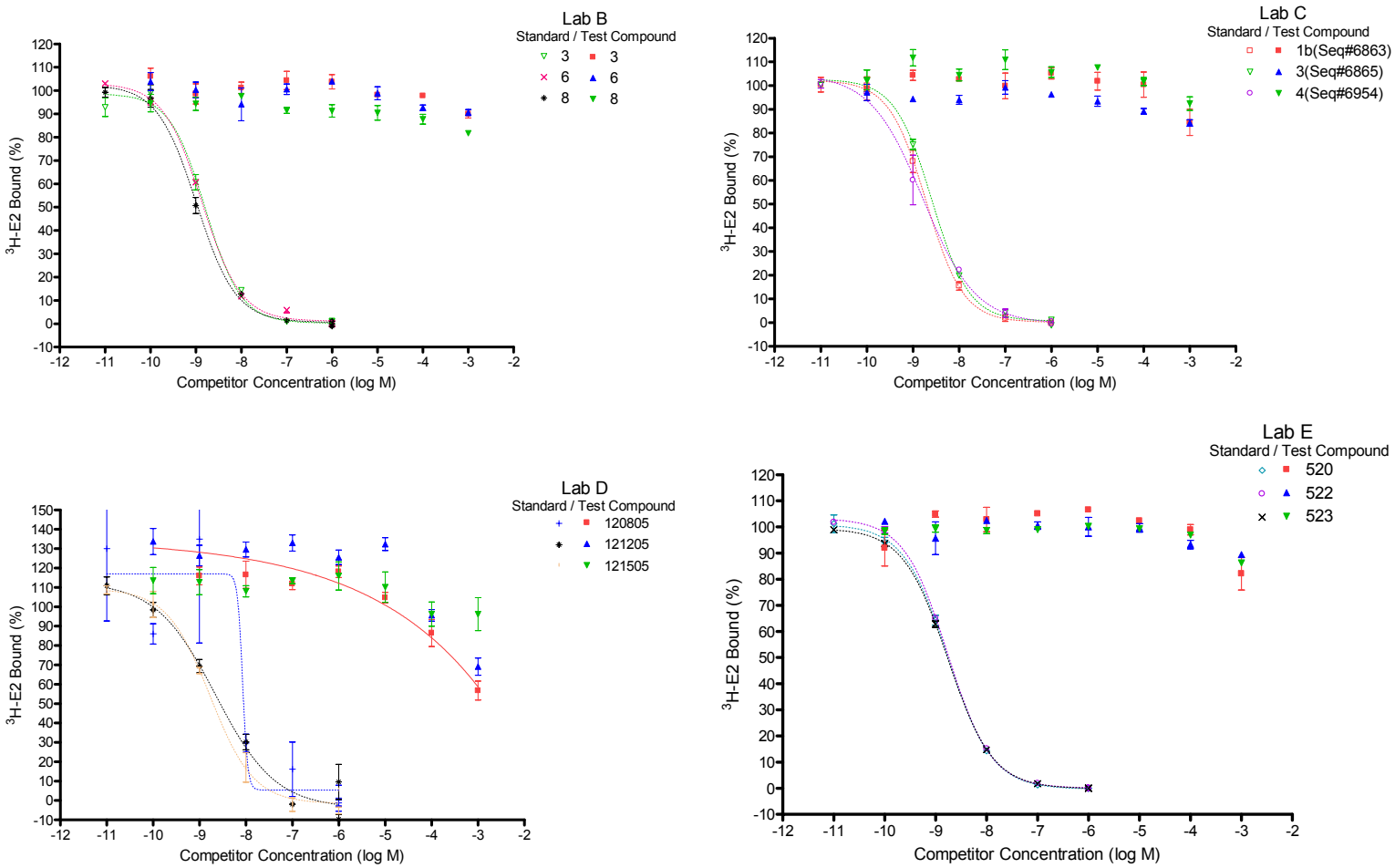


Figure 9.16 CR42409 Atrazine with corresponding standard curve runs. Note that individual runs are represented as means and SEM. Standard curves are dotted lines and open symbols, test compound is solid lines and closed symbols.

9.3 Intralaboratory Variability Analysis Using the Four Parameter Model

Statistical Method. The interlaboratory analysis combines the results across laboratories (and studies within laboratories) for each chemical, using a meta analysis approach. For each of the model parameters and the $\log_{10}$RBA, a one (or two) factor random effects analysis of variance model with heterogeneous variances among the (studies and) laboratories was fitted to the summary responses for each (study and) laboratory. For each endpoint, the within (study and) laboratory variances were based on the squares of the standard errors associated with the endpoint estimates obtained for the individual (studies and) laboratories. The analysis of variance provides an estimated weighted average across all the (studies and) laboratories and its associated standard error as well as an estimate of the laboratory-to-laboratory and study-to-study within laboratory components of variation. The weights included in the weighted averages incorporate both among (study and) laboratory variation and within (study and) laboratory variation. The degrees of freedom associated with the overall weighted average were approximated as

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

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

To describe the variability among the (study and) laboratory values relative to the overall average value, coefficient of variation (CV) among (studies and) laboratories and its associated 95 percent confidence intervals (CI) were calculated for the IC_{50}, the slope, the bottom and the top parameters, and the $\log_{10}$RBA. (Note that the CV was calculated for IC_{50} in the original domain, rather than for the $\log_{10}IC_{50}$). The coefficient of variation is defined as the standard deviation of the response divided by its mean. The method for calculating the CV and the associated 95 percent CI was different depending on the underlying assumption about the distribution of the endpoint parameter.

The within test run IC_{50} estimates were assumed to be approximately log normally distributed. The CV for IC_{50} is

$$CV = (10^{S^2(\ln 10)} - 1)^{1/2}$$

where S^2 is the total logarithmic (base 10) variance among the K (studies and) laboratories. S^2 is approximated as $K*(se)^2$ where se is the standard error of the pooled mean ($\log_{10}IC_{50}$) estimates. This would be exact if the within run components of variance were equal across (studies and) laboratories. The 95 percent confidence interval associated with the CV is based on the chi square distribution and is calculated as

$$[(10^{(df*S^2(\ln 10)/(\chi_{df,0.975}))} - 1)^{1/2}, (10^{(df*S^2(\ln 10)/(\chi_{df,0.025}))} - 1)^{1/2}]$$

where df is the estimated degree of freedom among the (studies and) laboratories, shown above.

For the other parameters, the measurements are assumed to be approximately normal. The CV is expressed as

$$CV=|S/M|$$

where M is the mean and S is the associated standard deviation. S is approximated as K(se) where se is the standard error of the pooled mean estimate and K is the number of (studies and) laboratories. The endpoints of the 95 percent confidence interval associated with the CV are related to the solutions to the two nonlinear equations:

$$t_{df}(NC)^{-1}(0.025) = M/(S/K) \text{ and } t_{df}(NC)^{-1}(0.975) = M/(S/K)$$

where $t_{df}(NC)$ is noncentral t distribution with noncentrality parameter NC ($= \mu/(\sigma/\sqrt{K})$) and degrees of freedom df is the estimated degree of freedom among the (studies and) laboratories, shown above. The solution to the first equation gives the lower bound of the CI, and the solution for the second equation gives the upper bound of the CI. A Newton-type algorithm is used to solve for the noncentrality parameters in these equations (Lehmann, 1986). Since for a full response curve fit the bottom is typically at or near 0, the CV is not meaningful for this parameter.

Interlaboratory Comparison. All of the runs in the interlaboratory validation were run in each laboratory in each run using a single batch of rat prostate cytosol allowing estimation of the laboratory-to-laboratory variability (Table 9-8). The average value, overall standard error and associated degrees of freedom, the laboratory-to-laboratory variance component, the ratio of the between to within variances, and the coefficient of variation among studies and laboratories are presented for $\log_{10}IC_{50}$, hill slope and for the $\log_{10}RBA$ ($\log_{10}IC_{50,\text{ standard}} - \log_{10}IC_{50,\text{ test}}$). A large value in the ratio column indicates that the variability in the particular laboratory in greater than the interlaboratory variability. For many of the test chemicals and model parameters, Laboratories C and D display greater variation among studies than the other laboratories.

In the course of the validation studies, the R1881 standard and weak positive dexamethasone were run many times (Table 9-9). While this does allow an estimation of the interlaboratory variability, the use of different cytosol preparations means that only the RBA comparisons are relevant since they compare the relative binding rather than lot-specific values. Tables 9-10 and 9-11 show the interlaboratory variability for R1881 and dexamethasone. The average log RBA confidence interval, -4.386(-4.539,-4.233), is relatively small, especially considering some of the difficulties of the Lab D data. The ratio of the inter to intra laboratory variability is approximately 4, indicating that most of the time the laboratories were able to consistently reproduce their results. Additionally, the average log IC_{50} and slope CIs are not large indicating that the cytosol preparation activities were relatively consistent.

A graphical representation of the parameter estimates and CIs can be helpfully in interpreting the intra and inter-laboratory variability. Figure 9-17 shows the $\log_{10}RBA$ variability for the weak positive dexamethasone. For most of the laboratories, the CI includes the overall $\log_{10}RBA$

estimate (-4.386). The size of the CI also provides an indication of the ability of the labs to produce consistent results with the standard and test chemicals.

Table 9-8. Inter-Laboratory Analysis for 10 Coded Chemicals

Parameter	Variance Between Laboratories /Pooled Within	Ratio (Between/Within)	CV (%) (95% CI)
CR42400 Dihydrotestosterone			
$log_{10}IC_{50}$	0.046/0.002	20.381	54.261(31.183,270.139)
β slope	0.010/0.003	3.396	11.833(6.546,51.552)
$log_{10}RBA$	0.009/0.004	1.955	828.051(91.066,1E38)
CR42401 Testosterone			
$log_{10}IC_{50}$	0.032/0.002	20.727	44.311(25.870,179.501)
β slope	0.001/0.002	0.234	4.336(2.602,12.396)
$log_{10}RBA$	0.000/0.004	0.000	4.766(3.712,6.665)
CR42402 17β-estradiol			
$log_{10}IC_{50}$	0.011/0.018	0.643	29.587(18.775,71.676)
β slope	0.003/0.005	0.632	7.718(3.879,65.914)
$log_{10}RBA$	0.000/0.020	0.017	2.689(1.638,7.232)
CR42403 MPA			
$log_{10}IC_{50}$	0.172/0.002	113.664	122.577(62.237,4838.618)
β slope	0.000/0.007	0.000	3.556(2.482,6.259)
$log_{10}RBA$	0.081/0.004	22.279	23.791(13.910,81.386)
CR42404 Linuron			
$log_{10}IC_{50}$	0.001/0.004	0.274	11.756(8.614,18.535)
β slope	0.041/0.071	0.572	28.493(19.525,54.015)
$log_{10}RBA$	0.003/0.006	0.485	1.712(0.955,6.945)
CR42405 pp'-DDE			
$log_{10}IC_{50}$	0.008/0.004	1.862	24.384(14.945,67.092)
β slope	0.014/0.013	1.143	17.000(10.232,49.342)
$log_{10}RBA$	0.061/0.007	9.323	6.885(4.094,20.527)
CR42406 Cyproterone acetate			
$log_{10}IC_{50}$	0.079/0.001	57.690	73.033(40.724,575.386)
β slope	0.000/0.005	0.000	3.976(3.304,4.995)
$log_{10}RBA$	0.027/0.004	7.826	8.729(5.210,25.696)
CR42407 Spironolactone			
$log_{10}IC_{50}$	0.014/0.001	10.136	28.876(16.902,102.826)
β slope	0.000/0.006	0.000	3.872(3.184,4.943)
$log_{10}RBA$	0.004/0.003	1.174	3.628(2.352,7.851)
CR42408 DEHP			

Parameter	Variance Between Laboratories /Pooled Within	Ratio (Between/Within)	CV (%) (95% CI)
$log_{10}IC_{50}$	0.000/61923.20	0.000	.(.,.)
β slope	0.000/0.389	0.000	84.783(46.102,541.723)
$log_{10}RBA$	0.000/61923.20	0.000	2709.603(98.344,1E38)
CR42409 Atrazine			
$log_{10}IC_{50}$	0.000/44316.99	0.000	7051.522(3075.668,22796.55)
β slope	0.000/0.158	0.000	70.094(41.419,229.532)
$log_{10}RBA$	0.000/44316.99	0.000	22.661(18.151,30.266)

Table 9-9. Number of Test Runs per Chemical in Validation Studies

Study	Chemical	Number of Runs				
		Lab A	Lab B	Lab C	Lab D	Lab E
i. Preliminary Validation Battelle Cytosol (Chapter 8)	R1881	3	3	3	3	3
	dexamethasone	3	3	3	3	3
ii. Preliminary Validation Lab Cytosol (Chapter 8)	R1881		3	3		3
	dexamethasone		3	3		3
iii. Inter-laboratory Validation (Chapter 9)	R1881		8	6	9	6
	dexamethasone		8	6	9	6
	CR42400-CR42409 [a]		30	30	30	30
iv. Supplemental Validation (Chapter 10)	R1881					18
	dexamethasone					18
Total	R1881	3	14	12	12	30
	dexamethasone	3	14	12	12	30

a: 3 runs each for 10 test chemicals for a total of 30 runs per lab

Table 9-10. Inter-Laboratory Analysis for Standard Curve

Laboratory Code	Study	$log_{10}IC_{50}$ (log M)			β slope		
		Estimate (95% CI)	Degree of Freedom	Standard Error	Estimate (95% CI)	Degree of Freedom	Standard Error
A	i	-8.803(-8.959,-8.647)	3.36	0.052	-0.887(-1.059,-0.715)	35.71	0.085
B	i	-8.833(-8.882,-8.785)	2.96	0.015	-1.078(-1.201,-0.956)	2.69	0.036
B	ii	-8.848(-8.938,-8.757)	3.26	0.030	-1.045(-1.145,-0.944)	49.25	0.050
B	iii	-8.853(-8.892,-8.815)	6.40	0.016	-0.961(-1.043,-0.879)	15.05	0.039
C	i	-8.864(-8.888,-8.839)	46.32	0.012	-1.070(-1.146,-0.994)	38.74	0.038
C	ii	-8.900(-9.148,-8.652)	4.93	0.096	-0.937(-1.093,-0.781)	33.72	0.077
C	iii	-8.539(-8.705,-8.373)	17.00	0.079	-1.041(-1.191,-0.891)	17.01	0.071
D	i	-8.747(-8.808,-8.685)	2.59	0.018	-0.989(-1.068,-0.909)	43.05	0.039
D	iii	-8.490(-8.580,-8.401)	17.00	0.042	-0.799(-0.907,-0.691)	17.00	0.051
E	i	-8.739(-8.771,-8.708)	3.55	0.011	-1.028(-1.066,-0.990)	46.07	0.019
E	ii	-8.813(-8.832,-8.795)	39.22	0.009	-0.998(-1.046,-0.950)	39.17	0.024
E	iii	-8.784(-8.824,-8.743)	7.01	0.017	-1.000(-1.032,-0.968)	61.07	0.016
E	iv	-8.822(-8.838,-8.807)	58.33	0.008	-0.998(-1.026,-0.971)	88.27	0.014
Average		-8.775(-8.846,-8.704)	7.68	0.031	-0.999(-1.031,-0.966)	24.03	0.016

Parameter	Random Laboratory Variance	Random Study (Within Laboratory) Variance	Variance Total Random /Pooled Within	Ratio (Random/Within)	CV (%) (95% CI)
$log_{10}IC_{50}$	0.001	0.009	0.009/0.002	5.445	25.819(17.175,52.734)
β slope	0.000	0.002	0.002/0.002	0.721	5.619(4.383,7.835)

Table 9-11. Inter-Laboratory Analysis for Weak Positive Binder

Laboratory Code	Study	$log_{10}IC_{50}$ (log M)			β slope			$log_{10}RBA$		
		Estimate (95% CI)	Degree of Freedom	Standard Error	Estimate (95% CI)	Degree of Freedom	Standard Error	Estimate (95% CI)	Degree of Freedom	Standard Error
A	i	-4.394(-4.841,-3.947)	17.00	0.212	-0.910(-1.258,-0.561)	17.00	0.165	-4.409(-4.866,-3.952)	18.77	0.218
B	i	-4.463(-4.507,-4.420)	15.00	0.020	-0.926(-1.019,-0.832)	15.00	0.044	-4.370(-4.424,-4.316)	14.11	0.025
B	ii	-4.231(-4.281,-4.180)	55.60	0.025	-1.001(-1.143,-0.858)	49.55	0.071	-4.617(-4.705,-4.529)	9.32	0.039
B	iii	-4.251(-4.288,-4.215)	107.26	0.018	-0.956(-1.052,-0.860)	113.02	0.048	-4.602(-4.652,-4.552)	30.83	0.024
C	i	-4.474(-4.516,-4.431)	57.35	0.021	-0.939(-1.033,-0.845)	56.05	0.047	-4.390(-4.438,-4.342)	88.91	0.024
C	ii	-4.157(-4.293,-4.021)	3.17	0.044	-0.946(-1.084,-0.808)	20.78	0.066	-4.743(-4.994,-4.491)	6.76	0.106
C	iii	-4.091(-4.140,-4.043)	36.36	0.024	-0.891(-1.247,-0.535)	7.13	0.151	-4.448(-4.619,-4.276)	20.16	0.082
D	i	-4.629(-4.696,-4.561)	20.00	0.032	-1.216(-1.515,-0.917)	20.00	0.144	-4.118(-4.195,-4.041)	19.98	0.037
D	iii	-4.340(-4.465,-4.215)	33.05	0.062	-1.134(-1.469,-0.798)	29.82	0.164	-4.150(-4.300,-4.000)	49.97	0.075
E	i	-4.385(-4.402,-4.368)	47.74	0.008	-0.918(-0.955,-0.881)	46.61	0.018	-4.354(-4.385,-4.323)	8.76	0.014
E	ii	-4.307(-4.327,-4.287)	26.34	0.010	-0.950(-1.000,-0.900)	25.35	0.024	-4.506(-4.533,-4.480)	60.84	0.013
E	iii	-4.439(-4.493,-4.384)	8.15	0.024	-1.006(-1.085,-0.927)	82.98	0.040	-4.345(-4.407,-4.283)	14.33	0.029
E	iv	-4.438(-4.470,-4.405)	20.44	0.016	-0.937(-1.006,-0.869)	40.42	0.034	-4.385(-4.420,-4.349)	31.38	0.017
Average		-4.352(-4.438,-4.266)	16.21	0.041	-0.943(-0.965,-0.920)	83.58	0.011	-4.386(-4.539,-4.233)	8.62	0.067

Parameter	Random Laboratory Variance	Random Study (Within Laboratory) Variance	Variance Total Random /Pooled Within	Ratio (Random/Within)	CV (%) (95% CI)
$log_{10}IC_{50}$	0.000	0.020	0.020/0.004	4.631	34.766(25.605,54.794)
β slope	0.000	0.000	0.000/0.009	0.000	4.242(3.679,5.009)
$log_{10}RBA$	0.015	0.010	0.025/0.006	4.175	5.520(3.770,10.274)

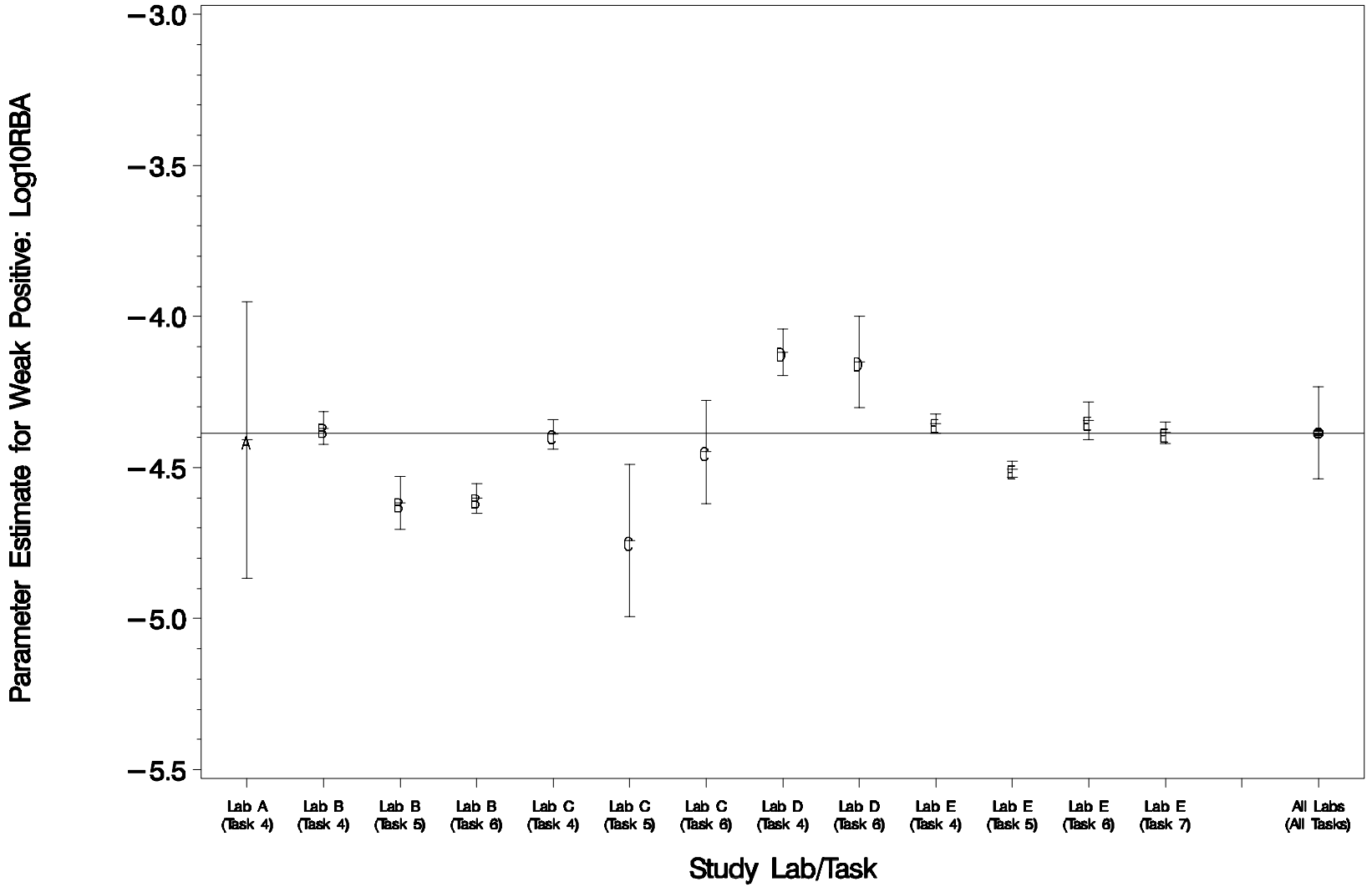


Figure 9-17. Intra and inter-laboratory variability for the log_{10}RBA for the weak positive dexamethasone. Error bars represent the 95% confidence interval of the parameter estimate.

10.0 SUPPLEMENTAL VALIDATION STUDIES

The objective for this task was to test approximately 30 test chemicals in one laboratory to expand the data base of binding information on chemicals for the validation of the AR binding assay. The assays used cytosol prepared by Battelle, Battelle-supplied R 1881 from a common-lot, Battelle-supplied "weak binder" dexamethasone from a common lot, and a common lot of 3H-R1881. Stock concentrations of R1881, and dexamethasone were supplied by Battelle, Sequim Chemical Repository to Battelle, Richland. A detailed protocol for androgen receptor (AR) saturation and competitive binding assays was followed to conduct these assays. Battelle, Richland conducted three separate experiments to evaluate the inhibition of androgen receptor binding of ^{3}H-R1881 by the test substances. Test chemicals were tested as coded unknowns. Some of the material in this chapter was adapted from the results of the prior EDSP Work Assignment 4-11 (USEPA, 2006e).

10.1 Confirmation of Standard Curves and Assay Performance

A total of three saturation experiments were conducted and the amount of cytosolic protein used for each incubation in the saturation binding experiments was 0.6 mg. The K_d and B_{max} values are shown in Table 10-1 and are consistent with those in earlier validation work. Rat ventral prostate cytosol was prepared by Battelle at a protein concentration of 5.532 mg/mL. A series of saturation and competitive binding experiments were conducted using 1.0 mg of cytosolic protein for each assay tube as previously described in Chapter 4. All runs were conducted by the same technician. Preliminary analysis of the competitive assay standard and weak positive was conducted with the 2 parameter model, as shown in Figure 10-2 for the R1881 standard and weak positive dexamethasone. The log IC_{50} values for R1881 and dexamethasone, along with log RBAs, are shown in Table 10-2. Figure 10-1 and Table 10-2 are included for completeness, note that means and standard errors are shown in Figures 10-3 and 10.4 and Table 10-3 with the 4 parameter model.

10.2 Data Analysis Using the Four Parameter Model

The data were then re-fit using the 4 parameter model (Tables 10-3 and 10-4). For the R1881 standard (18 runs) the range of $\log_{10}IC_{50}$ (log M) values was -8.9 to -8.7 with a median value of -8.8 (Table 10-3). The goodness of fit for all data sets was greater than 0.983, indicating an adequate model fit.

For the competitive binding assays with the weak positive dexamethasone (18 runs) the range of $\log_{10}IC_{50}$ (log M) values was -4.6 to -4.3 with a median value of -4.4 (Table 10-3). The goodness of fit for all data sets was greater than 0.952, indicating an adequate model fit. Fitted parameters are shown in Table 10-4 for the test compounds (also shown graphically in Figures 10-3 to10-5).

For 2,4,5-trichlorophenoxyacetic acid, estrone, ketoconazole, genistein and butylbenzyl phthalate the bottom parameter was arbitrarily set to a value of 49.95 to allow the model to fit. These runs are at the limits of the viability of the assay and the IC_{50} and RBA values are very suspect.

Phenobarbital, phorbol 12-myristate 13-acetate, and 2,4,5-trichlorophenoxyacetic acid did not exhibit any significant inhibition of the binding of [^{3}H] R1881 to the androgen receptor.

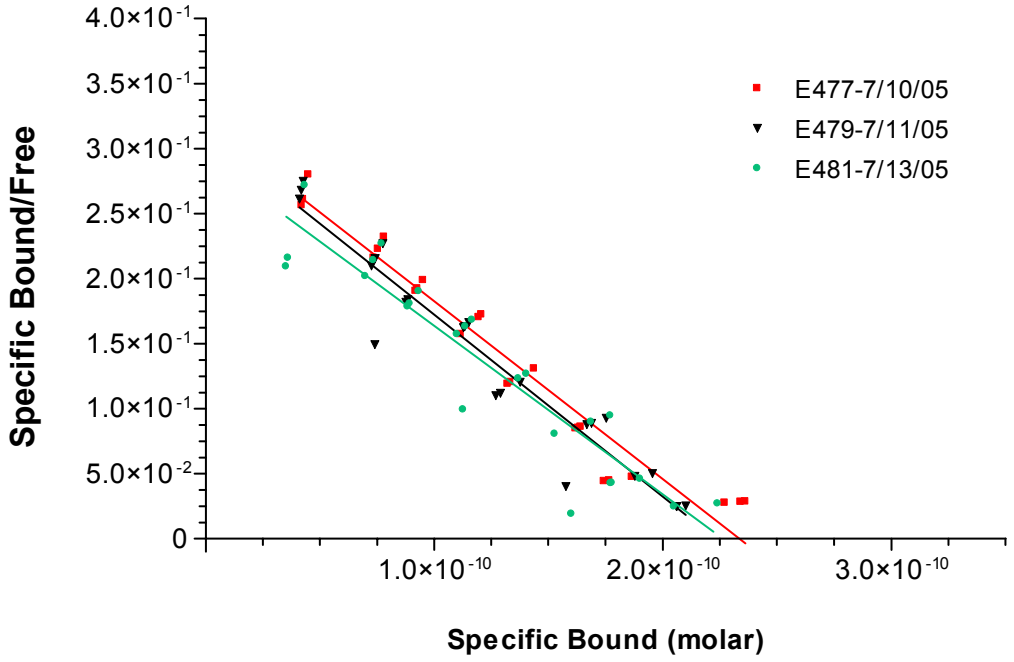


Figure 10-1. Scatchard plot for saturation assay. Colors represent different assay runs.

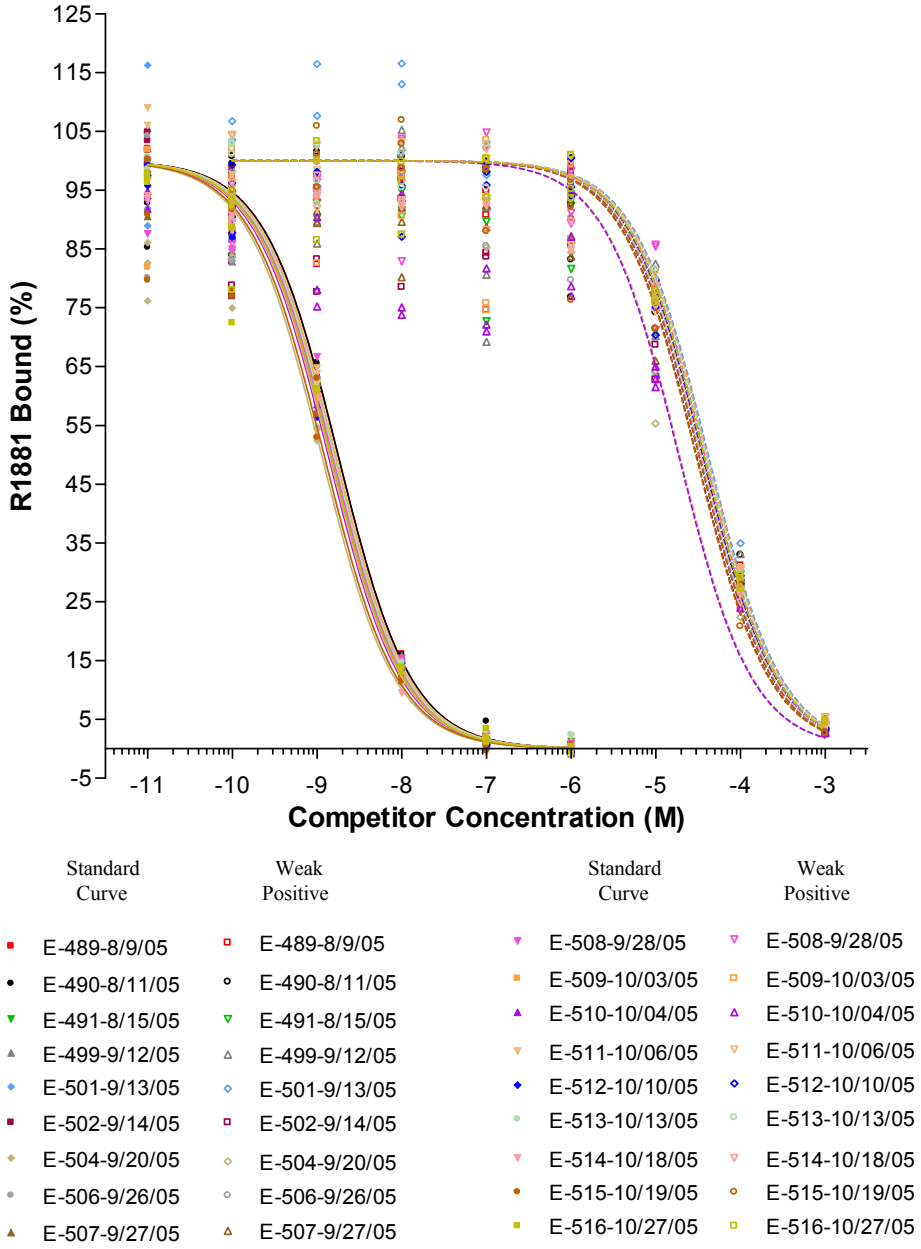


Figure 10-2. Competitive Binding of R1881 and Dexamethasone (Weak Positive) to the Androgen Receptor (2 parameter model)

Table 10-1. Saturation Binding Experiments

Run number	K_d (M)	B_{max} (fmoles/100 ug)
477	0.92×10^{-9}	11.85
479	0.90×10^{-9}	11.34
481	0.92×10^{-9}	11.11
Mean	0.91×10^{-9}	11.43
SD	0.01×10^{-9}	0.38

Table 10-2. Competitive Binding Experiments for Standard Chemicals (2 parameter model)

	R1881	Dexamethasone	
Run number	log IC_{50} (log M)	log IC_{50} (log M)	log RBA
489	-8.754	-4.398	-4.357
490	-8.752	-4.435	-4.317
491	-8.824	-4.455	-4.369
499	-8.824	-4.481	-4.342
501	-8.833	-4.378	-4.455
502	-8.799	-4.520	-4.279
504	-8.947	-4.719	-4.228
506	-8.804	-4.489	-4.315
507	-8.785	-4.491	-4.294
508	-8.815	-4.420	-4.395
509	-8.793	-4.394	-4.400
510	-8.870	-4.730	-4.139
511	-8.777	-4.460	-4.318
512	-8.851	-4.452	-4.399
513	-8.801	-4.407	-4.395
514	-8.857	-4.465	-4.392
515	-8.917	-4.526	-4.391
516	-8.842	-4.433	-4.409
Mean	-8.825	-4.481	-4.344
SE	-0.006	-0.022	-0.017

Table 10-3. Competitive assay fits for R1881 and dexamethasone using the 4 parameter model.

Run	$log_{10}IC_{50}$ (log M) ±SE	B	T	β slope	Goodness of Fit	Log RBA
Standard Curve – R1881						
489	-8.763±0.012	-0.1	102.1	-0.994	0.999	
490	-8.739±0.041	0.4	95.7	-1.106	0.991	
491	-8.816±0.009	-0.2	98.6	-0.997	1.000	
499	-8.830±0.039	-1.1	102.1	-0.805	0.993	
501	-8.817±0.060	-0.7	97.2	-0.933	0.983	
502	-8.805±0.014	-0.3	102.3	-0.967	0.999	
504	-8.892±0.055	0.6	86.0	-1.087	0.984	
506	-8.784±0.048	0.2	94.0	-1.105	0.990	
507	-8.775±0.018	0.1	97.1	-1.050	0.998	
508	-8.785±0.035	-0.3	93.6	-1.007	0.994	
509	-8.775±0.039	0.2	94.5	-1.095	0.992	
510	-8.843±0.010	-0.2	94.1	-1.027	1.000	
511	-8.791±0.029	-1.0	104.6	-0.949	0.996	
512	-8.837±0.015	-0.6	97.6	-0.944	0.999	
513	-8.801±0.014	-0.2	100.3	-0.965	0.999	
514	-8.832±0.011	-0.2	94.3	-1.084	0.999	
515	-8.865±0.053	-0.7	88.8	-0.983	0.987	
516	-8.818±0.046	-0.5	95.0	-0.923	0.990	
Mean	-8.809	-0.3	96.5	-1.001	0.994	
SE	-0.004	-1.7	0.0	-0.079		
Weak Positive - Dexamethasone						
489	-4.366±0.045	1.4	93.0	-1.112	0.989	-4.397
490	-4.421±0.068	-3.0	97.3	-0.836	0.974	-4.318
491	-4.402±0.057	-0.4	91.1	-1.080	0.979	-4.414
499	-4.434±0.090	-1.0	90.9	-0.936	0.952	-4.396
501	-4.376±0.064	-8.9	104.7	-0.708	0.980	-4.441
502	-4.438±0.099	0.3	86.6	-1.050	0.941	-4.367
504	-4.657±0.058	-1.0	92.8	-0.781	0.983	-4.235
506	-4.473±0.076	-4.8	98.3	-0.750	0.971	-4.311
507	-4.438±0.043	-0.2	90.7	-0.991	0.988	-4.337
508	-4.406±0.069	1.9	96.4	-1.141	0.970	-4.379
509	-4.358±0.084	0.8	92.8	-1.121	0.954	-4.417
510	-4.617±0.095	-2.6	82.3	-0.853	0.955	-4.226
511	-4.453±0.057	-1.2	99.5	-0.865	0.984	-4.338
512	-4.431±0.042	-0.3	96.2	-0.960	0.989	-4.406
513	-4.400±0.041	0.4	98.4	-1.027	0.989	-4.401
514	-4.429±0.030	1.1	92.8	-1.063	0.994	-4.403
515	-4.506±0.068	-4.8	98.1	-0.795	0.977	-4.359
516	-4.415±0.062	1.6	95.2	-1.043	0.976	-4.403
Mean	-4.446	-1.2	94.3	-0.951	0.975	-4.364
SE	-0.018	-2.4	0.1	-0.146		-0.014

Table 10-4. Competitive binding assay with the chemical unknowns using the 4 parameter model.

Chemical / Run	$log_{10}IC_{50}$ (log M) ±SE	SE of $log_{10}IC_{50}$	B	T	β slope	Goodness of Fit	Log RBA
Trenbolone							
506	-8.757	0.0260	0.166	107.7	-0.877	0.995	0.027
507	-8.697	0.0130	-0.097	99.1	-1.073	0.999	0.078
508	-8.682	0.0145	-0.610	107.6	-0.893	0.999	0.103
Mean	-8.712		-0.180	104.8	-0.948	0.998	0.069
SE	-0.005						0.559
Bicalutamide							
514	-6.288	0.0360	-1.156	99.7	-0.909	0.995	2.544
515	-6.141	0.0264	-0.640	103.6	-0.994	0.997	2.724
516	-6.127	0.0562	0.988	94.6	-1.128	0.983	2.691
Mean	-6.185		-0.269	99.3	-1.010	0.991	2.653
SE	-0.014						0.036
Mifepristone							
511	-7.291	0.0352	0.096	101.5	-1.582	0.995	1.500
512	-7.264	0.0193	-0.473	104.4	-1.134	0.998	1.573
513	-7.553	0.0326	1.039	105.5	-1.169	0.995	1.248
Mean	-7.369		0.221	103.8	-1.295	0.996	1.440
SE	-0.022						0.118
Nilutamide							
499	-5.595	0.0502	-0.814	97.6	-0.833	0.990	3.235
501	-5.405	0.0604	-0.587	117.6	-0.943	0.985	3.412
502	-5.615	0.0743	-1.402	93.2	-0.844	0.983	3.190
Mean	-5.538		-0.934	102.8	-0.873	0.986	3.279
SE	-0.021						0.036
17a-Ethynyl estradiol							
499	-5.483	0.0433	-0.110	105.9	-1.090	0.990	3.347
501	-5.407	0.0635	0.320	120.3	-1.150	0.981	3.410
502	-5.630	0.0662	1.282	94.9	-1.182	0.977	3.175
Mean	-5.507		0.497	107.0	-1.141	0.983	3.311
SE	-0.021						0.037
Hydroxyflutamide							
511	-5.697	0.0605	-0.372	101.0	-0.971	0.984	3.094
512	-5.738	0.0352	-0.124	101.1	-0.905	0.995	3.099
513	-5.736	0.0206	-0.192	106.3	-0.922	0.998	3.065
Mean	-5.724		-0.229	102.8	-0.932	0.992	3.086
SE	-0.004						0.006
Fluoxymestrone							
514	-7.029	0.0170	0.118	98.1	-0.992	0.999	1.803
515	-6.997	0.0149	-0.436	106.1	-0.999	0.999	1.868
516	-7.037	0.0413	-0.138	99.6	-1.019	0.991	1.781
Mean	-7.021		-0.152	101.3	-1.004	0.996	1.817
SE	-0.003						0.025
Estrone							
514	-4.213	0.3385	40.95	94.7	-0.659	0.862	4.619
515	-1.374	0.4157	49.95	106.1	-0.822	0.977	7.491
516	-2.820	0.4320	49.95	102.2	-1.252	0.965	5.998

Chemical / Run	$log_{10}IC_{50}$ (log M) ±SE	SE of $log_{10}IC_{50}$	B	T	β slope	Goodness of Fit	Log RBA
Mean	-2.802		46.95	101.0	-0.911	0.935	6.036
SE	-0.507						0.238
Flutamide							
499	-4.899	311.00	24.15	101.8	-5.528	0.927	3.931
501	-4.636	2.1320	25.01	115.4	-2.239	0.926	4.181
502	-4.870	4.3370	18.84	90.9	-3.594	0.930	3.935
Mean	-4.802		22.67	102.7	-3.787	0.928	4.016
SE	-0.030						0.036
Diethylstilbestrol							
499	-4.804	13.5700	12.46	98.0	-3.427	0.937	4.026
501	-4.390	0.3113	-1.35E+07	107.5	-0.564	0.853	4.427
502	-4.693	0.1392	-41.08	97.5	-0.763	0.947	4.112
Mean	-4.629		-4.51E+06	101.0	-1.585	0.912	4.188
SE	-0.046						0.050
o,p'DDT							
506	-4.802	47.95	33.51	104.5	-4.533	0.984	3.982
507	-4.569	0.3968	34.42	99.6	-1.807	0.977	4.206
508	-4.233	0.1279	-1.32E+06	101.4	-0.500	0.927	4.552
Mean	-4.535		-4.38E+05	101.8	-2.280	0.963	4.247
SE	-0.063						0.068
Kepone							
511	-3.988	2136.0	8.68	105.0	-7.985	0.968	4.803
512	-3.895	0.0273	-213.0	103.1	-1.061	0.985	4.942
513	-3.918	0.0206	-242.1	104.0	-1.022	0.991	4.883
Mean	-3.934		-148.8	104.0	-3.356	0.981	4.876
SE	-0.012						0.014
Bisphenol A							
504	-4.498	0.0887	-3.428	90.2	-0.898	0.955	4.394
509	-4.371	0.0875	-4.294	96.5	-0.972	0.952	4.404
510	-4.567	0.0677	-4.495	92.3	-0.852	0.975	4.276
Mean	-4.479		-4.072	93.0	-0.907	0.961	4.358
SE	-0.022						0.016
Fluoranthene							
504	-4.526	0.3652	12.61	89.2	-1.075	0.877	4.366
509	-4.542	0.2246	23.4	105.0	-1.300	0.955	4.233
510	-4.402	0.1146	-651.6	101.1	-0.406	0.947	4.441
Mean	-4.490		-205.2	98.4	-0.927	0.926	4.347
SE	-0.017						0.024
Ketoconazole							
508	-3.885	0.5839	-1555	99.7	-0.789	0.753	4.900
506	-3.329	22.88	49.95	94.2	-3.798	0.624	5.455
507	-2.382	0.4765	49.95	99.5	-1.482	0.859	6.393
Mean	-3.199		-485.0	97.8	-2.023	0.745	5.583
SE	-0.238						0.135
4-Nonylphenol							
514	-4.239	0.0599	-3.185	94.8	-1.299	0.980	4.593
515	-4.169	0.0209	-4.295	106.5	-1.249	0.998	4.696
516	-4.184	0.0176	-3.277	104.0	-1.281	0.998	4.634

Chemical / Run	$log_{10}IC_{50}$ (log M) ±SE	SE of $log_{10}IC_{50}$	B	T	β slope	Goodness of Fit	Log RBA
Mean	-4.197		-3.586	101.8	-1.276	0.992	4.641
SE	-0.009						0.011
Phenobarbital							
489	Did not converge						
490	Did not converge						
491	Did not converge						
Mean							
SE							
Phorbol 12-Myristate 13-Acetate							
511	Did not converge						
512	Did not converge						
513	Did not converge						
Mean							
SE							
2,4,5-Trichlorophenoxyacetic acid							
504	0.701	1.7930	49.95	87.7	-0.730	0.548	9.593
509	Did not converge						8.775
510	0.513	2.1200	49.95	96.0	-0.835	0.442	9.356
Mean	0.607		49.95	91.8	-0.782	0.495	9.241
SE	0.219						0.046
Bisphenol B							
504	-4.543	0.0432	-1.309	99.3	-1.254	0.989	4.349
509	-4.408	0.0310	-1.389	107.8	-1.244	0.994	4.367
510	-4.479	0.0454	-1.487	99.1	-1.254	0.988	4.364
Mean	-4.477		-1.395	102.1	-1.251	0.990	4.360
SE	-0.015						0.002
Genistein							
511	-3.531	0.0744	-81.48	113.2	-0.993	0.906	5.260
512	-2.954	0.2001	49.95	104.7	-2.838	0.983	5.883
513	-3.229	0.4183	49.95	97.9	-4.099	0.903	5.572
Mean	-3.238		6.140	105.3	-2.643	0.931	5.572
SE	-0.089						0.056
Butylbenzyl phthalate							
489	-2.186	0.3870	49.95	96.3	-1.281	0.952	6.577
490	-1.993	0.5852	49.95	100.0	-1.233	0.903	6.746
491	-1.741	0.5656	49.95	101.4	-1.139	0.911	7.075
Mean	-1.973		49.95	99.2	-1.218	0.922	6.799
SE	-0.113						0.037
Kaempferol							
506	-4.479	3.6460	19.23	100.3	-2.008	0.942	4.305
507	-4.394	0.2560	7.075	103.1	-1.170	0.973	4.381
508	-4.493	6.3450	24.7	103.3	-2.154	0.909	4.292
Mean	-4.455		17.00	102.2	-1.777	0.941	4.326
SE	-0.012						0.011
Norethynodrel							
489	-6.331	0.0187	-0.204	100.4	-0.943	0.998	2.432
490	-6.374	0.0479	-1.315	103.0	-0.823	0.991	2.365
491	-6.321	0.0393	-0.493	99.0	-1.019	0.993	2.495

Chemical / Run	$log_{10}IC_{50}$ (log M) ±SE	SE of $log_{10}IC_{50}$	B	T	β slope	Goodness of Fit	Log RBA
Mean	-6.342		-0.671	100.8	-0.928	0.994	2.431
SE	-0.004						0.027
Finasteride							
489	-3.473	0.2175	-3.665	96.8	-1.099	0.969	5.290
490	-3.558	6.3800	19.83	96.8	-2.326	0.926	5.181
491	-3.448	0.1148	-15.26	101.9	-0.937	0.982	5.368
Mean	-3.493		0.302	98.5	-1.454	0.959	5.280
SE	-0.017						0.018
17a-Estradiol							
489	-4.785	0.1246	-53.27	95.3	-0.567	0.947	3.978
490	-4.950	0.0638	21.24	100.8	-0.947	0.980	3.789
491	-4.814	0.0360	8.399	102.8	-0.940	0.994	4.002
Mean	-4.850		-7.877	99.6	-0.818	0.974	3.923
SE	-0.018						0.030
Econazole							
514	-4.492	0.0771	2.478	93.3	-1.585	0.971	4.340
515	-4.524	0.0520	1.293	104.3	-1.333	0.987	4.341
516	-4.517	0.0428	2.943	98.6	-1.603	0.992	4.301
Mean	-4.511		2.238	98.7	-1.507	0.983	4.327
SE	-0.004						0.005

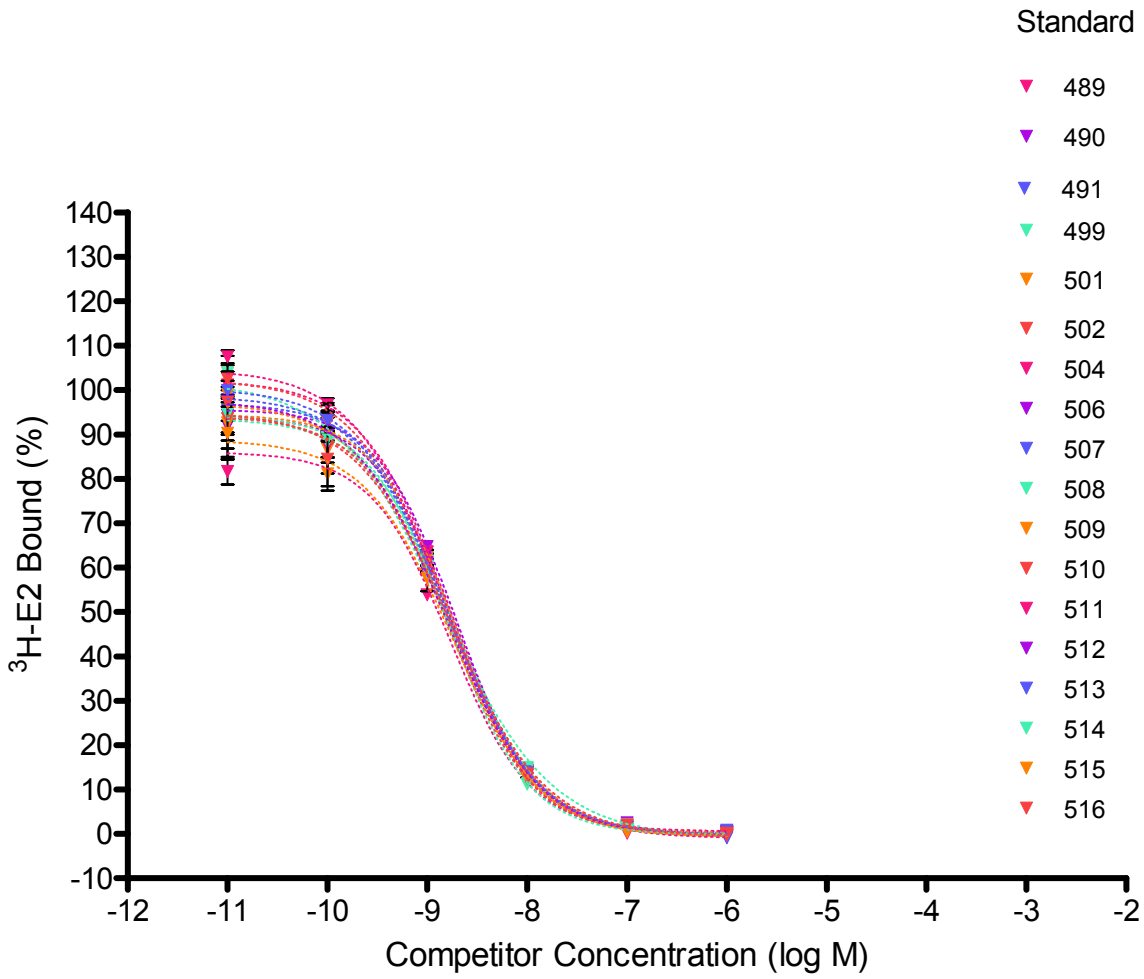


Figure 10-3. Competitive binding assay fits for standard R1881 using the 4 parameter model. Symbols designate individual runs.

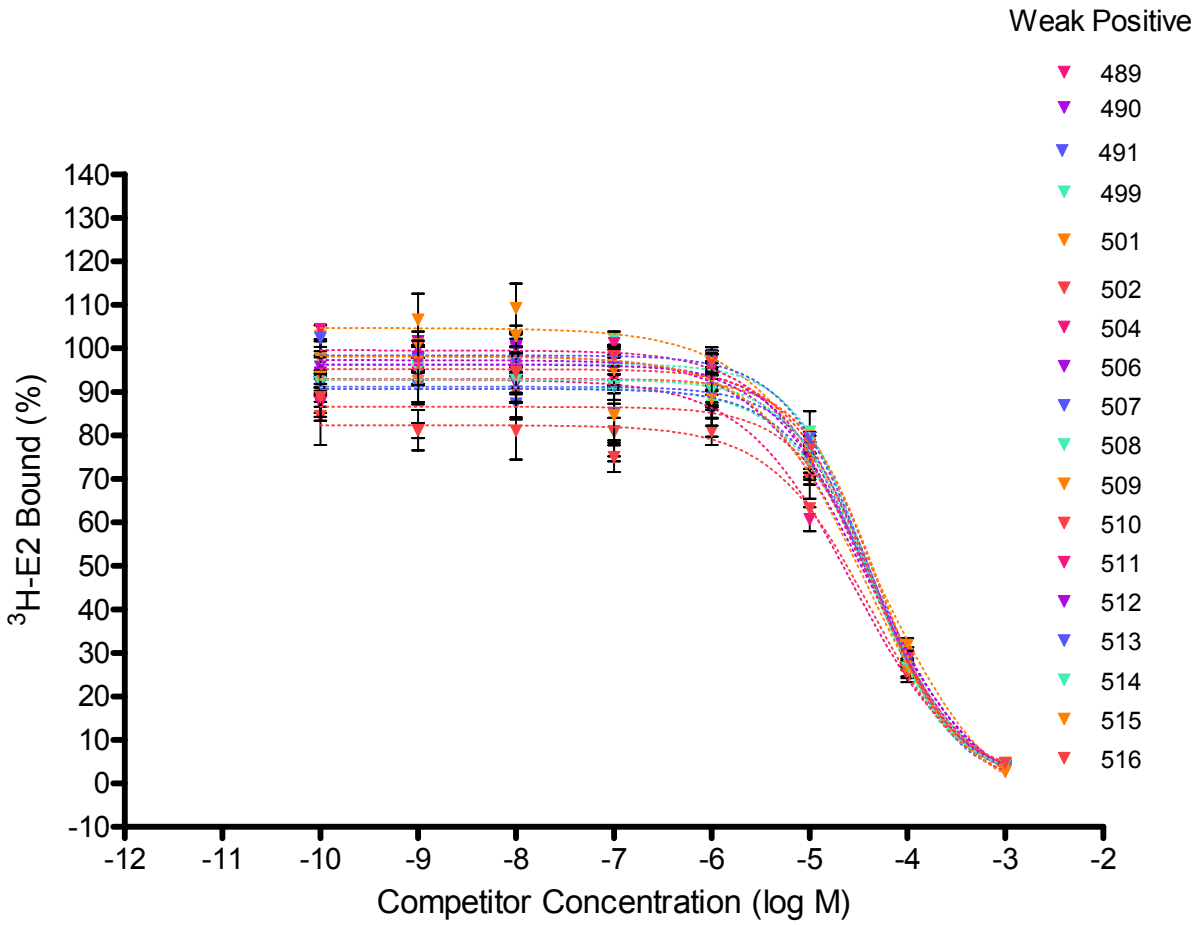


Figure 10-4. Competitive binding assay fits for the weak positive dexamethasone using the 4 parameter model. Symbols designate individual runs.

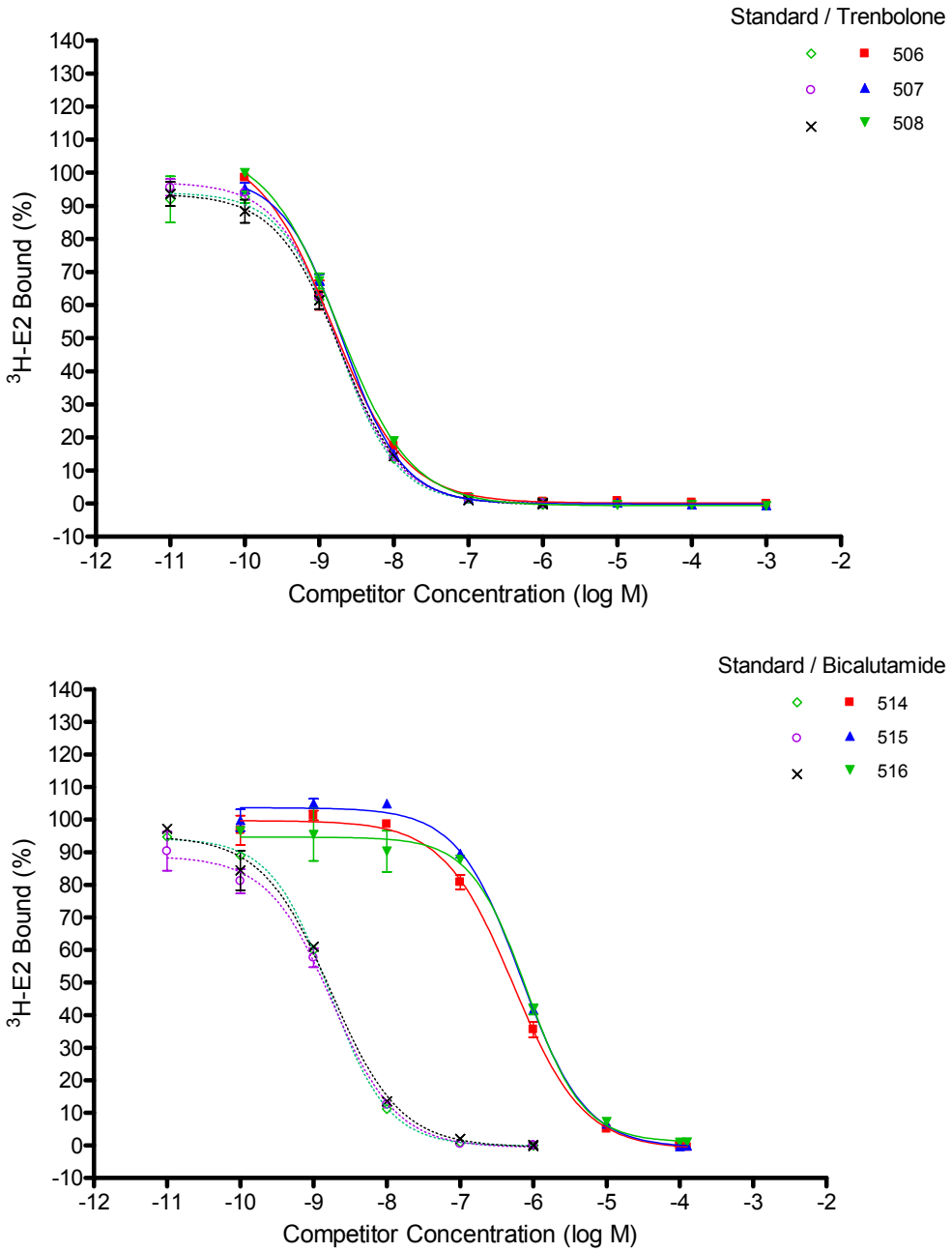
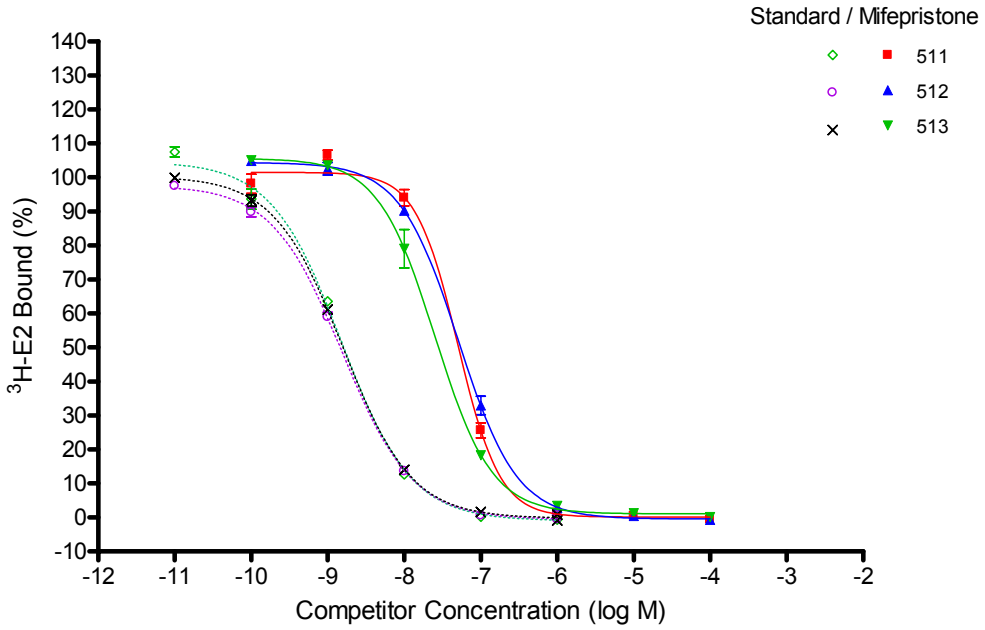


Figure 10-5. Competitive binding assay fits for test chemicals using the 4 parameter model. Standard curves run concurrently are shown as open symbols and dotted lines. Symbols designate individual runs.



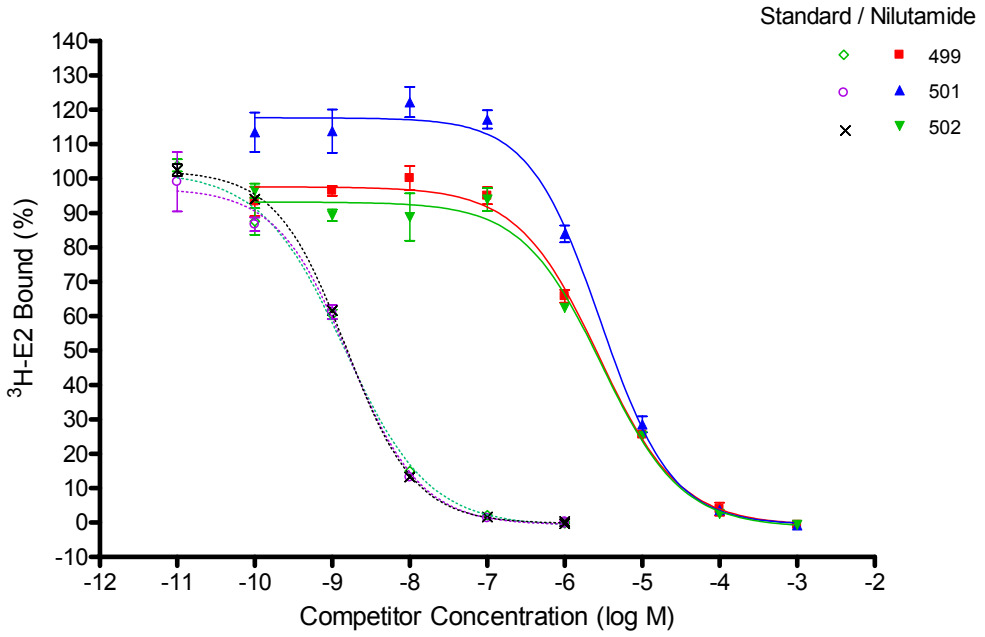


Figure 10-5. (continued)

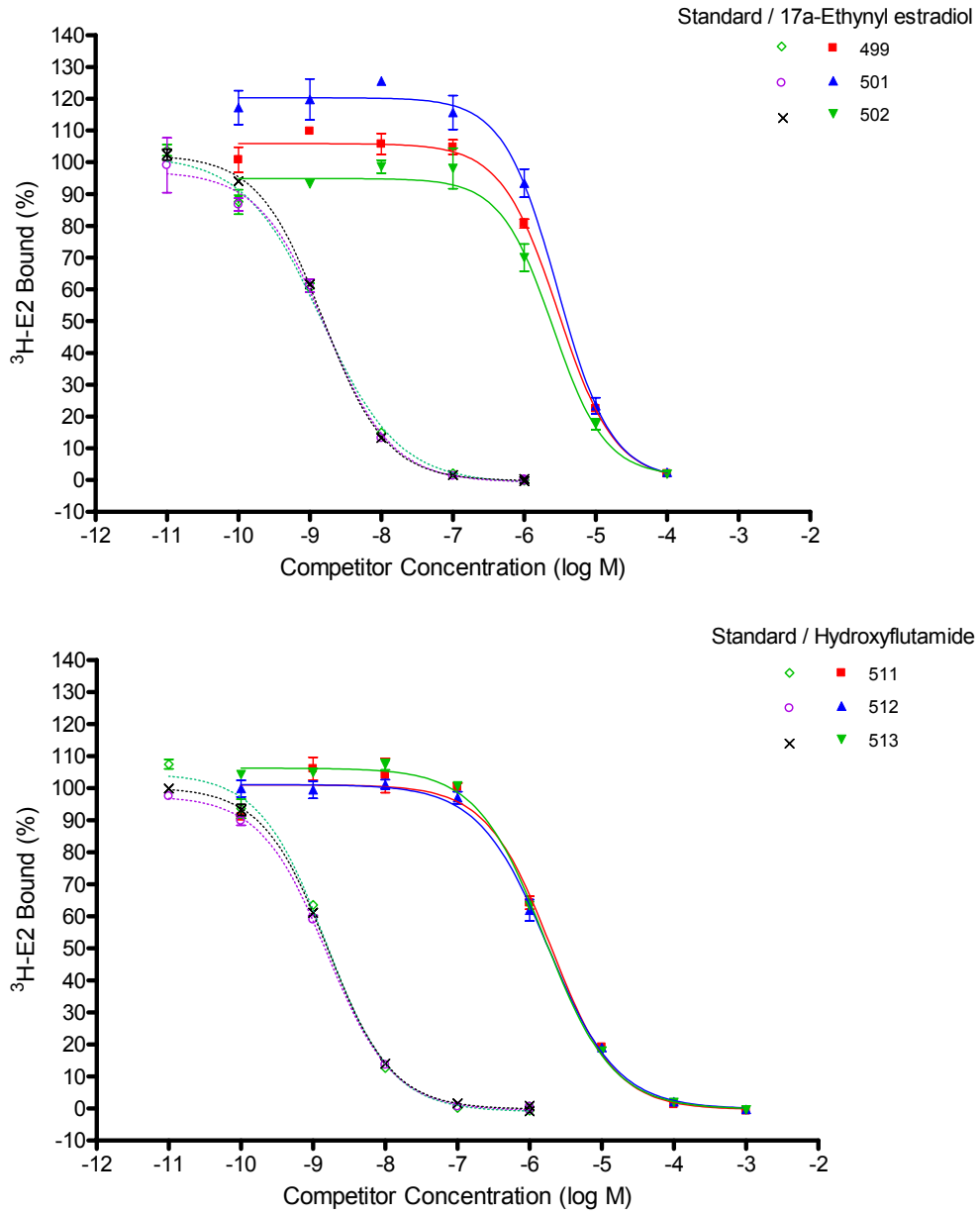
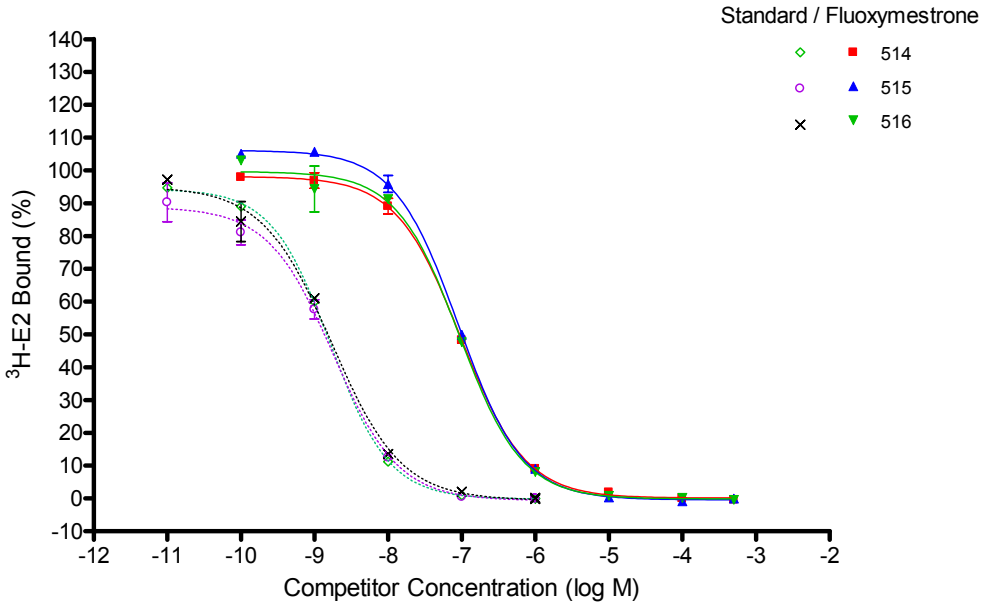


Figure 10-5. (continued)



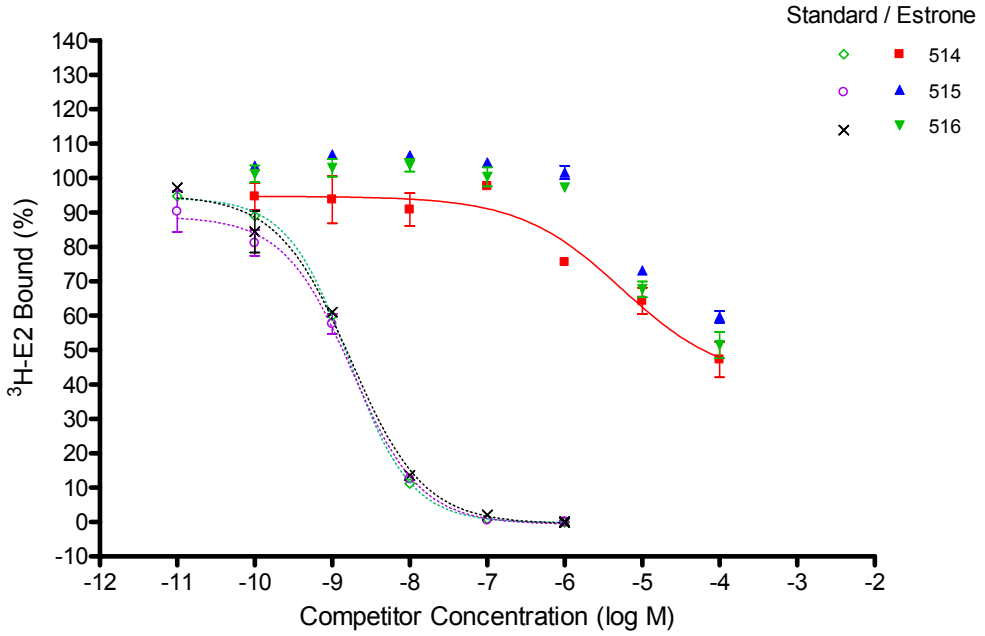
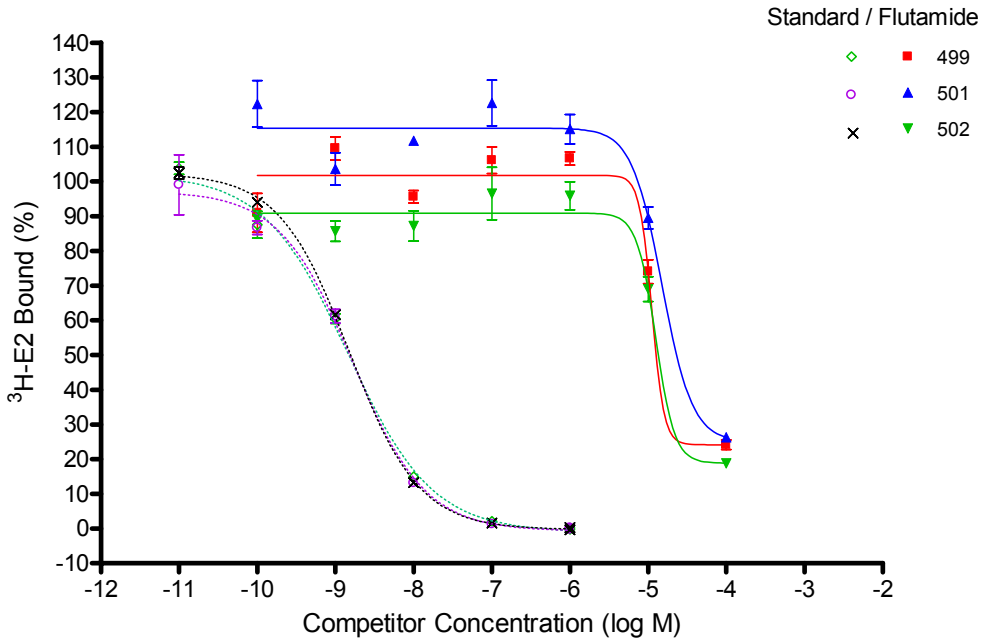


Figure 10-5. (continued)



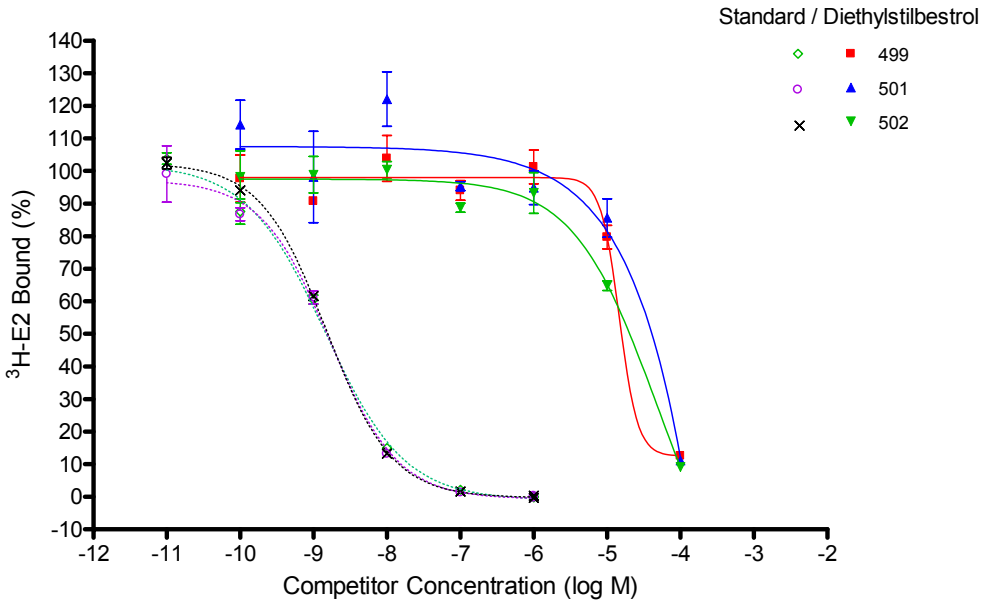
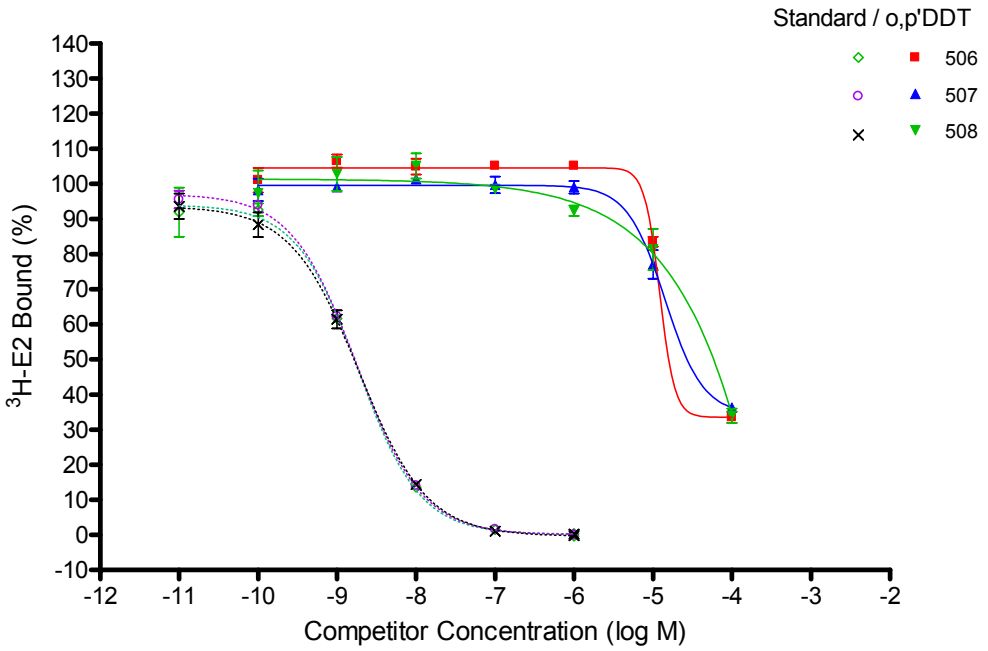


Figure 10-5. (continued)



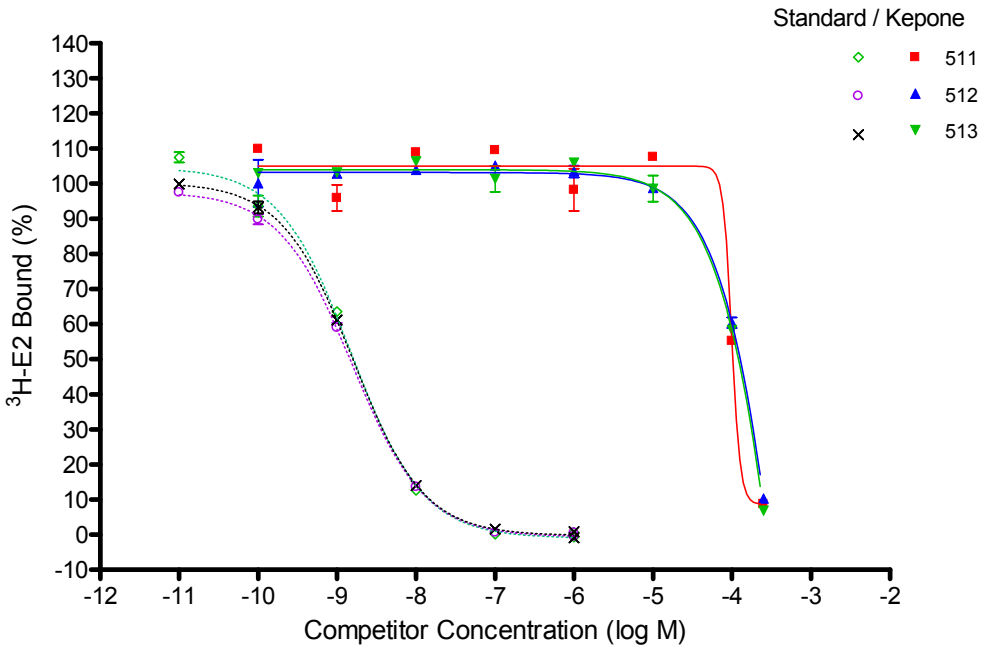
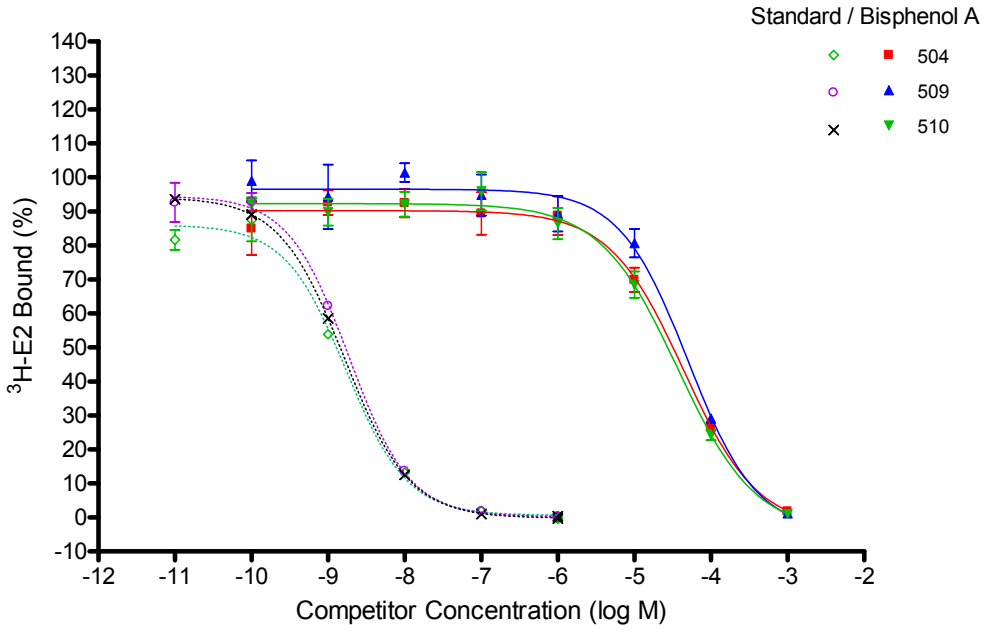
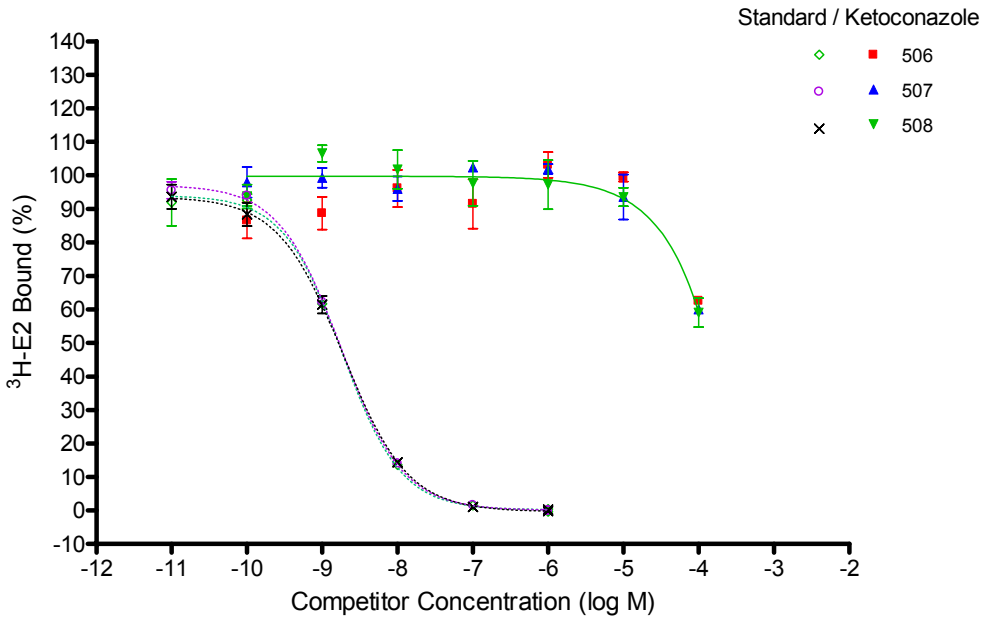


Figure 10-5. (continued)





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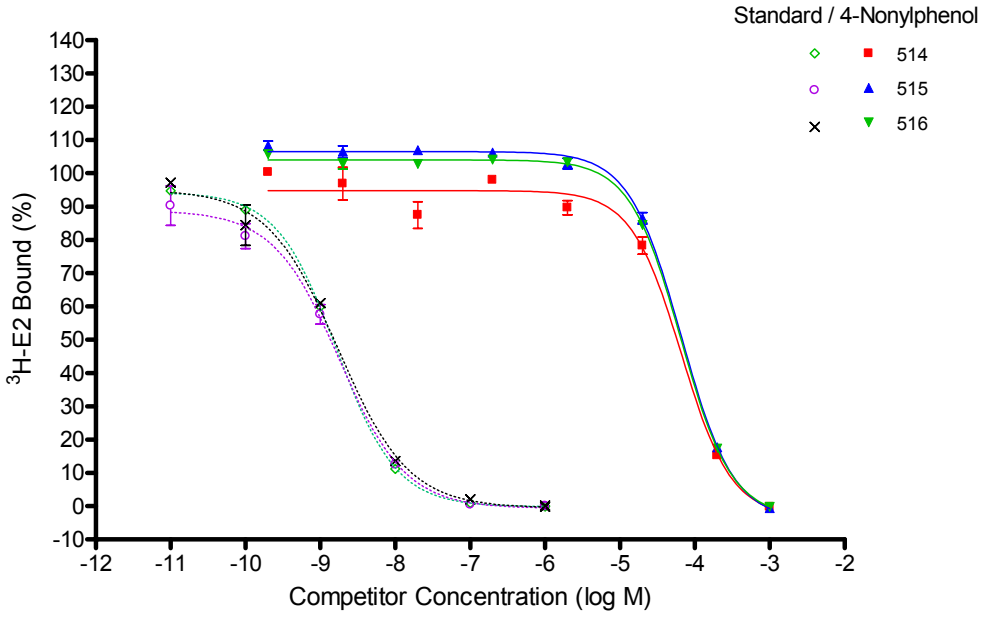


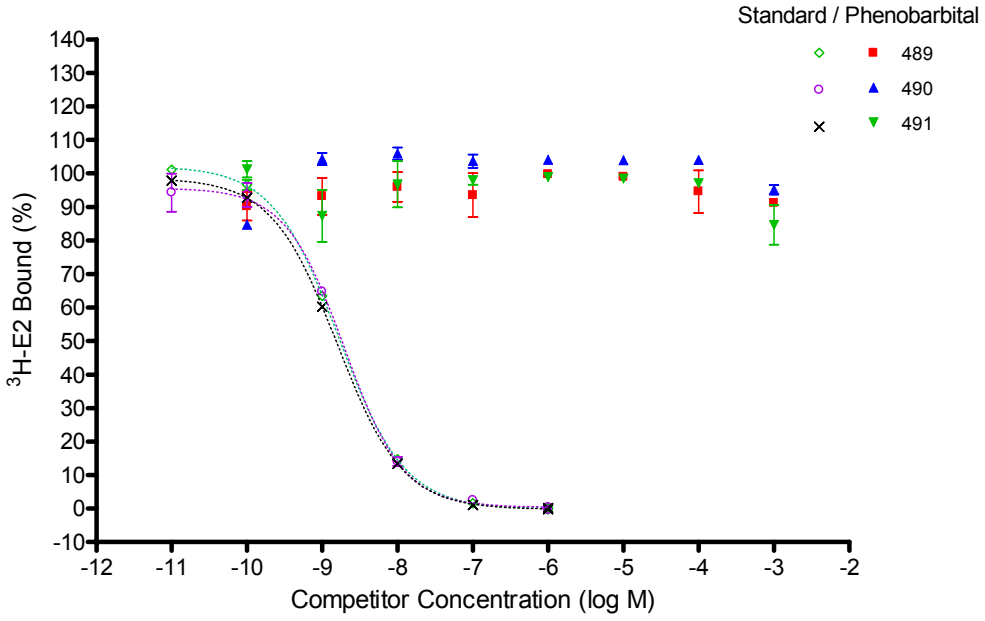
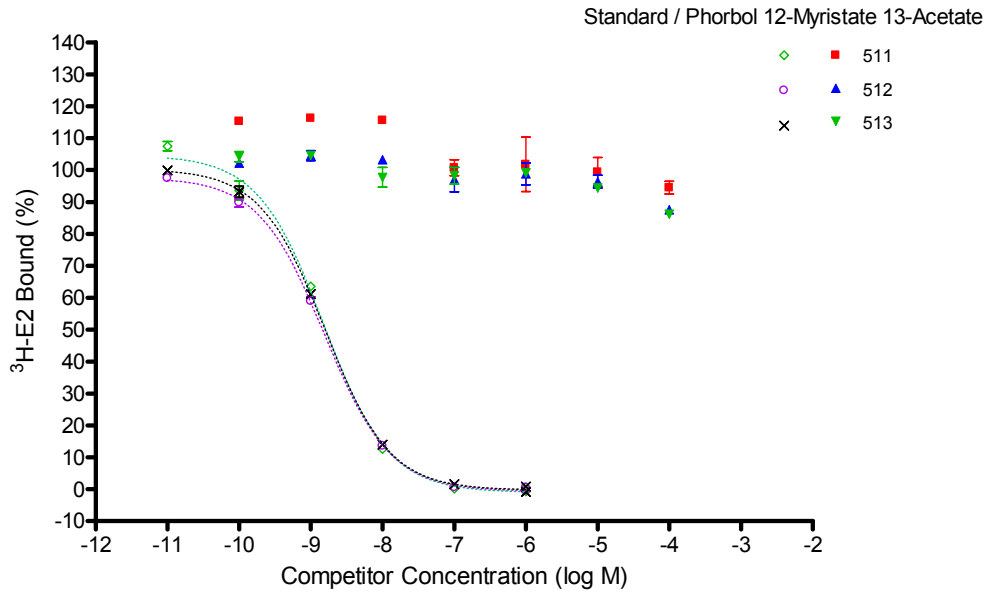


Figure 10-5. (continued)



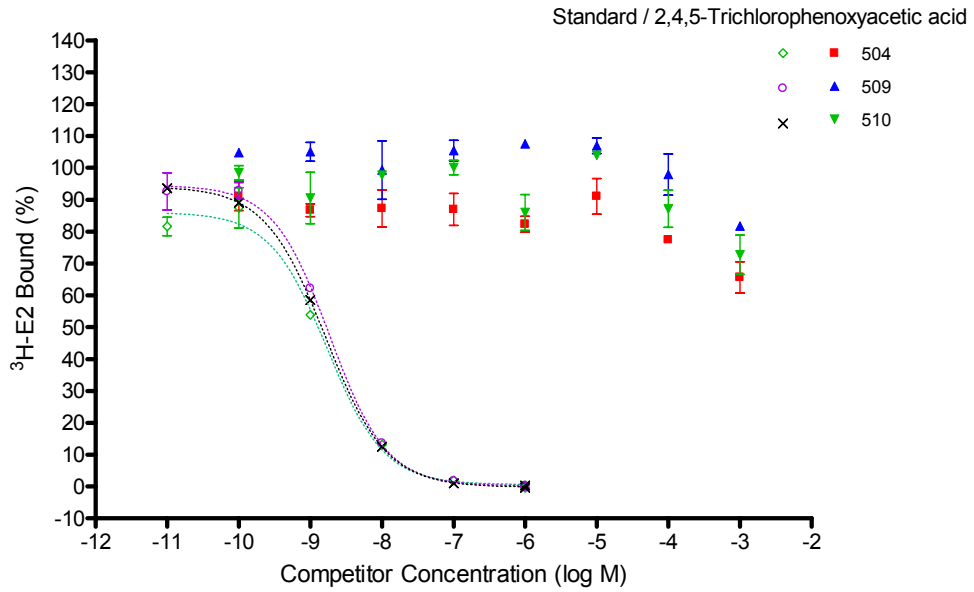
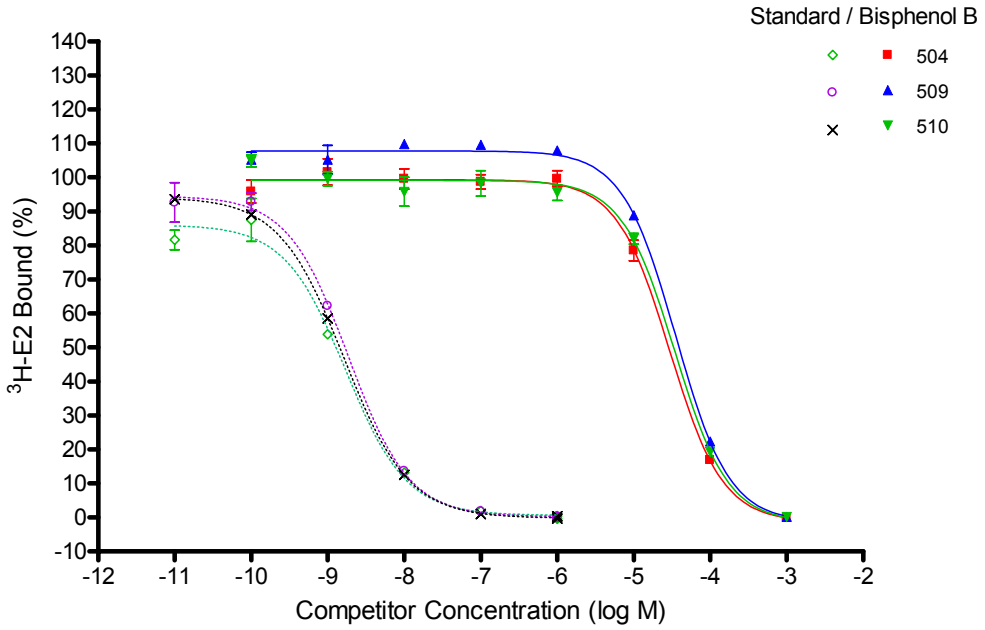


Figure 10-5. (continued)



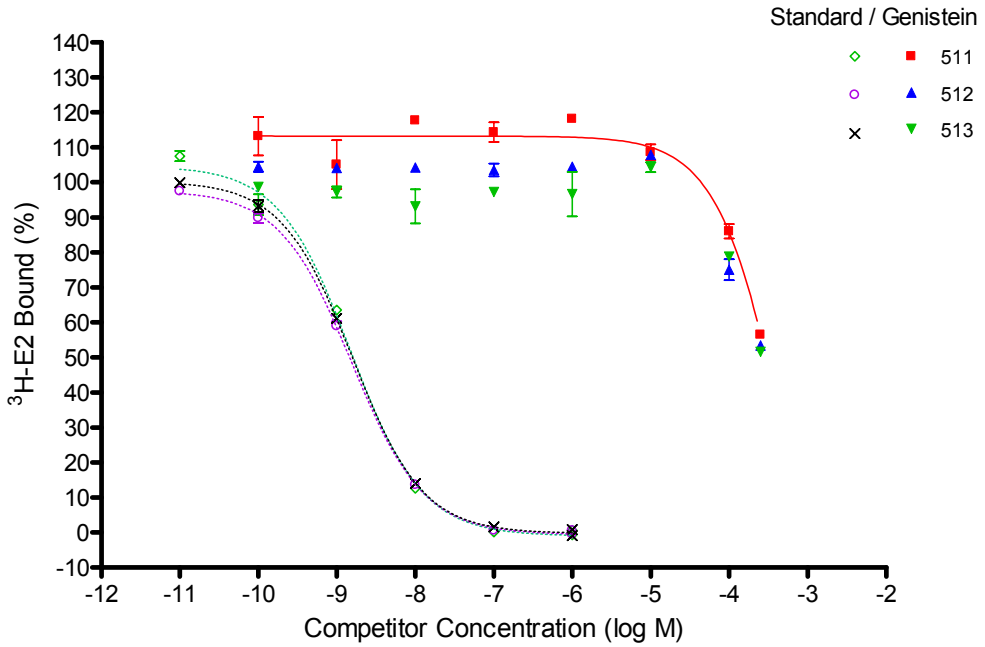


Figure 10-5. (continued)

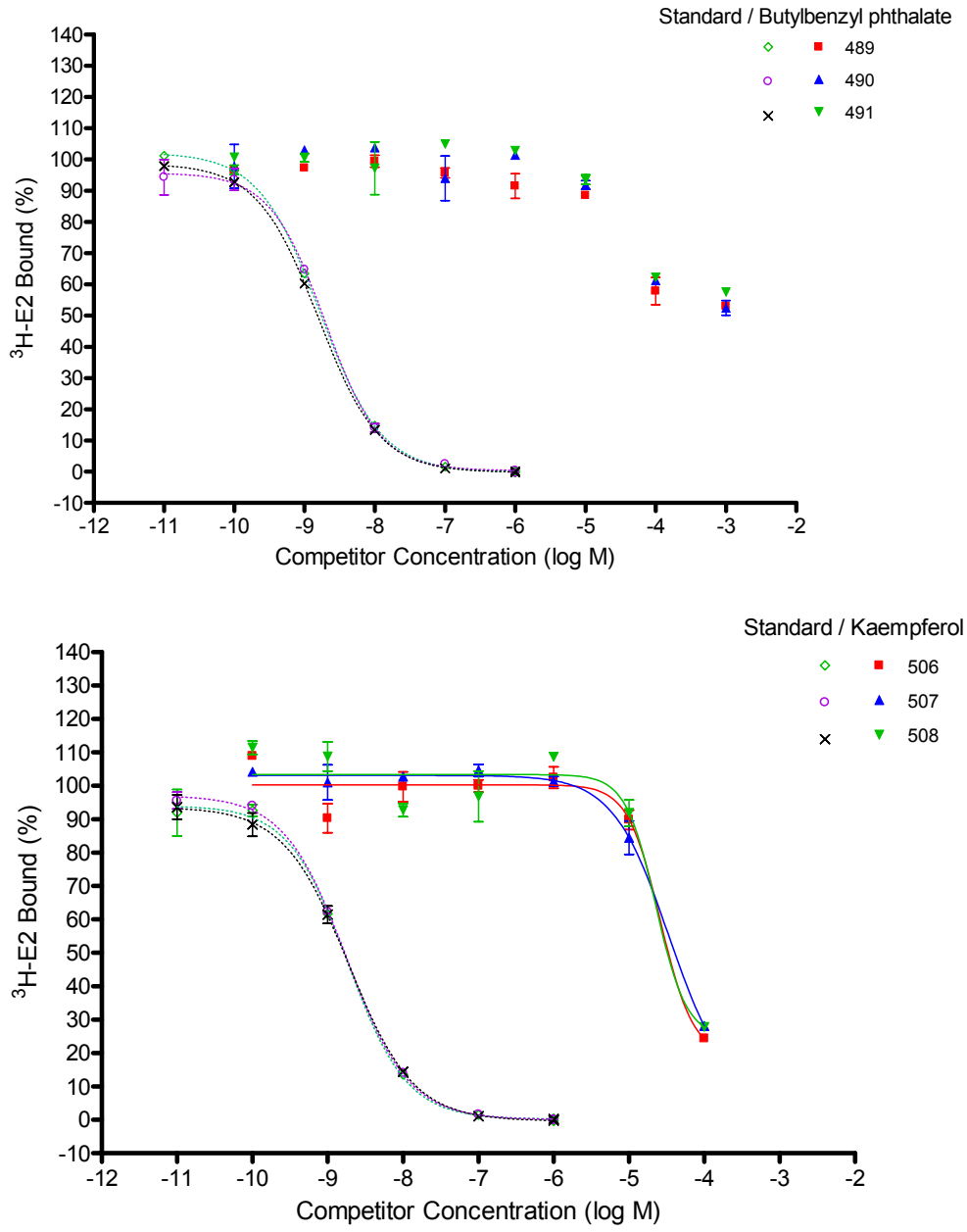
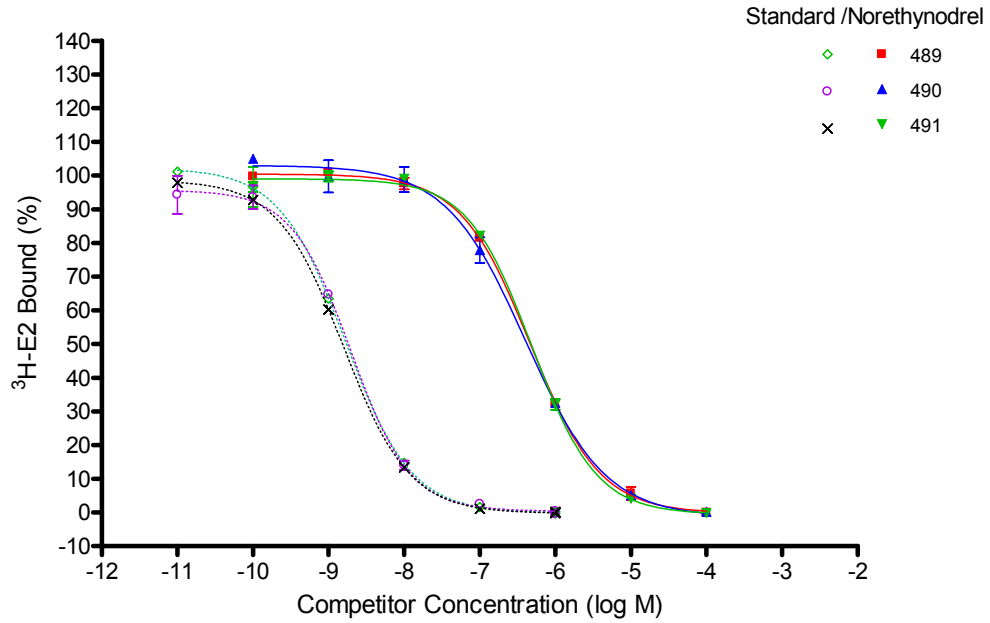


Figure 10-5. (continued)



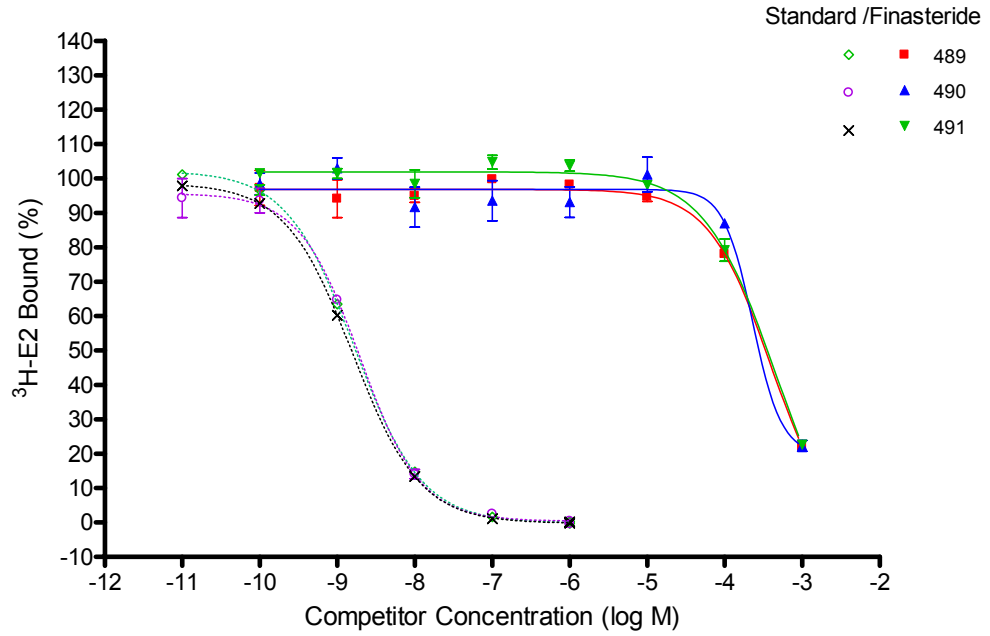
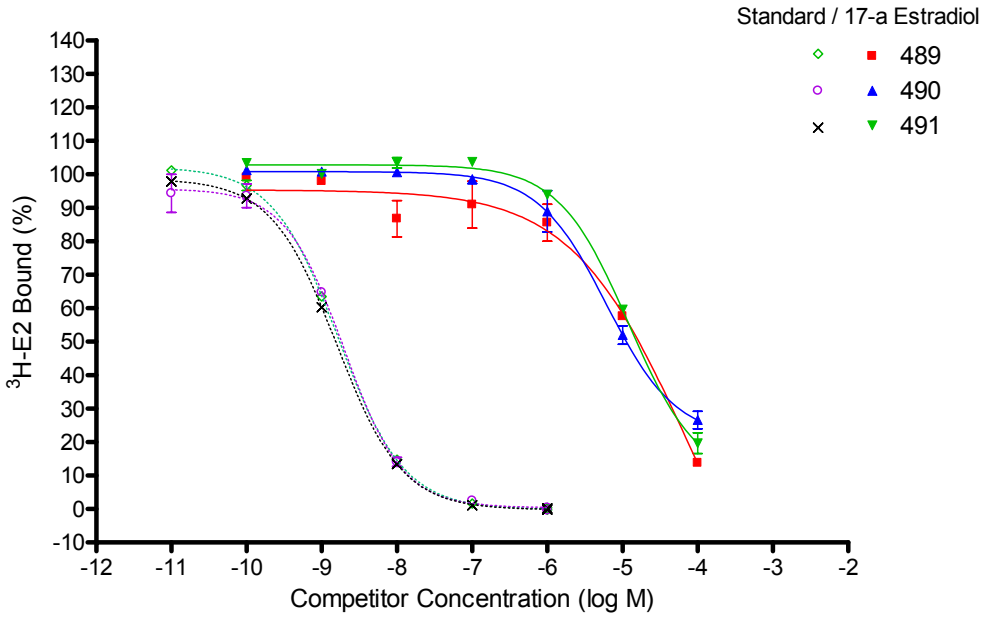


Figure 10-5. (continued)



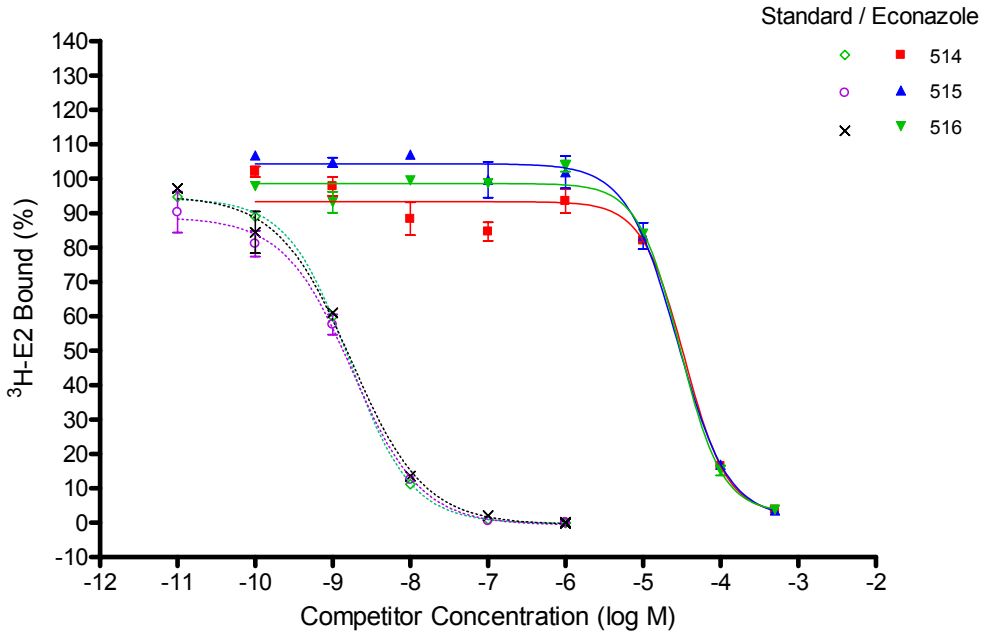


Figure 10-5. (continued)

11.0 PERFORMANCE CRITERIA AND DATA INTERPRETATION PROCEDURE

11.1 Performance Criteria

Performance criteria provide feedback to the testing laboratory and ensure reviewers of the data that the assay was conducted properly.

11.1.1 Saturation Binding Assay

Prior to conducting the competitive binding assay, the laboratory will conduct a saturation binding assay. When evaluating data from AR saturation binding assays, the following factors should be considered in judging the reasonableness of the results.

- As increasing concentrations of [3H]-R1881 were used, does the specific binding curve reach a plateau? Maximum specific binding must be reached indicating saturation of the AR with the ligand.

- Does the data produce a linear Scatchard plot (a plot of bound/free ligand as a function of specific binding)? Non-linear plots generally indicate a problem with the assay such as ligand depletion [concave plot] or incorrect assessment of non-specific binding [convex plot].

- Is the Kd within an acceptable range?. The values for Kd in the EPA validation program ranged from 0.8121 to 0.9698 nM.

- Is non-specific binding excessive? The non-specific binding for the assay optimization tasks ranged from 8.1 to 10.0%, well within the criteria.

Only after satisfactorily completing the saturation assay should the competitive binding assay be performed.

11.1.2 Competitive Binding Assay

The wide variation in results observed in the validation program lead EPA to conclude that performance criteria beyond the guidance listed above for the saturation binding assay, recommended by ICCVAM, were necessary to ensure that the assay generated valid results.

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. Criteria for the strong and weak positive controls and test chemicals were considered. In the end, EPA chose to specify tolerance intervals for just three parameters for the standard ligand (R1881) and the weak positive control (dexamethasone): top, bottom, and slope. No performance standards were adopted for test chemicals themselves due to the wide range of outcomes and variability possible for test chemicals: complete binding curves, partial binding curves, and no curve fit to the model.

To define a reasonable range for the top, bottom and slope of the strong and weak positive controls, EPA calculated tolerance intervals for each of these parameters based on the results of the validation studies in labs B, C, and E, Tasks 4 (determining the standard and weak positive competitive binding curves using the same cytosol supplied by the lead lab), 5 (determining the standard and weak positive competitive binding curves using different batches of cytosol prepared in participating labs) , and 6 (the interlaboratory validation studies conducted with the cytosol used in Task 5). This work was performed on the prior EDSP Work Assignment 4-11 (USEPA, 2006b,c).

The determination of performance criteria for the competitive binding assay is essentially 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. 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.[2] That is, using tolerance interval methodology, we are choosing performance criteria that we expect with 95% confidence that 80% of the laboratories will meet.

Tolerance intervals may either be two-sided or one-sided. Two-sided tolerance intervals are appropriate for "accuracy parameters" such as slope, top, and bottom 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 AR assay involves multiple "components of variation". Each laboratory in the reference set produced results in multiple tasks separated in time, in multiple runs within each task more closely bunched, and in variation within each run. Thus there were four components of variation:

[2] 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.

- 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 (both standard reference substance and the weak positive control) from the competitive binding assay. The tolerance intervals shown in Table 11-1 reflect the results of labs B, C, and E in the validation program. 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.

Dexamethasone, with a log IC_{50} of -4.3127, is a challenging weak positive concurrent control. Laboratories that produce consistently acceptable results with dexamethosone should have no trouble executing the assay.

Table 11-1. Tolerance Intervals

Chemical	Parameter	Lower limit	Upper Limit
Standard Curve	Slope	-1.2	-0.8
	Top (%)	82	114
	Bottom (%)	-2	+2
Weak Positive	Slope	-1.4	-0.6
	Top (%)	87	106
	Bottom (%)	-12	+12

11.1.3 Test Chemicals

Even though there are no standards for test chemicals themselves, professional judgment should be applied when reviewing the results of a test chemical run. Do the data appear to indicate that the chemical is a binder or a non-binder? Binders, in general, are characterized by curves that begin near 100% at low concentrations and fall from approximately 80% to 20% over two log units and asymptotically approach 0%; however, weak binders may produce only partial curves. Non-binders may produce more scatter that binders and would not be expected to fit the model.

If the data are unreasonable (e.g., there is a wide discrepancy between the values of triplicate points within a run or the mean of one set of triplicates is clearly an outlier) or vary too widely between runs, some troubleshooting may be in order to obtain more consistent results.

Data for a given run of test chemicals may be rejected if the performance criteria for the standards are not met, but EPA recognizes that test substances may give good data even if a control for a given run does not, and EPA will look at all of the data when making a judgment as to the acceptability or results

One problem that was frequently encountered during the validation program was insolubility of the test chemical at the highest concentration tested. The protocol calls for testing at a maximum of 10^{-3} M unless that is precluded by problems with solubility in which case a lower concentration should be used. Since obtaining a good fit for the bottom of the curve of weak binders depends on the results at the highest concentration tested, the protocol requires the highest dose upon retesting at $10^{-3.3}$ M (and if that fails at 10^{-4} M etc.) if the chemical is not soluble at 10^{-3} M unless a satisfactory binding curve can be obtained with lower concentrations.

Data quality should be assessed before and only acceptable data should be used in making any judgment as to whether the chemical is a binder or not.

11.2 Data Interpretation Procedure

The purpose of data interpretation procedures is to ensure that the data from the assay are assessed across laboratories in a scientifically sound and consistent manner. EPA is requiring that three independent runs be made for the assay and that only data of an acceptable quality should be used for making a binding determination. If a quantitative estimate of binding is desired the variance between logIC_{50} estimates of the triplicate runs should be small.

Test chemicals are classified as “binders”, “non-binders”, or “equivocal”. Intuitively if percent activity at the highest concentration is still relatively high (e.g. close to 100 percent) the test-chemical is considered to be a non-binder. If percent activity at the highest concentration is relatively low (e.g. close to 0 percent) the test-chemical is considered to be a binder

The **first option** considered were the recommendations of the ICCVAM Expert Panel:

- If a substance does not bind to the AR after testing to the limit concentration or to the maximum concentration based on its solubility, the substance is classified as “negative” for binding to the AR under the conditions of the test.

- A substance is classified as positive for binding to the AR if an IC50 value can be calculated. In general, the test substance should induce a sigmoid dose-response curve over at least a few log concentrations. A precipitous decline may reflect precipitation of the receptor rather than binding.

- Test substances that induce some reduction, but less than a 50% reduction in binding to the AR are classified as equivocal.

In reviewing this recommendation, it was not clear to EPA how the term “bind” would be defined in the first criterion. EPA presumed that, in combination with the sense of the third criterion, it meant that the test result is not significantly different from the controls. It was also

not clear what would be compared in determining binding. Would this involve a comparison of the response of each concentration level of the test substance to controls or a trend test?

With respect to the second criterion, an IC50 can be calculated by extrapolation when none of the data points, even at the highest concentration tested, lie below 50%. Given the definition of equivocal in the third criterion; however, it appears that some data points must actually lie below the 50% activity level. This definition is further confused by including the statement that a sigmoid dose-response curve running over a few log concentrations should be produced since this necessitates a nearly complete dose-response curve (i.e. running from approximately 100% to less than 20% activity). Clarification of these and other options for data interpretation criteria were explored by EPA.

A **second option** considered by EPA for data interpretation, like criterion 2 above, would require that for a chemical to be classified as a binder the data must fit a one-site competitive model with a complete dose-response curve having a top near 100% and a bottom near 0% with a slope such that the curve falls from about 80% to 20% over two log units. Chemicals failing to display a nearly complete curve but exceeding 50% displacement of the ligand would be classified as equivocal. Those with less than 50% displacement would be considered to be non-binders.

A **third option** examined by EPA focused principally on the binding curve generated by a four parameter model as the best estimator of binding. The guidance for data interpretation under this approach is as follows:

- If the best fit curve crosses 50%, the chemical is a binder.
- If the best fit curve is between 50% and 75%, the chemical is equivocal.
- If no curve can be fit and the data points are above 75%, the chemical is a non-binder.
- If no curve can be fit and the data points range below 75%, the chemical is equivocal.
- If there are no usable data points higher than 10^{-6}, the chemical is considered untestable.

A **fourth approach** studied by EPA would utilize the 95% confidence interval generated by the four-parameter Hill equation as the estimator of binding. In this approach the regression model can calculate the upper and lower 95% confidence limits for the dose-response curve generating a confidence interval after 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 upper confidence bound of the model at the highest concentration is less than 50%, the chemical will be classified as a "binder."
- If the lower confidence bound of the model at the highest concentration or of the highest concentration (if a model cannot be fit) is greater than 50%, the chemical will be classified as a "non-binder."

- If the CI of the model at the highest concentration or of the highest concentration (if the model cannot be fit) includes 50% as an interior point, the chemical will be classified as "equivocal."

EPA rejected the first approach as too vague and internally inconsistent. EPA also rejected the second approach in which a full curve was a requirement to define a binder. Such a stringent criteria would preclude identifying weak binders such as linuron and p,p'-DDE in all of the labs since only partial curves or curves flattening out before reaching 0% were obtained for these chemicals.

EPA analyzed two different ways of combining runs using the binding curve approach (Option 3) and three different ways of combining three runs using the 95% CI (Option 4).

In one sub-option under Option 3, the binding curve of each run is judged by the criteria under Option 3 to be a binder (B), equivocal (E), or non-binder (NB) and the two out of three that agree represent the overall classification. Thus the result B, B, E would be classed as a binder. Under the second sub-option, a simple numerical average of the activity levels in each of the three runs at the highest concentration tested is calculated and the result judged according to the criteria laid out under Option 3 above.

Like the first sub-option under Option 3, under Option 4 the simplest sub-option is one in which each run is evaluated against the criteria under Option 4 to be a binder (B), equivocal (E), or non-binder (NB) and the two out of three that agree represent the overall classification. A more sophisticated approach (the second sub-option) was to calculate the arithmetic average of the 95% CI of each run and apply the criteria to it. The third sub-option was most complex sub-option involved the calculation of an overall 95% confidence interval based on three runs by ANOVA While most of the results from Options 3 and 4 agreed, there were differences noted with some of the weak binders.

EPA concluded that although Option 4 had certain technical merits, it could not be used effectively unless criteria could be established for test chemicals themselves because large variances gave rise to wide confidence intervals and an unacceptably large number of chemicals would be judged equivocal under such circumstances. Of the variants under Option 3 the averaging method (sub-option 2) performed slightly better at identifying chemicals correctly under the interlaboratory validation study (Task 6) and also performed well for the testing of the supplementary chemicals. Thus EPA is establishing the data interpretation procedure shown in Table 11-2.

Table 11-2. Data Interpretation Procedure

Criteria		Classification
Data fit 4-parameter nonlinear regression model	Average curve across runs crosses 50%	Binder
	Average lower portion of curve across runs is between 50% and 75%	Equivocal
	Simple average lower portion of curve across runs is above 75%	Non-binder
Data do not fit the model	Simple average across runs of data points at highest concentration range below 75%	Equivocal
	Simple average across runs of data points at the highest concentration are above 75%	Non-binder
	No usable data points above 10^{-6} M	Un-testable

12.0 GENERAL CONCLUSIONS AND COMPLIANCE WITH VALIDATION CRITERIA

12.1 General Conclusions

The androgen receptor binding assay has long been in use but has not undergone optimization and standardization. Receptor binding is recognized as the key event in the intra and extra-cellular communication system known as the endocrine system. It has been well studied and the basic binding assay relies on competition between a test chemical and a natural or strong synthetic ligand. This interaction is modeled mathematically by a four parameter model based on the law of mass action. The studies conducted by EPA demonstrate conformity to the model and the biological and mathematical understanding of this assay are powerful reasons for its acceptance and use.

EPA has optimized and standardized a protocol for AR binding assay using cytosol from the rat prostate. Laboratory transferability of the test method was satisfactorily demonstrated by having four laboratories conduct saturation binding assay and competitive binding assays with R1881, the standard ligand chosen for the assay, with cytosol furnished by the lead laboratory, and with dexamethasone, which was chosen to be a challenging, weak positive control to be run concurrently with the test chemicals. It is clear from the data from these experiments that laboratory proficiency in the assay is challenged by the weak positive control and that more proficient laboratories are able to obtain consistent results across multiple runs of the assay.

Because cytosol could not be purchased, part of the protocol involved the preparation of cytosol by each participating laboratory. Of the four participating laboratories, only three successfully made active cytosol which was used in subsequent tasks and in the main interlaboratory study with 10 reference chemicals. Four laboratories, including the lead laboratory, participated in the main interlaboratory study. Laboratories had little trouble with the strong binders which produced full binding curves. As expected from the results with the weak positive control, laboratories had more difficulty with the weaker binders, especially linuron and p,p'-DDE. The shape of the curve was the expected sigmoid curve for linuron and p,p'-DDE for some laboratories and appeared truncated for others, but none of the laboratories reached 0% activity for all three runs. To obtain a high quality binding curve for weak binders, it is necessary to provide good quality data at high concentrations in order to define the bottom of the binding curve. The highest dose required in the protocol is 10-3 M, but this concentration exceeded the limits of solubility of some test substances. In these cases the protocol permits using a lower concentration than the standard "limit concentration" of 10^{-3} M. Choosing a half-log lower concentration in these circumstances proved to be a more effective strategy in obtaining a high quality binding curve than choosing a whole log interval. Thus, the requirement to try the 10-3.3 M concentration, if the chemical is insoluble at 10-3 M, has been added to the protocol.

The supplementary studies with 27 additional chemicals showed that a wide variety of chemicals could be tested with this protocol. Test substances included steroids, non-steroidal antiandrogens, synthetic androgens and antiandrogens, chlorinated pesticides, PAHs, flavanoids, phenols, and heterocyclic compounds. Most of these chemicals were selected with some indications that they were binders and most were found to be. Others were clearly non-binders in the assay with a few that were equivocal. However, since authoritative data were not available

for many of these chemicals, they could not be used to determine the specificity or selectivity of the assay.

Various options for performance criteria were explored for both standards and test chemicals. Based upon results of the validation study and supplementary chemicals, it was concluded that no universal criteria were applicable to test chemicals. EPA developed performance criteria for the strong and weak positive controls only, as consistent results on the positive controls and especially on the weak positive control, are good indicators that the laboratory is proficient in conducting the assay. Performance criteria were defined for the top, slope, and bottom of both the standard curve and the weak positive control. The performance criteria should be met for each run; however, it is important that test chemicals be subjected to a reasonableness test as well. In some cases data on test chemicals may judged acceptable when the standards do not meet criteria and vice versa.

EPA investigated several different options for data interpretation criteria. There is no perfect system. Some would insist on a complete curve for positive chemicals. EPA concluded that this was too restrictive and would miss weak positives. EPA concludes that the criterion of 50% or greater displacement of the binding curve provides a reasonable balance between false negatives and false positive results.

12.2 Validation Criteria Compliance

Table 12-1 lists the nine criteria for validation as outlined by ICCVAM (NIEHS, 1997), and whether or not each criterion has been met, along with discussion and explanation.

Table 12-1. Status of validation criteria

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.	Binding of an androgen to the androgen receptor a fundamental process in the endocrine system and is one potential mode by which chemicals can affect the endocrine system, so an assay that detects chemicals that bind to the AR and either mimic the natural ligand or competitively interfere with its binding is needed to identify such chemicals.

Principles	Criteria met/ not met: explanation and justification
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 39 additional chemicals were also tested in the lead laboratory and variability over time within the lead lab was determined for these chemicals, as discussed in Chapters 7.0 and 10.0.
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 49 chemicals representing a variety of chemical classes were tested under code (Chapters 7.0, 9.0, 10.0). 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 sources--*in vitro* competitive binding assays and transcriptional activation 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 distinguish between androgens and antiandrogens or indicate whether the chemical can initiate transcription; denaturation of the receptor may give false positive results; it cannot test poorly soluble chemicals; it utilizes animal tissues; and the assay has limited to no ability to metabolize xenobiotics. (Chapter 3.6)
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.

Principles	Criteria met/ not met: explanation and justification
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.

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APPENDIX A

Assay Protocol for the *In Vitro* Androgen Receptor (AR) Saturation and Competitive Binding Assay Using Rat Ventral Prostate Cytosol (RVPC)

OP No. NHEERL-H/RTD/EB/VW/2002-03-000

1.0 Purpose and Applicability

Determine ability of compound to compete with [^{3}H] ligand for binding in rat ventral prostate tissue homogenate.

2.0 Safety and Operating Precautions

All procedures with radioisotopes should follow the regulations and procedures as described in the Hazardous Agent Protocol and in the Radiation Safety Manual and Protocols for US EPA.

3.0 Animal Use

Follow U.S. EPA approved animal use protocols

4.0 Equipment and Materials

4.1 Equipment
Corning Stir/hot Plates
Pipettes
Balance
Polytron PT 35/10 Tissue Homogenizer
Vacuum Concentrator
Refrigerated General Laboratory Centrifuge
High-Speed Refrigerated Centrifuge (up to 30,000 x *g*)
pH Meter with Tris Compatible Electrode
Scintillation Counter
refrigerators

4.2 Chemicals
Tris HCL & Tris Base
Phenylmethylsulfonyl Fluoride (PMSF)
Glycerol 99%+
Sodium Molybdate
Ethylenediaminetetraacetic acid (EDTA); Disodium salt
-Dithiothreitol (DTT)
Potassium Chloride

Hydroxylapatite (HAP; BIO-RAD)
Scintillation Cocktail (Flow Scint III)
Ethyl Alcohol, anhydrous
Negative Control (Corticosterone)
[^{3}H]-R1881 (NEN; Purity >97%)
Radioinert R1881 (NEN)
Triamcinolone Acetonide
Steroids (Steraloids - recrystallized)
Optifluor

4.3 Supplies
20 ml Polypropylene Scintillation Vials
12 x 75 mm Borosilicate Glass Test Tubes
1000 ml graduated cylinders
500 ml Erlenmeyer flasks
pipette tips

5.0 Stock Preparations

5.1 Preparation of Stock Solutions for making TEDG Buffer

5.1.1 *EDTA Stock Solution*: Add 7.444g disodium EDTA to 100 ml ddH_2O = 200mM. Store at 4^{o}C. Use 750 ul/100ml TEDG buffer = 1.5 mM.

5.1.2 PMSF Stock Solution: Add 1.742 g PMSF to 100 ml ethanol = 100 mM. Store at 4oC. Use 1.00 ml/100ml TEDG buffer = 1.0 mM.

5.1.3 Sodium Molybdate Stock: Add 2.419 g sodium molybdate to 8.0 ml ddH2O in a 10 ml volumetric flask; bring the total volume to 10 mls = 1.0 M. Store at 4oC. Use 100ul/100ml TEDG buffer = 1.0 mM.

5.1.4 1 M Tris Buffer: Add 147.24 g Tris-HCL + 8.0 g Tris base to 800mls ddH2O in a volumetric flask; bring the final volume to 1.0 liter. Refrigerate to 4oC and pH (using 4oC pH standardizing solutions) the cooled solution to 7.4. Store at 4oC. Use 1.0 ml/100 ml TEDG buffer = 10mM. (50 mM Tris = 50 ml 1 M Tris/1 L ddH2O)

5.1.5 Potassium Chloride Stock Solution: Add 298.2 g KCL to 600 ml ddH2O in a 1000 ml volumetric flask; bring the total volume to 1000 ml = 4.0 M. Store at room temperature. Use 10.0 ml per 100 ml high-salt TEDG buffer = 0.4 M.

5.1.6 Add 15.4 mg DTT directly to 100 ml TEDG buffer the morning of the receptor isolation = 1.0 mM.

5.2 Preparation of Low-Salt TEDG Buffer (pH 7.4)

To make 100 ml of low-salt TEDG buffer add the following together in this order:

87.15 ml ddH_2O
1.0 ml 1M TRIS
10.0 ml glycerol
100 ul 1 M sodium molybdate
7 50 ul 200mM EDTA
1.0 ml 100mM PMSF
15.4 mg DTT (add immediately before use)

Check pH of the final solution to make sure it is 7.4 at 4^{o}C.

5.3 Preparation of 50 mM TRIS Buffer

Add 50.0 ml of 1.0 M TRIS to 950 ml ddH_2O. Store at 4^{o}C. Check pH of the final solution to make sure it is 7.4 at 4^{o}C.

5.4 Preparation of 60% Hydroxylapatite (HAP) Slurry

5.4.1 Shake BIO-RAD HT-GEL until all the HAP is in suspension (i.e., looks like milk). The evening before the receptor extraction, pour 100 ml (or an appropriate volume) into a 100 ml graduated cylinder, parafilm seal the top and place in the refrigerator for at least 2h.

5.4.2 Pour off the phosphate buffer supernatant, and bring the volume to 100 ml with 50 mM TRIS. Suspend the HAP by parafilm sealing the top of the graduated cylinder and inverting the cylinder several times. Place in the refrigerator overnight.

5.4.3 The next morning, repeat the washing steps x 2 with fresh 50 mM TRIS buffer.

5.4.4 After the last wash, add enough 50 mM TRIS to make the final solution a 60% slurry (i.e., if the volume of the settled HAP is 60 ml bring the final volume of the slurry to 100 mls with 50 mM TRIS).

5.4.5 Store at 4oC until ready for use in the extraction.

5.5 Preparation of [3H]-R1881 Stock Solutions

Dilute the original 1.0 mCi/ml stock of [^{3}H]-R1881 to 0.1 µM (i.e., 1 x 10^{-7} M). This is most easily accomplished by pipetting 1 ul of the stock solution for every specific activity unit (Ci/mmol) and diluting this to 10.0 mls with ethanol. Thus, if the specific activity of the stock vial is 86 Ci/mmol, then pipette 86.0 µl into an amber colored vial (i.e., R1881 is photosensitive) and add 10.0 ml ethanol to the vial; this solution is 1 x 10^{-7}M.

Note: [^{3}H]-R1881 stock solution and dilutions should be stored at -20^{0}C. Store stock solution in original protective vial and store dilutions in amber glass vials. This product is light-sensitive; care should be taken to minimize exposure to light.

5.6 Calculation Check and Dilutions

86 µl x 1.0 mCi/1000 µl = 86 x 10^{-3} mCi R1881 = 86 x 10^{-6} Ci R1881
86 x 10^{-6} Ci ÷ 86.0 Ci/mmol = 1 x 10^{-6} mmol R1881 = 1 x 10^{-9} moles R1881
1 x 10^{-9} moles R1881 ÷ 0.010 liters = 1 x 10^{-7} moles/liter = 0.1 µM

To prepare the 1 x 10^{-8} M stock simply make a 10-fold dilution of the 1 x 10^{-7} M stock (i.e., pipette 1.0 ml of the 1 x 10^{-7} M stock into a clean amber colored vial and add 9 ml ethanol = 0.01 uM).

To prepare the 1 x 10^{-9} M stock simply make a 10-fold dilution of the 1 x 10^{-8} M stock (i.e., pipette 1.0 ml of the 1 x 10^{-8} M stock into a clean amber colored vial and add 9 ml ethanol = 0.001 uM).

5.7 Preparation of 100X Radioinert R1881 Solutions

The R1881 comes as a 5.00 mg quantity. Dilute the original stock to 5.0 ml with ethanol = 3.52 mM. Take 56.82 µl and dilute to 20 ml in an amber vial with ethanol = 1 x 10^{-5} M R1881. This is the 10 µM radioinert R1881 stock.

To make the 1.0 µM radioinert R1881 stock, pipette 2 ml of the 10 µM stock into an amber vial and dilute to 20 ml with ethanol = 1 x 10^{-6}M = 1.0 µM radioinert R1881 stock. To make the 0.10 µM radioinert R1881 stock, pipette 2 ml of the 1 µM stock into an amber vial and dilute to 20 ml with ethanol = 1 x 10^{-7}M = 0.10 µM radioinert R1881 stock.

5.8 Compound Stock Preparations

5.8.1 Make stocks 30X above desired final concentration (this accounts for the use of 10 µl stock in 300 µl cytosol). Initial Stock of each test chemical solution will be diluted in 100% ethanol at a concentration of 3.0 x 102 M (i.e., 30 mM).

EXAMPLE:

4 (t) octyl phenol FW 206.33
1M = 206.33 g/L
1mM= 0.20633 mg/ml x 30 (30 mM desired final stock conc.) = 6.1899 mg/ml

2 ml Stock = 6.1899 mg x 2 = 12.37mg/2ml ethanol

5.8.2 Prepare serial dilutions of R1881 for standard curve in ethanol (100%) to yield the Initial Concentrations as indicated in Table 1.

Table 1: Standard Curve		
Standards	*Initial R1881 Concentration (Molar)*	**Final R1881 Concentration (Molar) in AR assay tube*
Negative Control	0	
0	0 (EtOH)	0
NSB	1×10^{-5}	1×10^{-6}
S1	3×10^{-6}	1×10^{-7}
S2	3×10^{-7}	1×10^{-8}
S3	3×10^{-8}	1×10^{-9}
S4	3×10^{-9}	1×10^{-10}
S5	3×10^{-10}	1×10^{-11}
** Final concentration = 10 ul of each standard is added to the assay tube, except for the NSB which is 30 ul.*		

5.8.3 Prepare serial dilutions of the test chemicals as indicated in Table 2.

Table 2: Test Chemical Concentrations (this subject to adjustment)		
Serial Dilutions of the Test Chemical	*Initial Concentration (Molar)*	**Final Concentration (Molar) in AR assay tube*
Concentration 1	3×10^{-3}	1×10^{-4}
Concentration 2	3×10^{-4}	1×10^{-5}
Concentration 3	3×10^{-5}	1×10^{-6}
Concentration 4	3×10^{-6}	1×10^{-7}
Concentration 5	3×10^{-7}	1×10^{-8}
Concentration 6	3×10^{-8}	1×10^{-9}
Tube 7	0 (vehicle only)	0
** Final concentration = 10 ul of each Initial Concentration of test chemical is added to the assay tube along with 300 ul of ventral prostate cytosol.*		

6.0 Tissue Homogenate Collection

6.1 Castrate 90 day old rats (60-90 day old acceptable; 90 day old preferred) as per laboratory animal protocols.

6.2 24 hours after castration, make low salt TEDG buffer and place in an ice-water bucket.

6.3 Kill rat and excise ventral prostate. Tissue should be trimmed of fat, weighed and the weights recorded.

6.4 Add low-salt TEDG buffer at 10ml/g tissue.

6.5 Mince tissues with Metzenbaum scissors until all pieces are small 1-2 mm cubes. Then homogenize the tissues at 4°C with a Polytron homogenizer using 5-sec bursts of the Polytron. [Note: place probe of the Polytron in TEDG buffer in an ice-water bath to cool it down prior to its use for homogenization. Recool probe as needed.]

6.6 Transfer homogenates to pre-cooled centrifuge tubes, balance, and centrifuge at 30,000x g for 30 minutes (i.e., 15, 262 rpm using JA-17/JA-21 Beckman rotors).

6.7 The supernatant contains the low-salt cytosolic receptor. Pool the supernatant from all rats. Aliquot into 5 ml and store -80°C until needed for assay. Discard after 6 months.

6.8 Determine the protein content for each batch of cytosol according to the method by Bradford (1976) using the commercially available BioRad Protein Assay Kit (BioRad Chemical Division, Richmond, CA). Protein concentrations usually range from 5.5 - 8 mg/ml in undiluted cytosol.

7.0. Saturation Radioligand Binding Assay

Prior to routinely conducting the AR competitive binding assays, the methods should be standardized within each laboratory. A series of saturation radioligand binding assays should be conducted to demonstrate AR specificity and saturation. Nonlinear regression analysis of these data and subsequent Scatchard plots will document AR binding affinity (K_d) and maximum specific binding number (B_{max}). Scatchard assay is to be conducted as follows:

Day 1

7.1 Set up tubes: 12x75 glass tubes and label for 8 concentrations in triplicate each with and without 100X inert (48 tubes total 1 through 48 below).

7.2 Add [3H] R1881 from the appropriate stock solutions to tubes as listed below:

7.3 Place 50 µl of 60 mM stock triamcinolone acetonide to ALL tubes.

7.4 An aliquot of each concentration of [^{3}H]R1881 should also be counted on scintillation counter to determine total counts added (tube # 49-72 below).

Saturation Assay Tube Layout

Position	Replicate	Tube Type Code	Hot Initial Concentration (nM)	Hot R1881 Volume (uL)	Hot Final Concentration (nM)	Cold Initial Concentration (nM)	Cold Volume (uL)	Cold Final Concentration (nM)	Triamcelenone Acetate (uL)	Cytosol (ul)
	1	H	10.0	7.5	0.25	—	—	—	50	300
	2	H	10.0	7.5	0.25	—	—	—	50	300
	3	H	10.0	7.5	0.25	—	—	—	50	300
	1	H	10.0	15	0.50	—	—	—	50	300
	2	H	10.0	15	0.50	—	—	—	50	300
	3	H	10.0	15	0.50	—	—	—	50	300
	1	H	10.0	21	0.70	—	—	—	50	300
	2	H	10.0	21	0.70	—	—	—	50	300
	3	H	10.0	21	0.70	—	—	—	50	300
	1	H	10.0	30	1.00	—	—	—	50	300
	2	H	10.0	30	1.00	—	—	—	50	300
	3	H	10.0	30	1.00	—	—	—	50	300
	1	H	10.0	45	1.50	—	—	—	50	300
	2	H	10.0	45	1.50	—	—	—	50	300
	3	H	10.0	45	1.50	—	—	—	50	300
	1	H	100.0	7.5	2.50	—	—	—	50	300
	2	H	100.0	7.5	2.50	—	—	—	50	300
	3	H	100.0	7.5	2.50	—	—	—	50	300
	1	H	100.0	15	5.00	—	—	—	50	300
	2	H	100.0	15	5.00	—	—	—	50	300
	3	H	100.0	15	5.00	—	—	—	50	300
	1	H	100.0	30	10.00	—	—	—	50	300
	2	H	100.0	30	10.00	—	—	—	50	300
	3	H	100.0	30	10.00	—	—	—	50	300
	1	HC	10.0	7.5	0.25	1.00	7.5	25	50	300
	2	HC	10.0	7.5	0.25	1.00	7.5	25	50	300
	3	HC	10.0	7.5	0.25	1.00	7.5	25	50	300
	1	HC	10.0	15	0.5	1.00	15	50	50	300
	2	HC	10.0	15	0.5	1.00	15	50	50	300
	3	HC	10.0	15	0.5	1.00	15	50	50	300
	1	HC	10.0	21	0.7	1.00	21	70	50	300
	2	HC	10.0	21	0.7	1.00	21	70	50	300
	3	HC	10.0	21	0.7	1.00	21	70	50	300
	1	HC	10.0	30	1	1.00	30	100	50	300
	2	HC	10.0	30	1	1.00	30	100	50	300

Saturation Assay Tube Layout

Position	Replicate	Tube Type Code	Hot Initial Concentration (nM)	Hot R1881 Volume (uL)	Hot Final Concentration (nM)	Cold Initial Concentration (nM)	Cold Volume (uL)	Cold Final Concentration (nM)	Triamcelenone Acetate (uL)	Cytosol (ul)
	3	HC	10.0	30	1	1.00	30	100	50	300
	1	HC	10.0	45	1.5	1.00	45	150	50	300
	2	HC	10.0	45	1.5	1.00	45	150	50	300
	3	HC	10.0	45	1.5	1.00	45	150	50	300
	1	HC	100.0	7.5	2.5	10.00	7.5	250	50	300
	2	HC	100.0	7.5	2.5	10.00	7.5	250	50	300
	3	HC	100.0	7.5	2.5	10.00	7.5	250	50	300
	1	HC	100.0	15	5	10.00	15	500	50	300
	2	HC	100.0	15	5	10.00	15	500	50	300
	3	HC	100.0	15	5	10.00	15	500	50	300
	1	HC	100.0	30	10	10.00	30	1000	50	300
	2	HC	100.0	30	10	10.00	30	1000	50	300
	3	HC	100.0	30	10	10.00	30	1000	50	300
	1	Hot	10.0	7.5	0.03	—	—	—	—	—
	2	Hot	10.0	7.5	0.03	—	—	—	—	—
	3	Hot	10.0	7.5	0.03	—	—	—	—	—
	1	Hot	10.0	15	0.06	—	—	—	—	—
	2	Hot	10.0	15	0.06	—	—	—	—	—
	3	Hot	10.0	15	0.06	—	—	—	—	—
	1	Hot	10.0	21	0.08	—	—	—	—	—
	2	Hot	10.0	21	0.08	—	—	—	—	—
	3	Hot	10.0	21	0.08	—	—	—	—	—
	1	Hot	10.0	30	0.10	—	—	—	—	—
	2	Hot	10.0	30	0.10	—	—	—	—	—
	3	Hot	10.0	30	0.10	—	—	—	—	—
	1	Hot	10.0	45	0.30	—	—	—	—	—
	2	Hot	10.0	45	0.30	—	—	—	—	—
	3	Hot	10.0	45	0.30	—	—	—	—	—
	1	Hot	100.0	7.5	0.60	—	—	—	—	—
	2	Hot	100.0	7.5	0.60	—	—	—	—	—
	3	Hot	100.0	7.5	0.60	—	—	—	—	—
	1	Hot	100.0	15	1.00	—	—	—	—	—
	2	Hot	100.0	15	1.00	—	—	—	—	—
	3	Hot	100.0	15	1.00	—	—	—	—	—
	1	Hot	100.0	30	3.00	—	—	—	—	—
	2	Hot	100.0	30	3.00	—	—	—	—	—
	3	Hot	100.0	30	3.00	—	—	—	—	—

7.5 Place tubes in speed-vac (Tubes 1-48) and dry the tubes according to instructions. Remove when dry and place on ice.

7.6 Cytosol should be diluted with the low salt TEDG buffer to a protein concentration of 1.2 mg per 300 ul assay (in our laboratory this was about a 1:1 dilution). Add 300 ul of diluted prostate cytosol to all tubes (1-48). Keep tubes and cytosol on ice at all times during this procedure. Gently vortex and place tubes in refrigerator overnight in rotor (20hr).

7.7 Before leaving for the day, prepare the first wash of the HAP slurry as described in section 5.4 above. If desired, label the HAP tubes and the scintillation vials to be used the following day.

Day 2

7.8 Continue as with Day 2 protocol for binding assay below in section 9.0.

8.0 Assay procedure for chemicals: Day 1

8.1 Set up tubes: 12x75 mm glass tubes

8.1.1 Label sufficient glass tubes as needed for the assay.

8.1.2 Add 30ul of 0.01uM [3H] R1881 (1 x 10-8M) and 50ul triamcinolone acetonide (60mM stock) to ALL tubes

8.1.3 For 3 tubes at beginning of assay and at end of assay, also add 100x inert R1881 (30ul of 1.0uM, i.e., 1 x 10-6M). These tubes are for determining nonspecific binding.

8.1.4 Place tubes in speed-vac and dry the tubes according to instructions. Remove when dry.

8.2 Add 10ul of compound stocks (see 5.8 for concentrations 1-7 in duplicate)

8.3 Remove aliquot of prostate cytosol and thaw on ice. Cytosol should be diluted with ice-cold low-salt TEDG buffer to give a protein concentration of 1.2 mg per 300 ul assay tube. (In our lab this is usually about a 1:1 dilution or 150 ul cytosol:150 ul TEDG buffer)

8.4 Add 300 ul of diluted cytosol to every tube ON ICE. Gently vortex and place tubes in refrigerator overnight in rotor (20hr).

8.5 Before leaving for the day, prepare the first wash of the HAP slurry as described in section 5.4 above.

Competitive Assay Tube Layout - One Test Chemical (Weak Positive)

Position	Replicate	Competitor	Competitor Code	Concentration Code	Competitor Initial Concentration (M)	Cytosol (uL)	Tracer (Hot R1881) Volume (uL)	Competitor Volume (uL)	triamcelenone Volume (uL)	Competitor Final Concentration (M)	Aliquot (uL)	HAP (500 ul)
1		ethanol	EtOH	0	—	300	30	10	50	—	100	500
2		ethanol	EtOH	0	—	300	30	10	50	—	100	500
3		ethanol	EtOH	0	—	300	30	10	50	—	100	500
4		Inert R1881	NSB		1.00E-05	300	30	30	50	1.0E-06	100	500
5		Inert R1881	NSB		1.00E-05	300	30	30	50	1.0E-06	100	500
6		Inert R1881	NSB		1.00E-05	300	30	30	50	1.0E-06	100	500
7		Inert R1881	S	1	3.00E-06	300	30	10	50	1.0E-07	100	500
8		Inert R1881	S	1	3.00E-06	300	30	10	50	1.0E-07	100	500
9		Inert R1881	S	1	3.00E-06	300	30	10	50	1.0E-07	100	500
10		Inert R1881	S	2	3.00E-07	300	30	10	50	1.0E-08	100	500
11		Inert R1881	S	2	3.00E-07	300	30	10	50	1.0E-08	100	500
12		Inert R1881	S	2	3.00E-07	300	30	10	50	1.0E-08	100	500
13		Inert R1881	S	3	3.00E-08	300	30	10	50	1.0E-09	100	500
14		Inert R1881	S	3	3.00E-08	300	30	10	50	1.0E-09	100	500
15		Inert R1881	S	3	3.00E-08	300	30	10	50	1.0E-09	100	500
16		Inert R1881	S	4	3.00E-09	300	30	10	50	1.0E-10	100	500
17		Inert R1881	S	4	3.00E-09	300	30	10	50	1.0E-10	100	500
18		Inert R1881	S	4	3.00E-09	300	30	10	50	1.0E-10	100	500
19		Inert R1881	S	5	3.00E-10	300	30	10	50	1.0E-11	100	500
20		Inert R1881	S	5	3.00E-10	300	30	10	50	1.0E-11	100	500
21		Inert R1881	S	5	3.00E-10	300	30	10	50	1.0E-11	100	500
22		Weak Positive	P	1	3.00E-02	300	30	10	50	1.E-03	100	500
23		Weak Positive	P	1	3.00E-02	300	30	10	50	1.E-03	100	500
24		Weak Positive	P	1	3.00E-02	300	30	10	50	1.E-03	100	500
25		Weak Positive	P	2	3.00E-03	300	30	10	50	1.E-04	100	500
26		Weak Positive	P	2	3.00E-03	300	30	10	50	1.E-04	100	500
27		Weak Positive	P	2	3.00E-03	300	30	10	50	1.E-04	100	500
28		Weak Positive	P	3	3.00E-04	300	30	10	50	1.E-05	100	500
29		Weak Positive	P	3	3.00E-04	300	30	10	50	1.E-05	100	500
30		Weak Positive	P	3	3.00E-04	300	30	10	50	1.E-05	100	500
31		Weak Positive	P	4	3.00E-05	300	30	10	50	1.E-06	100	500
32		Weak Positive	P	4	3.00E-05	300	30	10	50	1.E-06	100	500
33		Weak Positive	P	4	3.00E-05	300	30	10	50	1.E-06	100	500

Competitive Assay Tube Layout - One Test Chemical (Weak Positive)

Position	Replicate	Competitor	Competitor Code	Concentration Code	Competitor Initial Concentration (M)	Cytosol (uL)	Tracer (Hot R1881) Volume (uL)	Competitor Volume (uL)	triamcelenone Volume (uL)	Competitor Final Concentration (M)	Aliquot (uL)	HAP (500 ul)
34		Weak Positive	P	5	3.00E-06	300	30	10	50	1.E-07	100	500
35		Weak Positive	P	5	3.00E-06	300	30	10	50	1.0E-07	100	500
36		Weak Positive	P	5	3.00E-06	300	30	10	50	1.0E-07	100	500
37		Weak Positive	P	6	3.00E-07	300	30	10	50	1.0E-08	100	500
38		Weak Positive	P	6	3.00E-07	300	30	10	50	1.0E-08	100	500
39		Weak Positive	P	6	3.00E-07	300	30	10	50	1.0E-08	100	500
40		Weak Positive	P	7	3.00E-08	300	30	10	50	1.0E-09	100	500
41		Weak Positive	P	7	3.00E-08	300	30	10	50	1.0E-09	100	500
42		Weak Positive	P	7	3.00E-08	300	30	10	50	1.0E-09	100	500
43		Weak Positive	P	8	3.00E-09	300	30	10	50	1.0E-10	100	500
44		Weak Positive	P	8	3.00E-09	300	30	10	50	1.0E-10	100	500
45		Weak Positive	P	8	3.00E-09	300	30	10	50	1.0E-10	100	500
46		unknown 1	C	1	3.00E-02	300	30	10	50	1.0E-03	100	500
47		unknown 1	C	1	3.00E-02	300	30	10	50	1.0E-03	100	500
48		unknown 1	C	1	3.00E-02	300	30	10	50	1.0E-03	100	500
49		unknown 1	C	2	3.00E-03	300	30	10	50	1.0E-04	100	500
50		unknown 1	C	2	3.00E-03	300	30	10	50	1.0E-04	100	500
51		unknown 1	C	2	3.00E-03	300	30	10	50	1.0E-04	100	500
52		unknown 1	C	3	3.00E-04	300	30	10	50	1.0E-05	100	500
53		unknown 1	C	3	3.00E-04	300	30	10	50	1.0E-05	100	500
54		unknown 1	C	3	3.00E-04	300	30	10	50	1.0E-05	100	500
55		unknown 1	C	4	3.00E-05	300	30	10	50	1.0E-06	100	500
56		unknown 1	C	4	3.00E-05	300	30	10	50	1.0E-06	100	500
57		unknown 1	C	4	3.00E-05	300	30	10	50	1.0E-06	100	500
58		unknown 1	C	5	3.00E-06	300	30	10	50	1.0E-07	100	500
59		unknown 1	C	5	3.00E-06	300	30	10	50	1.0E-07	100	500
60		unknown 1	C	5	3.00E-06	300	30	10	50	1.0E-07	100	500
61		unknown 1	C	6	3.00E-07	300	30	10	50	1.0E-08	100	500
62		unknown 1	C	6	3.00E-07	300	30	10	50	1.0E-08	100	500
63		unknown 1	C	6	3.00E-07	300	30	10	50	1.0E-08	100	500
64		unknown 1	C	7	3.00E-08	300	30	10	50	1.0E-09	100	500
65		unknown 1	C	7	3.00E-08	300	30	10	50	1.0E-09	100	500
66		unknown 1	C	7	3.00E-08	300	30	10	50	1.0E-09	100	500
67		unknown 1	C	8	3.00E-09	300	30	10	50	1.0E-10	100	500

Competitive Assay Tube Layout - One Test Chemical (Weak Positive)

Position	Replicate	Competitor	Competitor Code	Concentration Code	Competitor Initial Concentration (M)	Cytosol (uL)	Tracer (Hot R1881) Volume (uL)	Competitor Volume (uL)	triamcelenone Volume (uL)	Competitor Final Concentration (M)	Aliquot (uL)	HAP (500 ul)
68		unknown 1	C	8	3.00E-09	300	30	10	50	1.0E-10	100	500
69		unknown 1	C	8	3.00E-09	300	30	10	50	1.0E-10	100	500
70		ethanol	EtOH	0	—	300	30	10	50	—	100	500
71		ethanol	EtOH	0	—	300	30	10	50	—	100	500
72		ethanol	EtOH	0	—	300	30	10	50	—	100	500
73		Inert R1881	NSB		1.00E-05	300	30	30	50	1.0E-06	100	500
74		Inert R1881	NSB		1.00E-05	300	30	30	50	1.0E-06	100	500
75		Inert R1881	NSB		1.00E-05	300	30	30	50	1.0E-06	100	500
76		none	Hot		—	—	30	—	—	—	—	—
77		none	Hot		—	—	30	—	—	—	—	—
78		none	Hot		—	—	30	—	—	—	—	—
79		none	Hot		—	—	30	—	—	—	—	—
80		none	Hot		—	—	30	—	—	—	—	—
81		none	Hot		—	—	30	—	—	—	—	—

8.6 Label the HAP tubes and the scintillation vials to be used the following day - see underlines below.

9.0 Assay Procedure: Day 2

9.1 The following morning, wash the HAP as described in section 5.4 above, dilute with 50 mM TRIS to yield a 60% slurry, and transfer contents to a 100 ml Erlenmeyer flask. Place a stir bar in the flask and place the flask into a beaker containing ice-water; stir the HAP slurry by placing the beaker on a magnetic stir plate.

9.2 While the HAP slurry is constantly being stirred, pipette 500 µl of the HAP slurry into clean pre-labelled 12 x 75 mm glass test tubes. Place these tubes in a rack in an ice-water bath prior to pipetting the HAP slurry and keep them in the ice-water bath for the remainder of the assay.

9.3 One HAP tube should be prepared for each incubation tube.

9.4 Take the incubation tubes from the refrigerator and place them in an ice-water bath with the HAP tubes. Pipette 100 µl from each of the incubation tubes into the appropriate pre-labelled tubes containing HAP. Repeat for all tubes. Quickly take each rack from the ice-water bath and vortex each rack of tubes using the whole-rack vortex unit. Place racks back into the ice-water bath and vortex as above every 5 minutes for 20 minutes.

9.5 Centrifuge the HAP tubes for 2-3 minutes at 4oC and 600 x g (1780 rpm in a Beckman GLC refrigerated centrifuge). Place the tubes back into the rack and into the ice-water bath.

9.6 While the tubes remain in the ice-water bath, aspirate the supernatant from each tube using a 9 inch pipette connected to an aspiration apparatus as per the radiation safety protocol.

9.7 Add 2 ml of 50 mM TRIS to each tube, vortex and centrifuge at 600 x g as above. Place the tubes into decanting racks in an ice-water bath and decant the supernatant TRIS wash into the radiation safety container. Gently tap the tube openings on a clean adsorbent diaper, place the rack back in the ice-water bath and add 2 mls of 50 mM TRIS.

9.8 Repeat the TRIS washing procedure 3 or 4 times (to be determined empirically) keeping the tubes at 4oC at all times.

9.9 Following the last wash and decanting, add 2 mls of ethanol to each tube, vortex 3 times at 5 minute intervals and centrifuge the tubes at 600 x g for 10 minutes. Decant the supernatants into pre-labelled 20 ml scintillation vials. Add 14 ml of Optifluor scintillation cocktail and count samples using the single label DPM program with quench correction.

10.0 Data Processing

10.1 Free Concentration of [3H]-R1881

Multiply the DPM in the total counts tubes by 1.8047×10^{-5}. This value will yield the free concentration (i.e., nM) of [^{3}H]-R1881 initially present in each incubation tube.

Calculation Check -

$$\frac{\text{X DPM}}{\text{2.22 X } 10^{12} \text{ DPM/CI *83.2 CI/MMOLE}} = \text{X * 5.4141 X } 10^{-15} \text{ MMOLE}$$

$$\frac{\text{X* 5.4141 X } 10^{-15} \text{ MOLES}}{\text{1000 MMOLE/MOLE}} = \text{X * 5.4141 X} 10^{-18} \text{ MOLES}$$

$$\frac{\mathbf{X * 5.4141\ X10^{-18}\ MOLES}}{\mathbf{0.0003\ LITERS}} = \mathbf{X * 1.8047\ X\ 10^{-14}\ MOLES/LITER}$$

$$\frac{\mathbf{X * 1.8047\ X\ 10^{-14}\ MOLES/LITER}}{\mathbf{1\ X\ 10^{-9}\ MOLES/NMOLE}} = \mathbf{X * 1.8047\ X\ 10^{-5}\ NM}$$

*Note this value will be the Specific activity of the radioligand ([^{3}H]R1881) used in the assay.

10.2 Calculation of Total, Nonspecific and Specific [3H]-R1881 Binding

10.2.1 Total binding is calculated by multiplying the DPM from the tubes that contained only radiolabelled R1881 x (1.6242 x 10-2). This value will be total binding in fmoles.

10.2.2 Nonspecific binding is calculated by multiplying the DPM from the tubes containing radiolabelled R1881 + 100-fold molar excess of radioinert R1881 x (1.6242 x 10-2). This value will be nonspecific binding in fmoles.

10.2.3 Specific binding is calculated by subtracting nonspecific binding from total binding i.e., fmoles total binding - fmoles nonspecific binding = specific binding in fmoles.

10.3 Graphical Presentation of the Data

10.3.1 Standard Curve and Test Chemical Competitive Binding Curves: Data for the standard curve and each test chemical will be plotted as the percent 3H_R1881 bound versus the molar concentration. Estimates of the IC50s will be determined using appropriate non linear curve fitting software such as GraphPad PRISM (GraphPad Software, Inc., San Diego, CA). A Scatchard Analysis may also be performed for the standard curve using R1881 to demonstrate that the assay meets acceptable QA standards.

10.3.2 Relative Binding Affinity: The RBA for each competitor should be calculated by dividing the IC50 for R1881 by the IC50 of the competitor and expressing as a percent (e.g., RBA for R1881 =100 %).

10.3.3 Maximal binding capacity (Bmax) and association/dissociation constants (Ka / Kd) can be estimated using a number of commercially available iterative nonlinear regression analysis programs. One of the better programs was developed by Munson and Rodbard and is called LIGAND.

11. Performance Criteria

11.1 Reasonableness checks for the saturation binding assay. When evaluating data from AR saturation binding assays, the following factors should be considered in judging the reasonableness of the results.

11.1.1 As increasing concentrations of [3H]-R1881 were used, does the specific binding curve reach a plateau? Maximum specific binding must be reached indicating saturation of the AR with the ligand.

11.1.2 Does the data produce a linear Scatchard plot (a plot of bound/free ligand as a function of specific binding)?

11.1.3 Is the Kd within an acceptable range? The values for Kd in the EPA validation program ranged from 0.8121 to 0.9698 nM.

11.1.4 Is non-specific binding excessive? The non-specific binding for the assay in the EPA validation program ranged from 8.1 to 10.0%. The value for non-specific binding should be less than 50% of the total binding.

11.2 Performance standards for the standard curve and weak positive control. For a run to be considered acceptable, the output from the model should fall within the following tolerance intervals; however, minor deviations will not invalidate an assay.

Chemical	Parameter	Lower limit	Upper Limit
Standard Curve	Slope	-1.2	-0.8
	Top (%)	82	114
	Bottom (%)	-2	+2
Weak Positive	Slope	-1.4	-0.6
	Top (%)	87	106
	Bottom (%)	-12	+12

12. Sources

1. Nonneman, D.J., Ganjam, V.K., Welshons, W.V., and Vom Saal, F.S. (1992). *Biol Reprod* 47: 723-729.

2. Segel, I.H. (1975). Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems; First edition. New York: John Wiley & Sons, Inc.

3. Munson, P.J., and Rodbard, D. (1980). *Anal Biochem.* 107: 220-239.

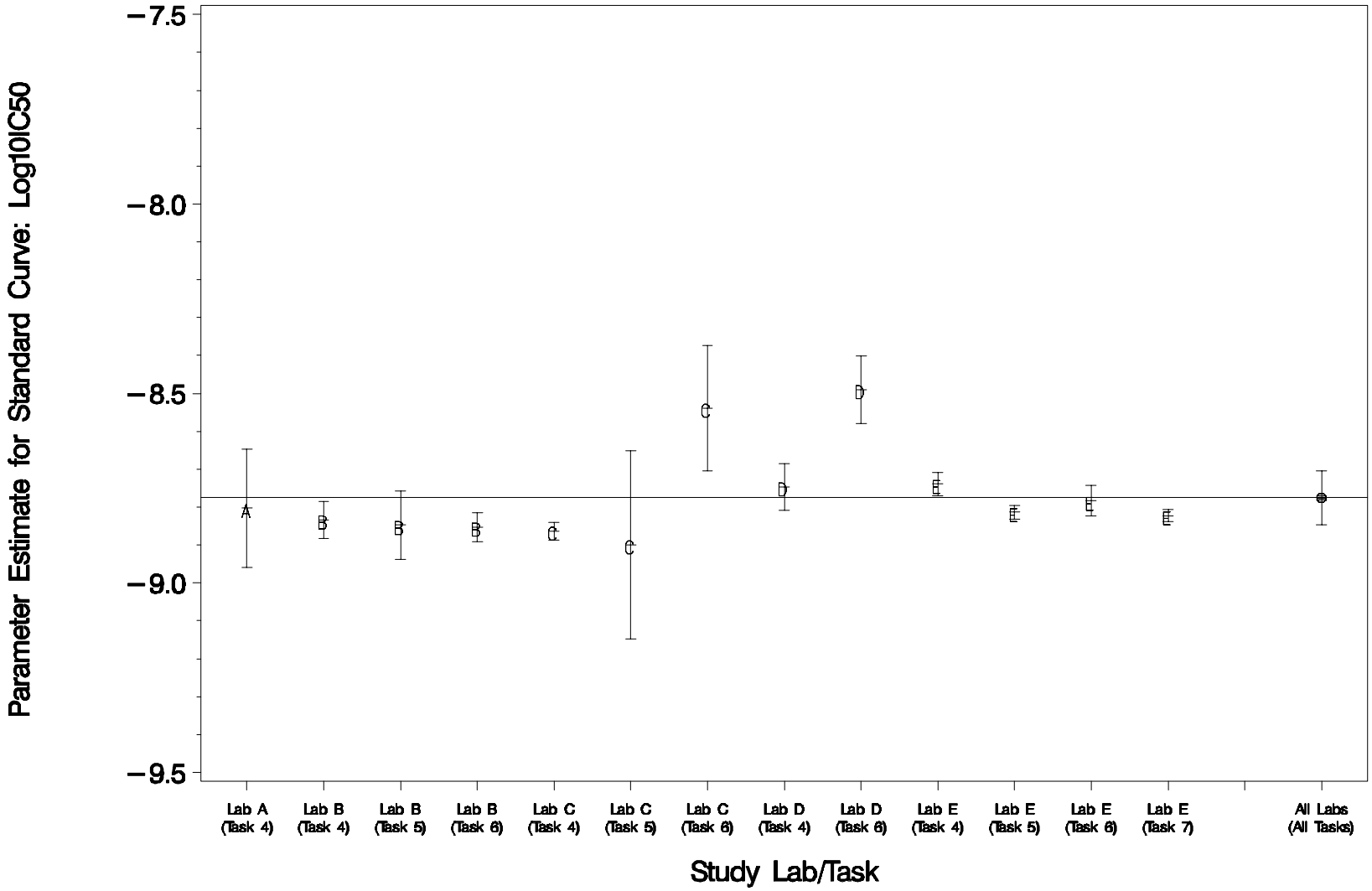
4. Tekpetey, F.R., and Amann, R.P. (1988). *Biol Reprod* 38: 1051-1060.

5. Wilson, V.S., Lambright, C.S., Ostby, J., and Gray, Jr., L.E. (2002). *In vitro* and *in vivo* effects of 17β-trenbolone: A feedlot effluent contaminant. *Toxicol Sci* 70(2): 202-11.

6. ICCVAM (2006). Addendum to ICCVAM Evaluation of *In Vitro* Test Methods for Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays. NIH Publication No: 03-4503.

APPENDIX B

Interlaboratory Analysis for the Standard Chemical, the Weak Positive Binder, and Test Chemicals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−8.5
−9.0
−9.5
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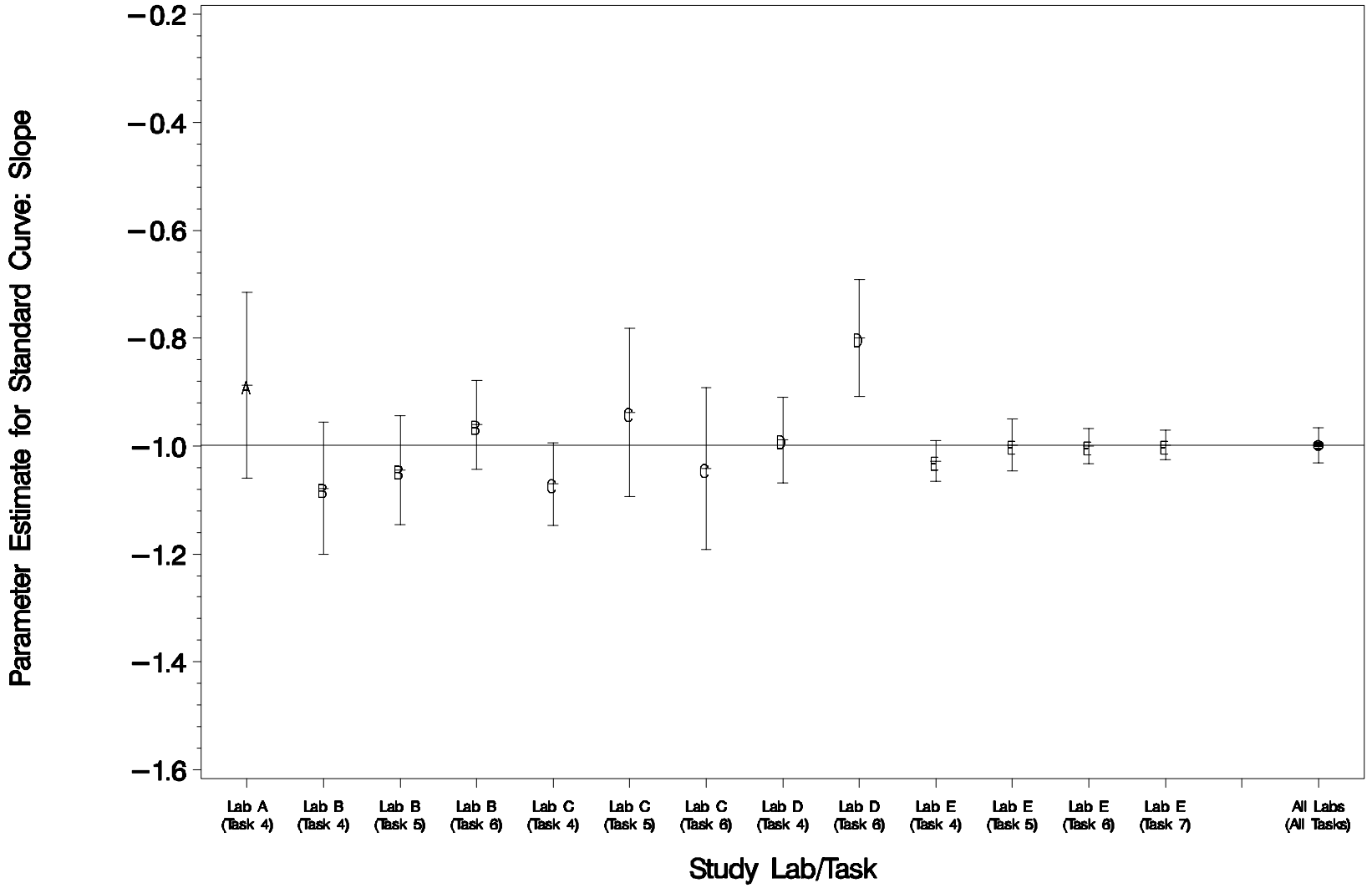
Lab B (Task 4)
Lab B (Task 5)
Lab B (Task 6)
Lab C (Task 4)
Lab C (Task 5)
Lab C (Task 6)
Lab D (Task 4)
Lab D (Task 6)
Lab E (Task 4)
Lab E (Task 5)
Lab E (Task 6)
Lab E (Task 7)
All Labs (All Tasks)
Study Lab/Task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−0.6
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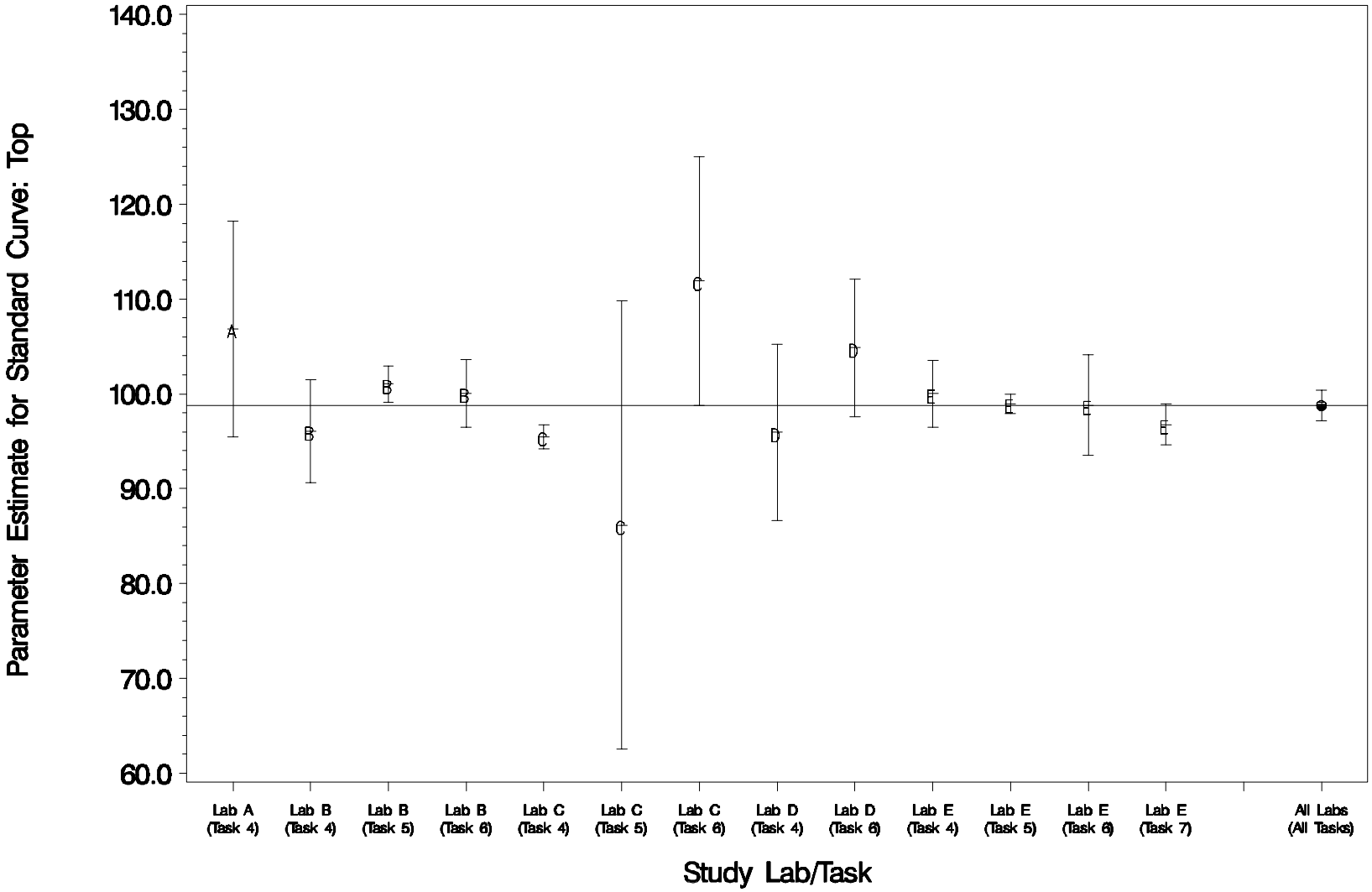
−1.4
−1.6
A
B
B
B
C
C
C
D
D
E
E
E
E
Lab A (Task 4)
Lab B (Task 4)
Lab B (Task 5)
Lab B (Task 6)
Lab C (Task 4)
Lab C (Task 5)
Lab C (Task 6)
Lab D (Task 4)
Lab D (Task 6)
Lab E (Task 4)
Lab E (Task 5)
Lab E (Task 6)
Lab E (Task 7)
All Labs (All Tasks)
Study Lab/Task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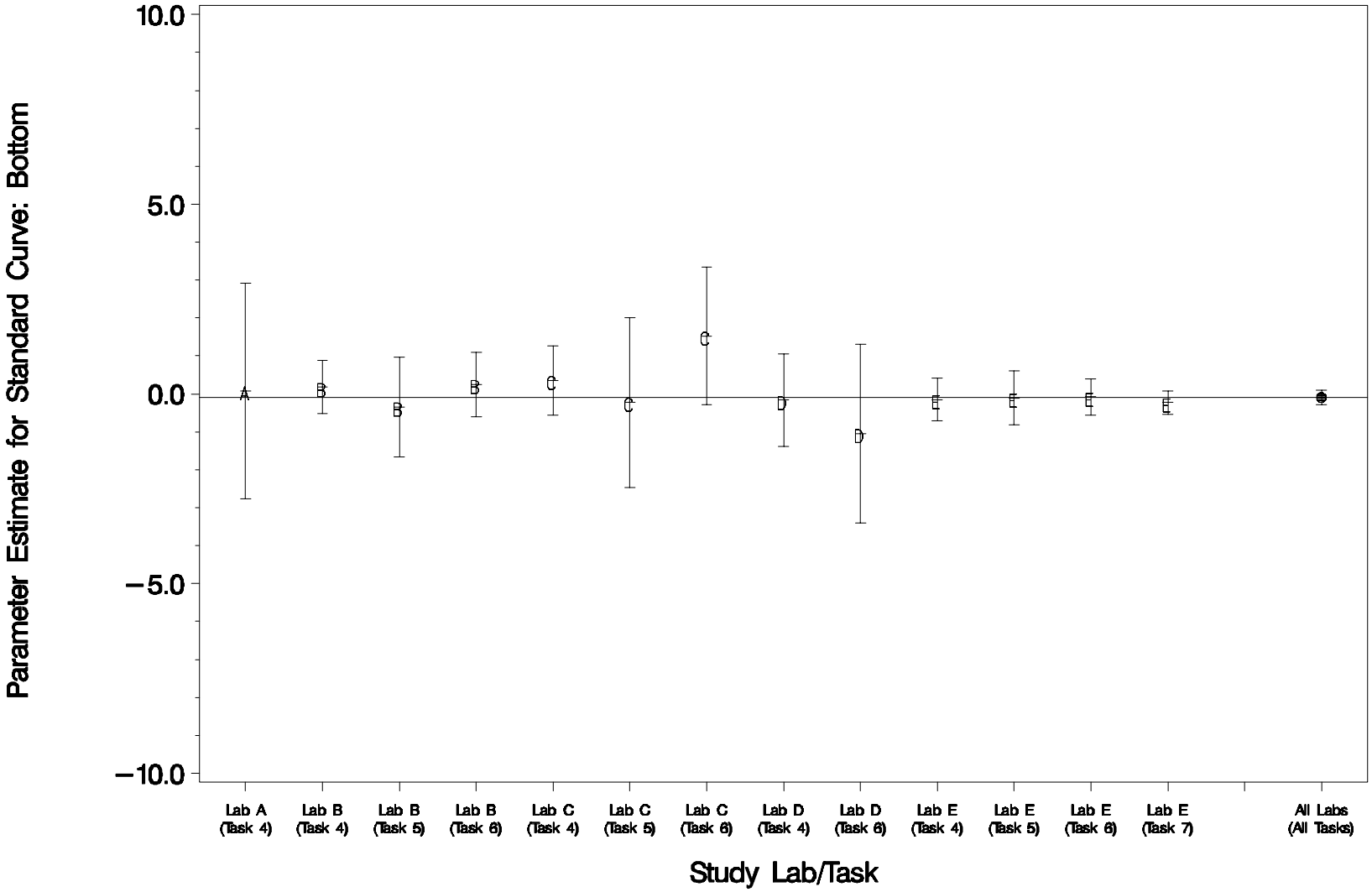
120.0
110.0
100.0
90.0
80.0
70.0
60.0
Lab A (Task 4)
Lab B (Task 4)
Lab B (Task 5)
Lab B (Task 6)
Lab C (Task 4)
Lab C (Task 5)
Lab C (Task 6)
Lab D (Task 4)
Lab D (Task 6)
Lab E (Task 4)
Lab E (Task 5)
Lab E (Task 6)
Lab E (Task 7)
All Labs (All Tasks)
Study Lab/Task


Parameter Estimate for Standard Curve: Bottom
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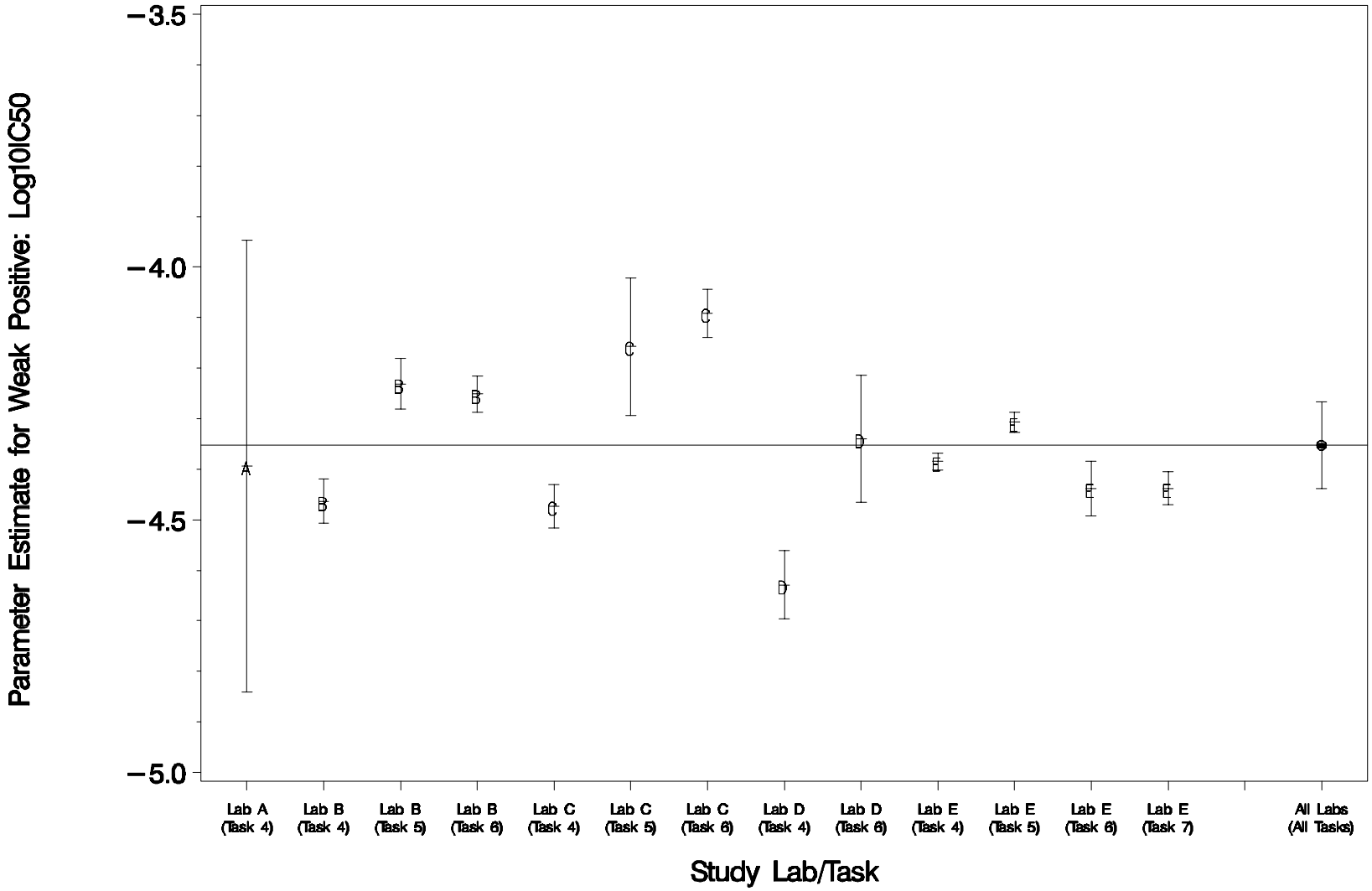
5.0
0.0
−5.0
−10.0
Lab A (Task 4)
Lab B (Task 4)
Lab B (Task 5)
Lab B (Task 6)
Lab C (Task 4)
Lab C (Task 5)
Lab C (Task 6)
Lab D (Task 4)
Lab D (Task 6)
Lab E (Task 4)
Lab E (Task 5)
Lab E (Task 6)
Lab E (Task 7)
All Labs (All Tasks)
Study Lab/Task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−5.0
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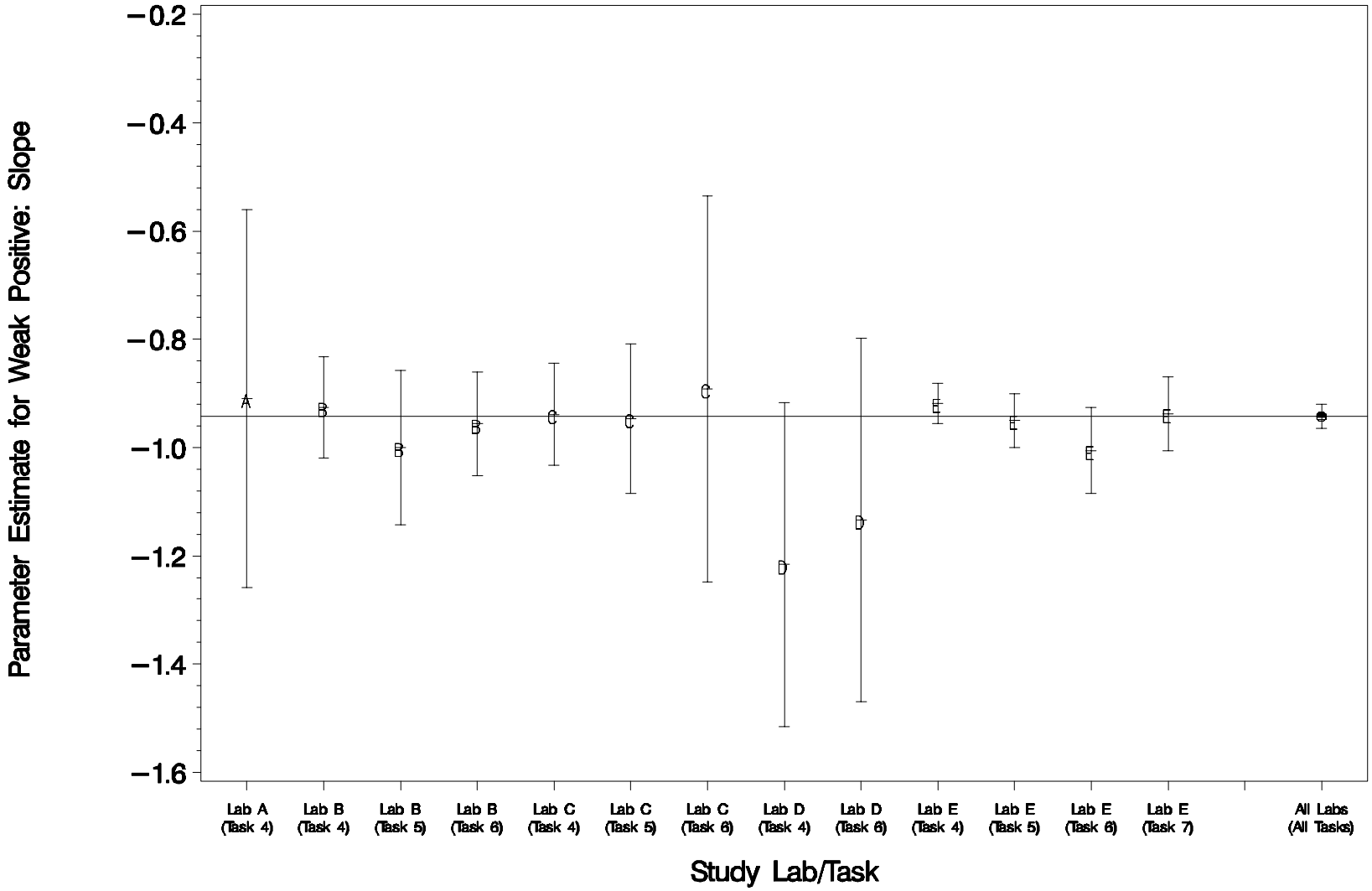
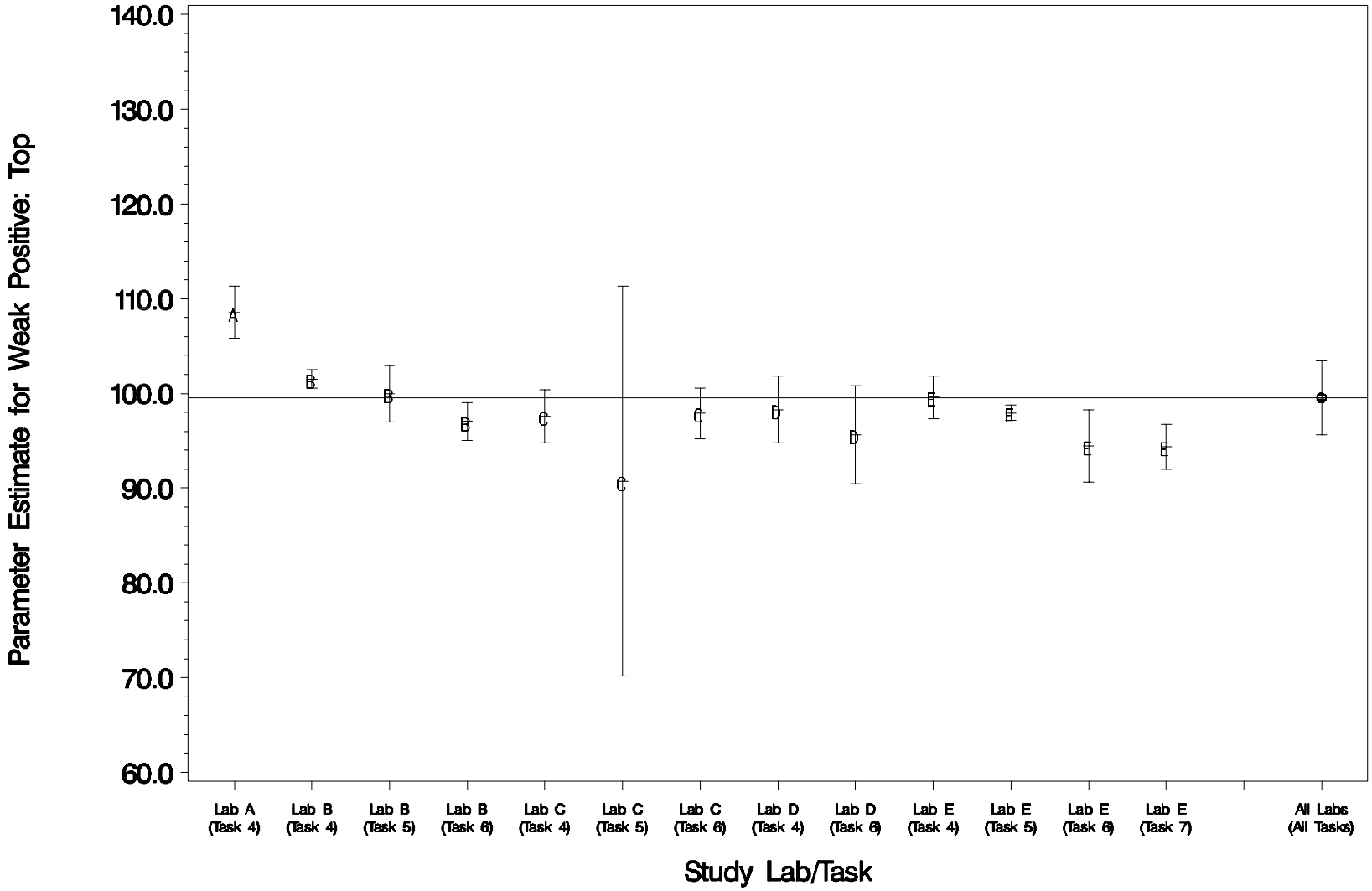
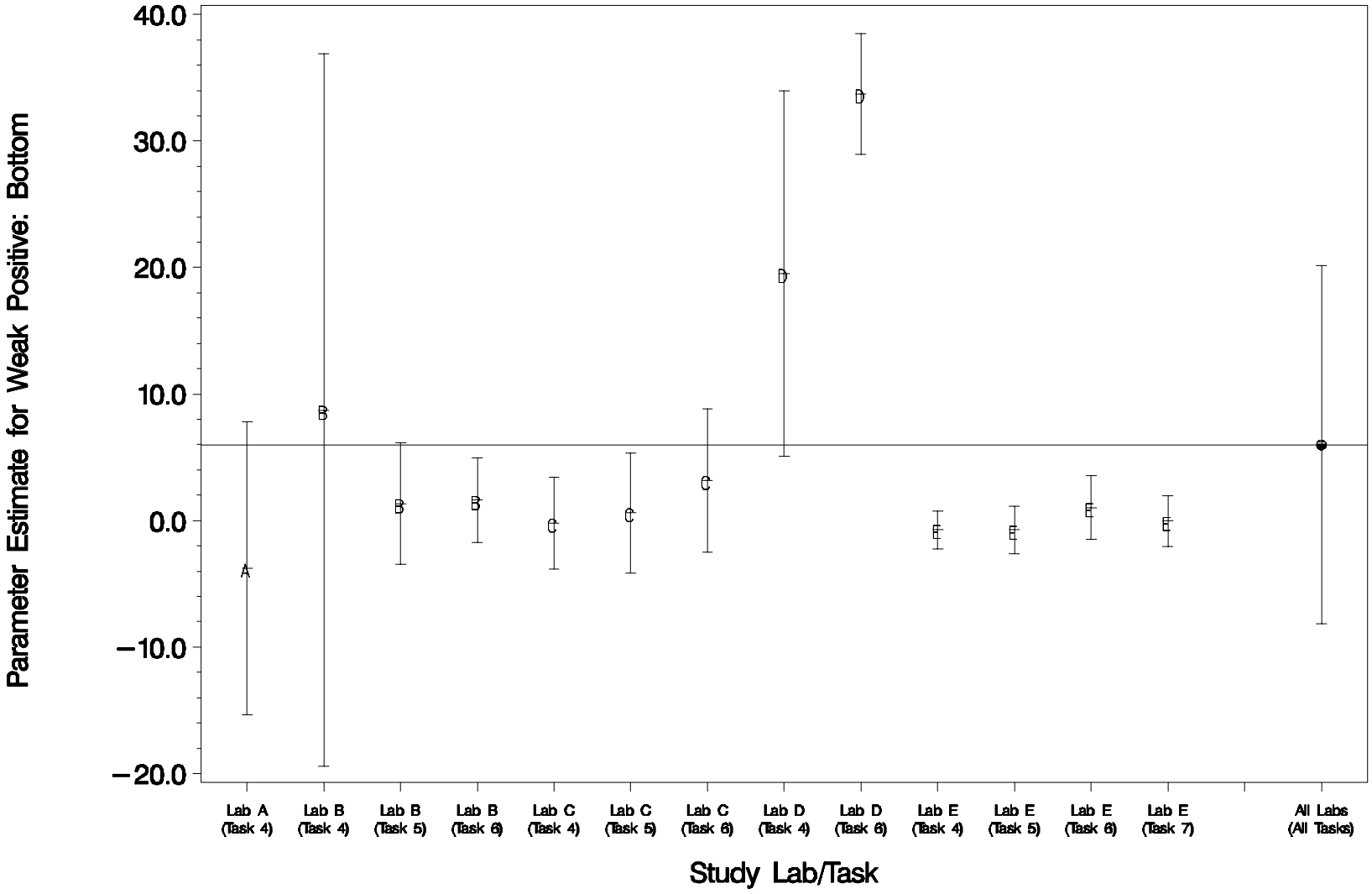
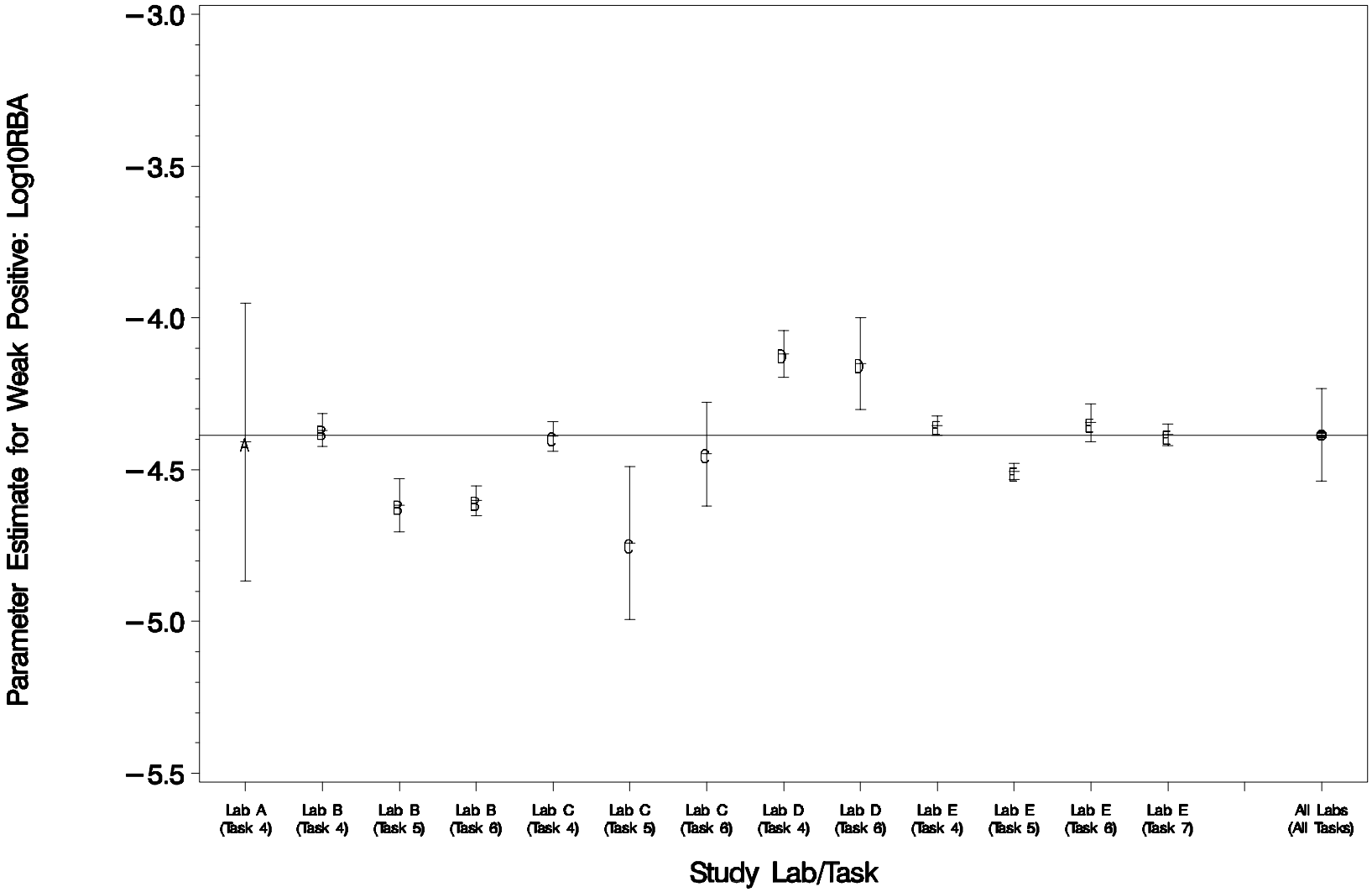
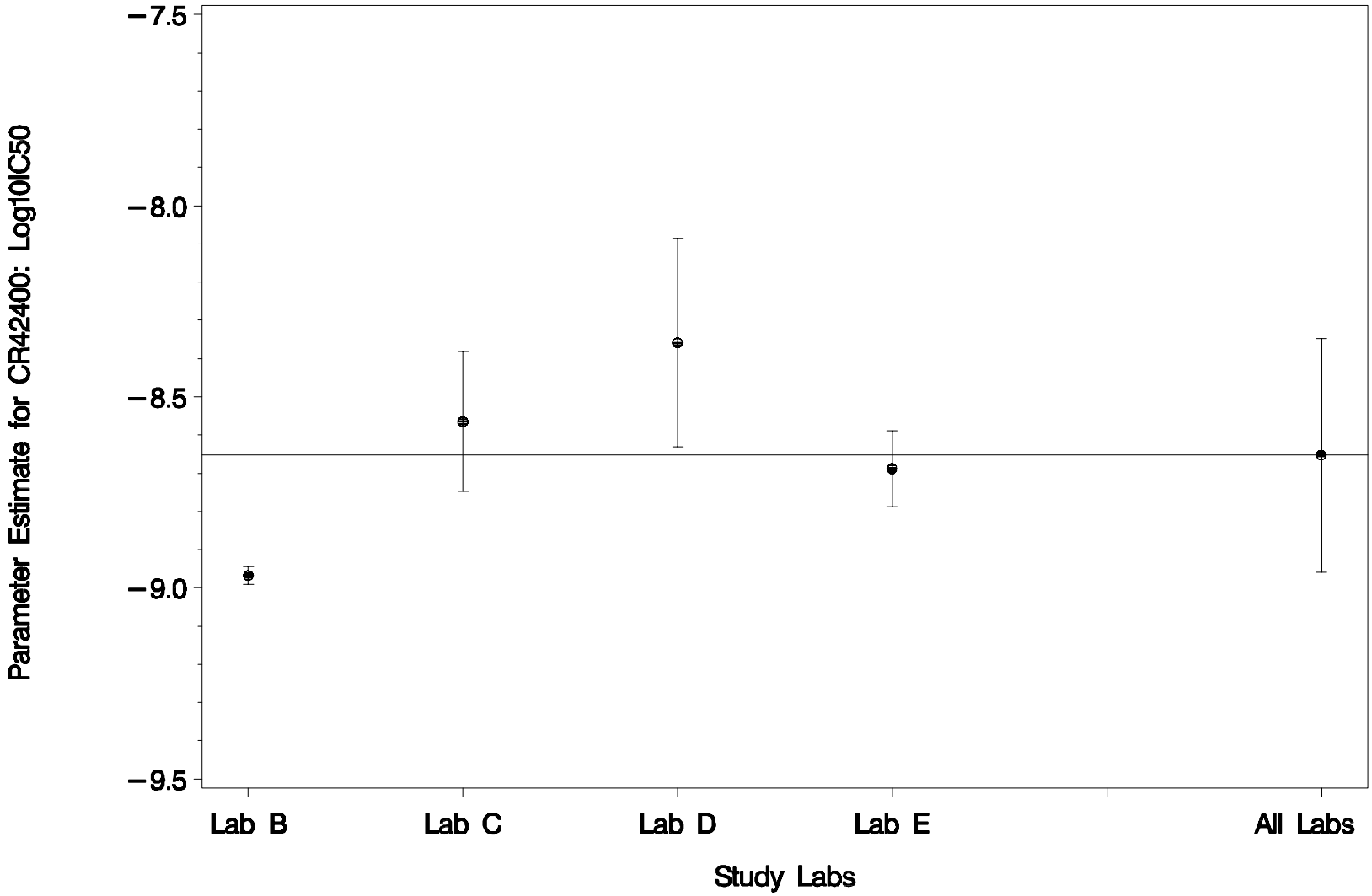
Lab B (Task 4)
Lab B (Task 5)
Lab B (Task 6)
Lab C (Task 4)
Lab C (Task 5)
Lab C (Task 6)
Lab D (Task 4)
Lab D (Task 6)
Lab E (Task 4)
Lab E (Task 5)
Lab E (Task 6)
Lab E (Task 7)
All Labs (All Tasks)
Study Lab/Task

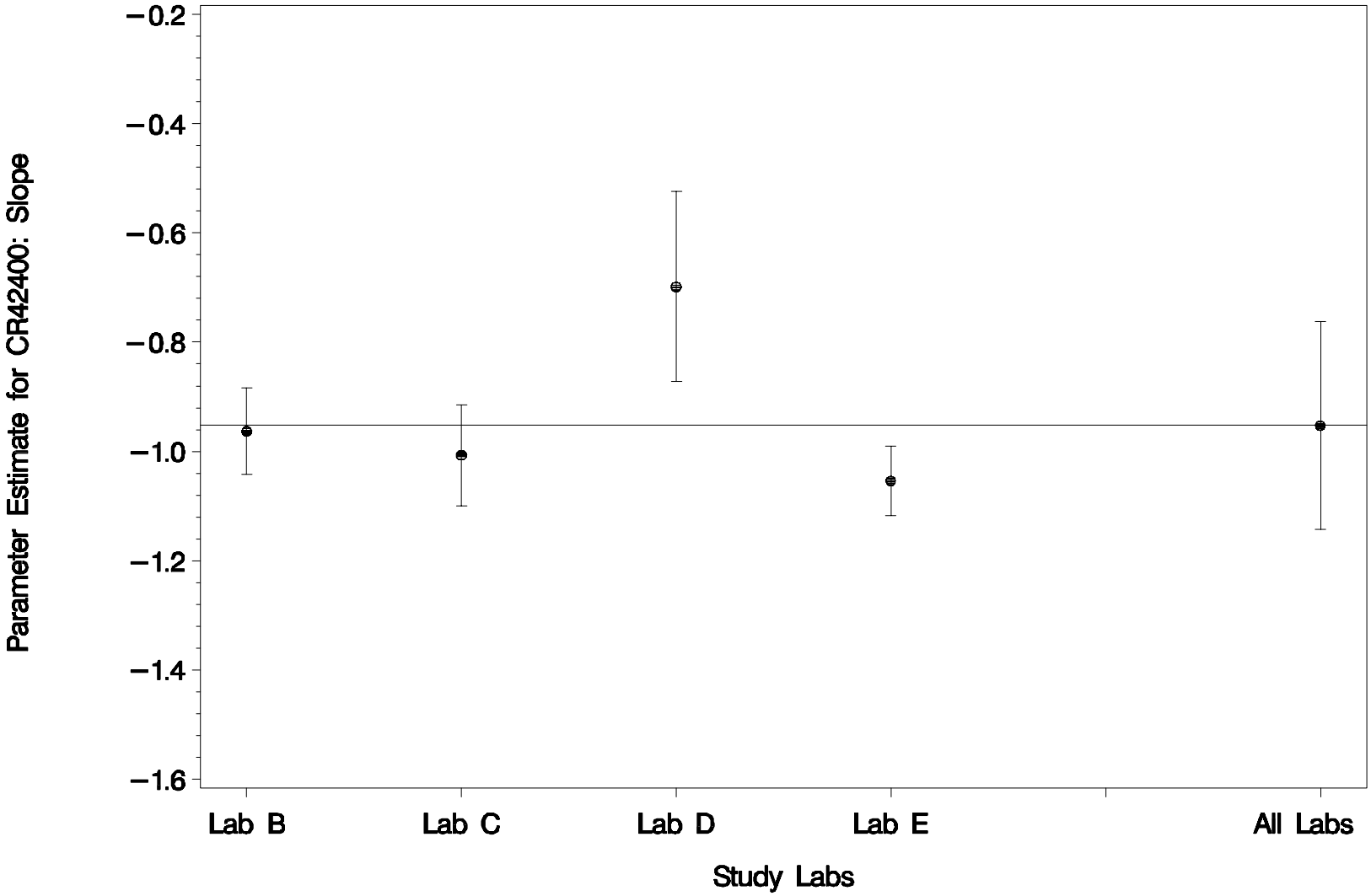

Parameter Estimate for Weak Positive: Slope
−0.2
−0.4
−0.6
−0.8
−1.0
−1.2
−1.4
−1.6
Lab A (Task 4)
Lab B (Task 4)
Lab B (Task 5)
Lab B (Task 6)
Lab C (Task 4)
Lab C (Task 5)
Lab C (Task 6)
Lab D (Task 4)
Lab D (Task 6)
Lab E (Task 4)
Lab E (Task 5)
Lab E (Task 6)
Lab E (Task 7)
All Labs (All Tasks)
Study Lab/Task

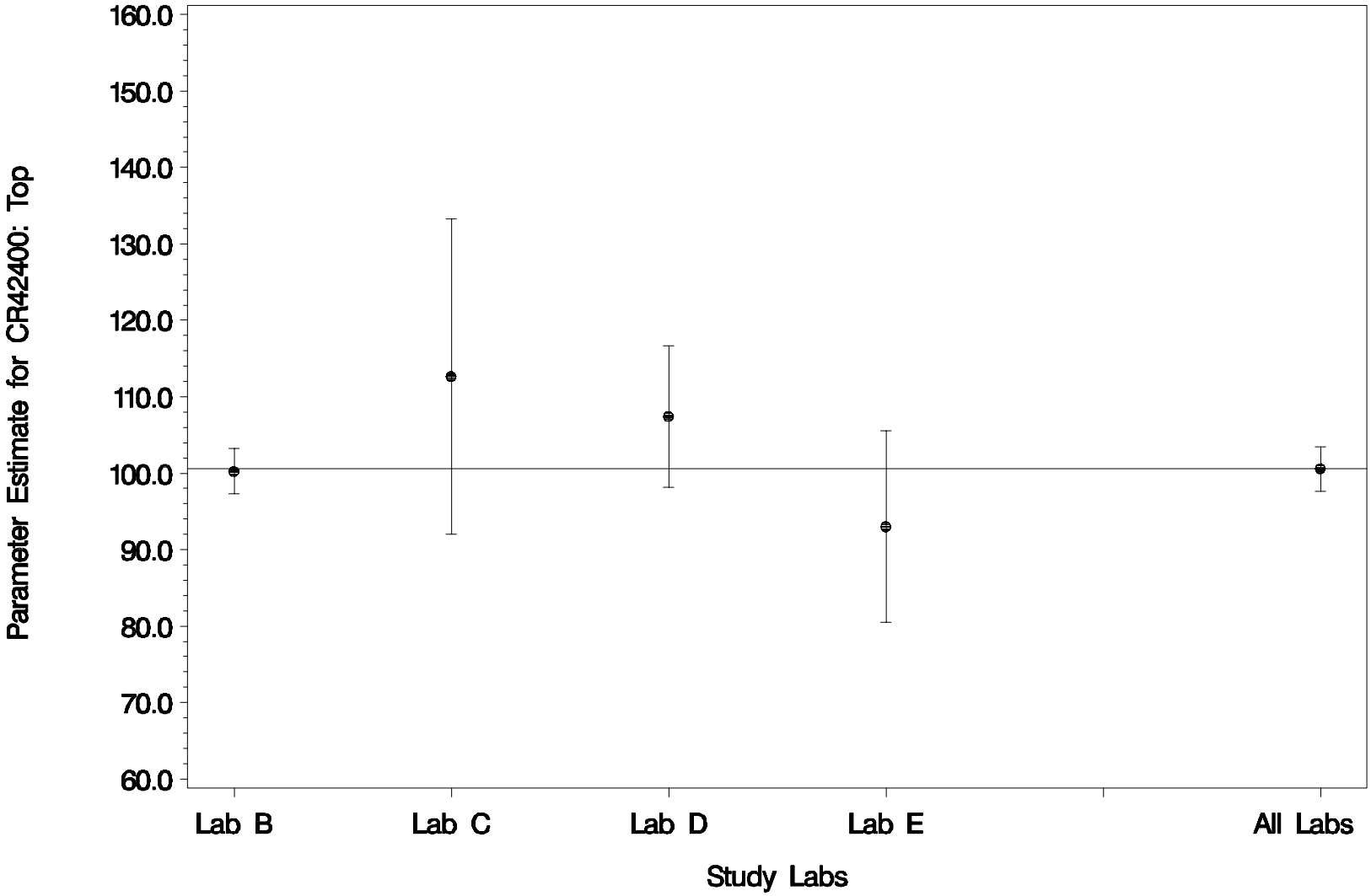

Parameter Estimate for Weak Positive: Top
140.0
130.0
120.0
110.0
100.0
90.0
80.0
70.0
60.0
Lab A (Task 4)
Lab B (Task 4)
Lab B (Task 5)
Lab B (Task 6)
Lab C (Task 4)
Lab C (Task 5)
Lab C (Task 6)
Lab D (Task 4)
Lab D (Task 6)
Lab E (Task 4)
Lab E (Task 5)
Lab E (Task 6)
Lab E (Task 7)
All Labs (All Tasks)
Study Lab/Task

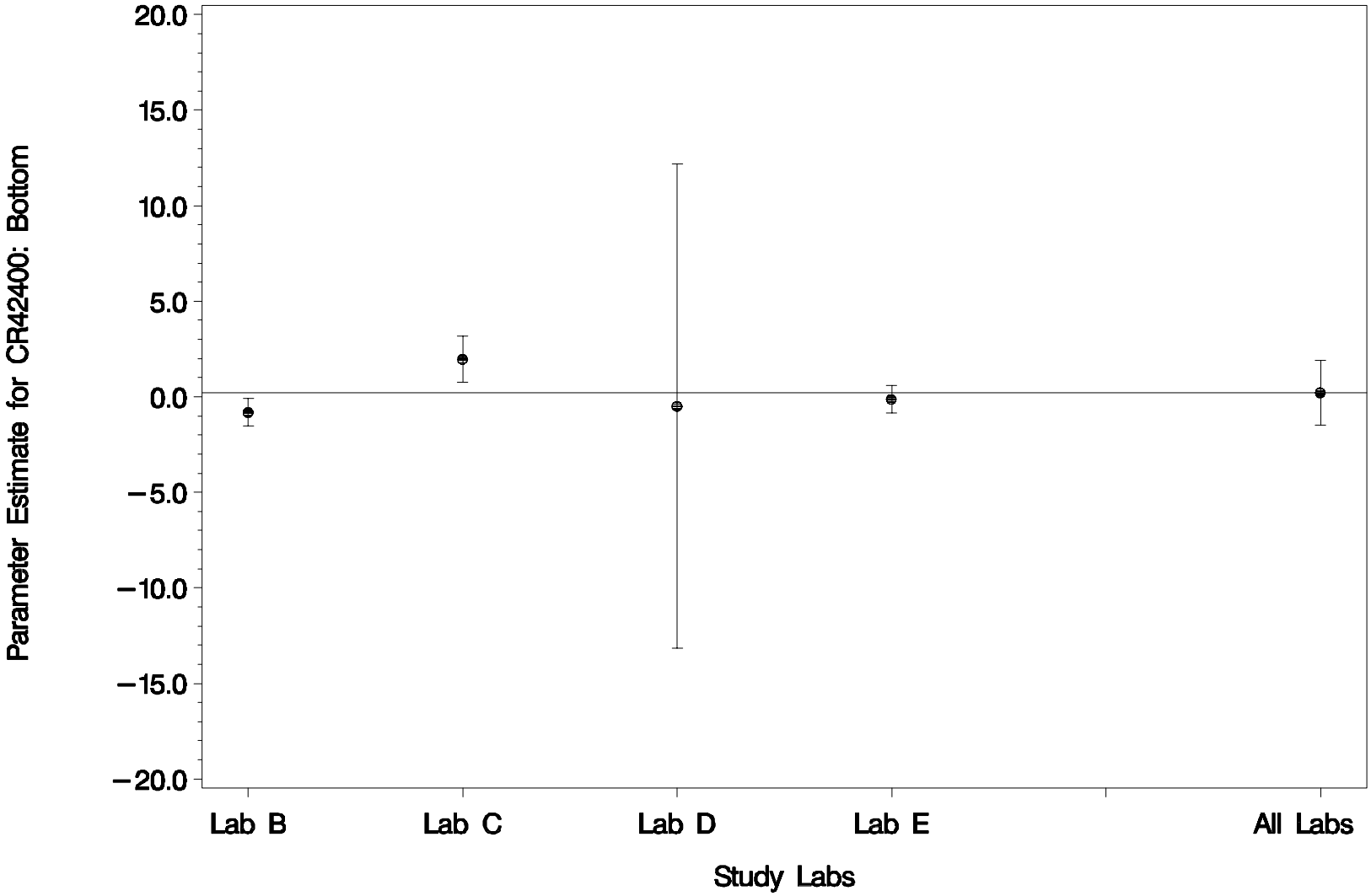

Parameter Estimate for Weak Positive: Bottom
40.0
30.0
20.0
10.0
0.0
−10.0
−20.0
Lab A (Task 4)
Lab B (Task 4)
Lab B (Task 5)
Lab B (Task 6)
Lab C (Task 4)
Lab C (Task 5)
Lab C (Task 6)
Lab D (Task 4)
Lab D (Task 6)
Lab E (Task 4)
Lab E (Task 5)
Lab E (Task 6)
Lab E (Task 7)
All Labs (All Tasks)
Study Lab/Task

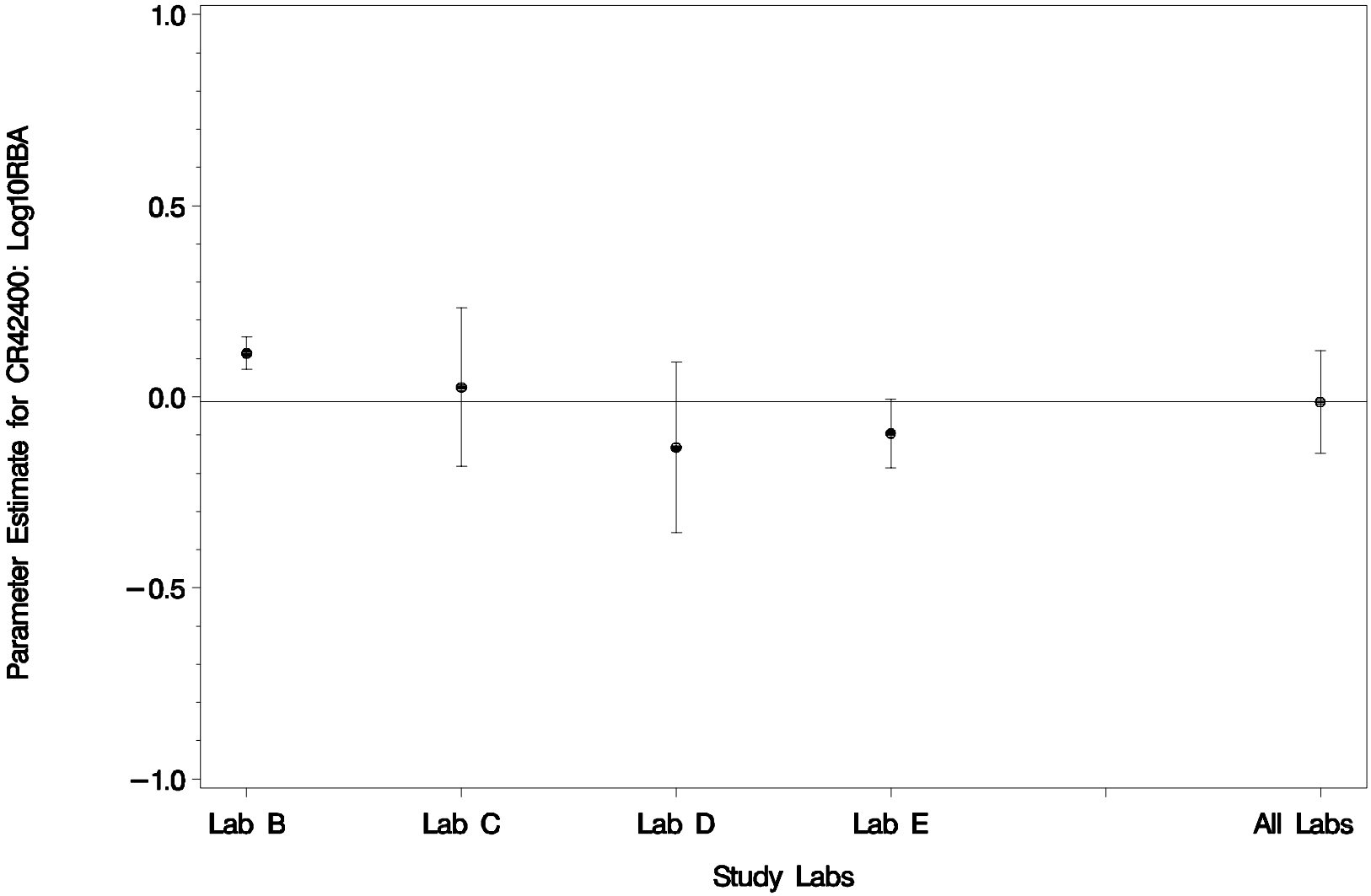

Parameter Estimate for Weak Positive: Log10RBA
−3.0
−3.5
−4.0
−4.5
−5.0
−5.5
Lab A (Task 4)
Lab B (Task 4)
Lab B (Task 5)
Lab B (Task 6)
Lab C (Task 4)
Lab C (Task 5)
Lab C (Task 6)
Lab D (Task 4)
Lab D (Task 6)
Lab E (Task 4)
Lab E (Task 5)
Lab E (Task 6)
Lab E (Task 7)
All Labs (All Tasks)
Study Lab/Task

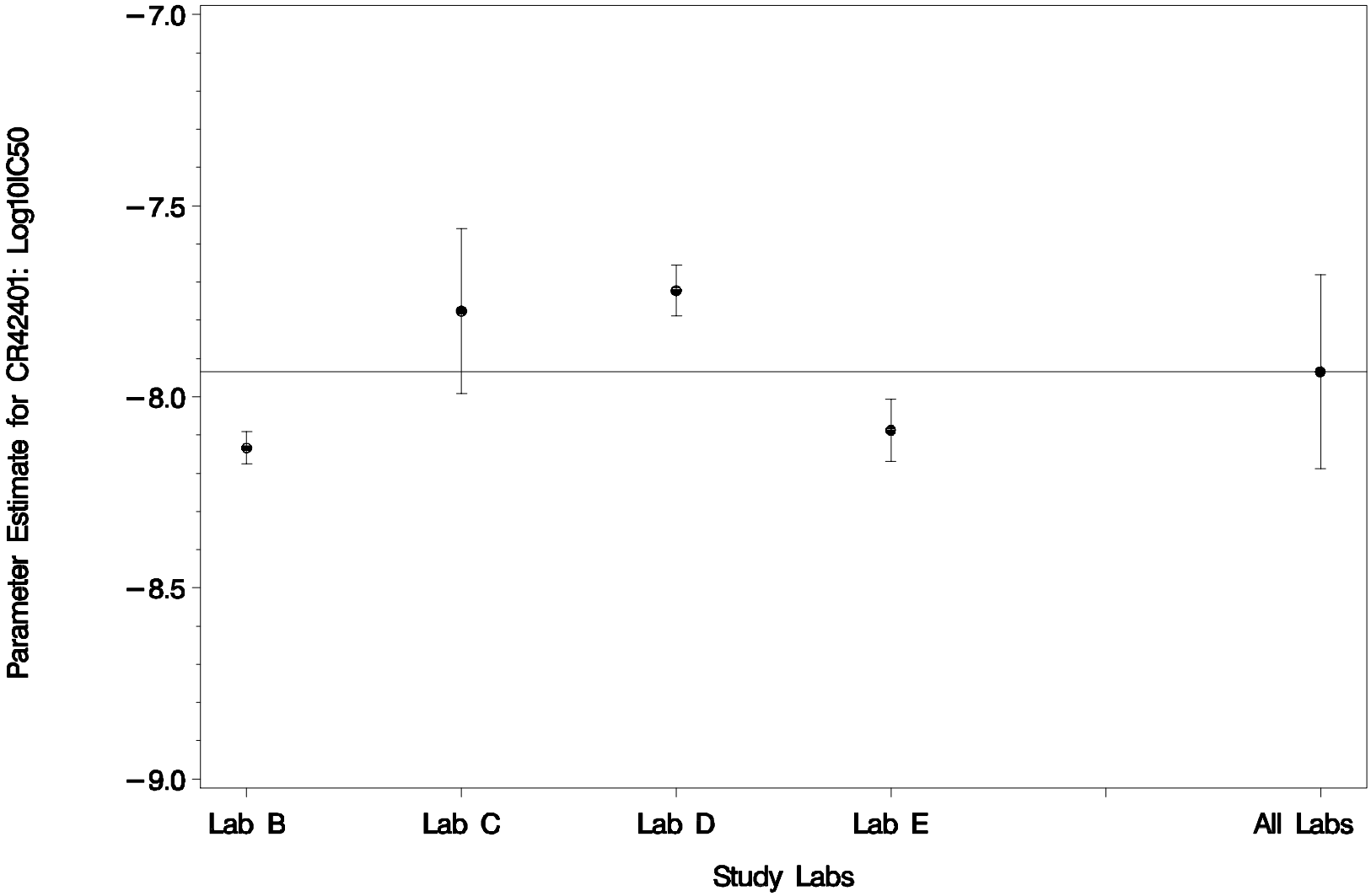

Parameter Estimate for CR42400: Log10IC50
−7.5
−8.0
−8.5
−9.0
−9.5
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

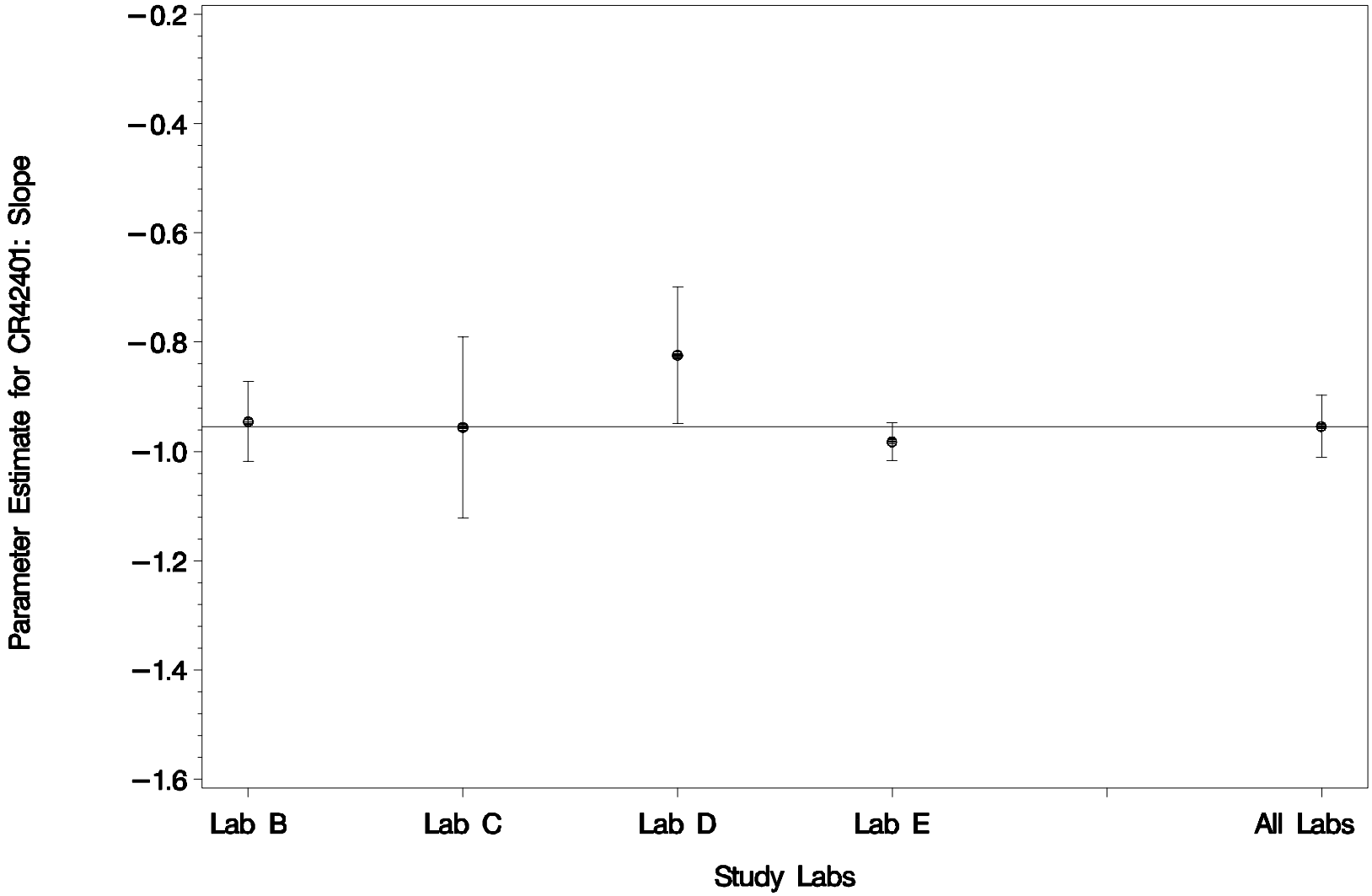

Parameter Estimate for CR42400: Slope
−0.2
−0.4
−0.6
−0.8
−1.0
−1.2
−1.4
−1.6
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

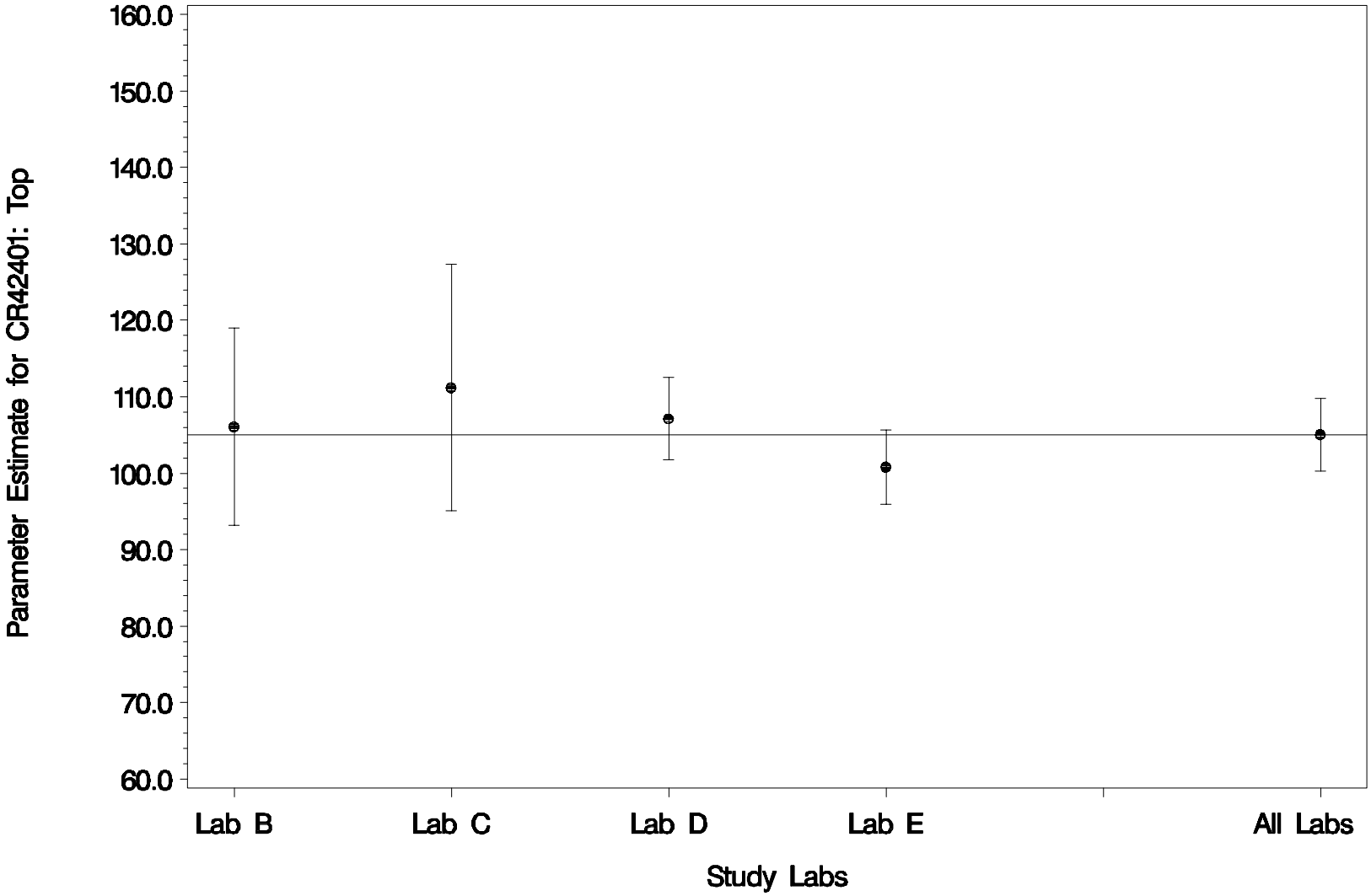

160.0
150.0
140.0
130.0
120.0
110.0
100.0
90.0
80.0
70.0
60.0
Parameter Estimate for CR42400: Top
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

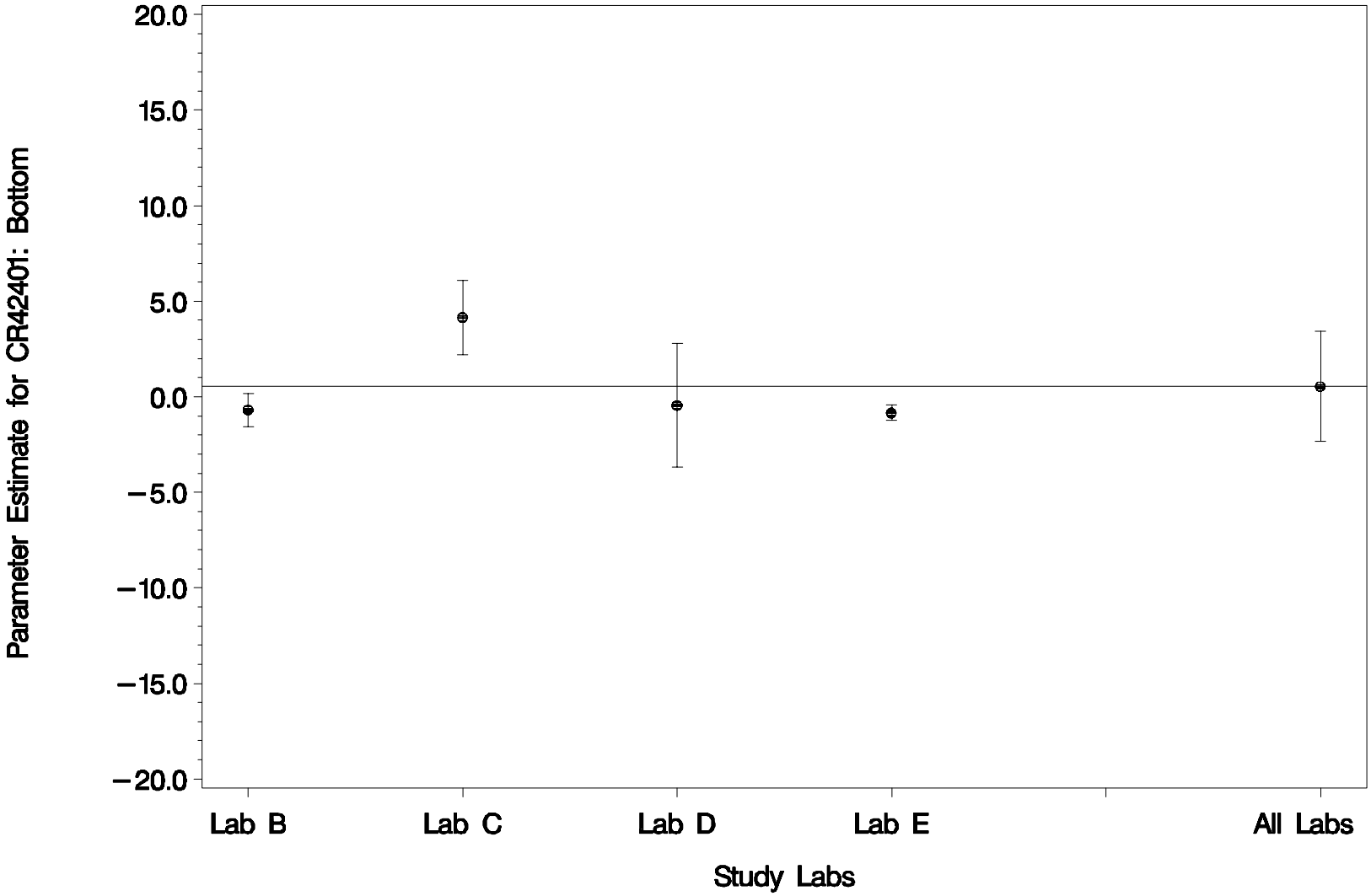

Parameter Estimate for CR42400: Bottom
20.0
15.0
10.0
5.0
0.0
−5.0
−10.0
−15.0
−20.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

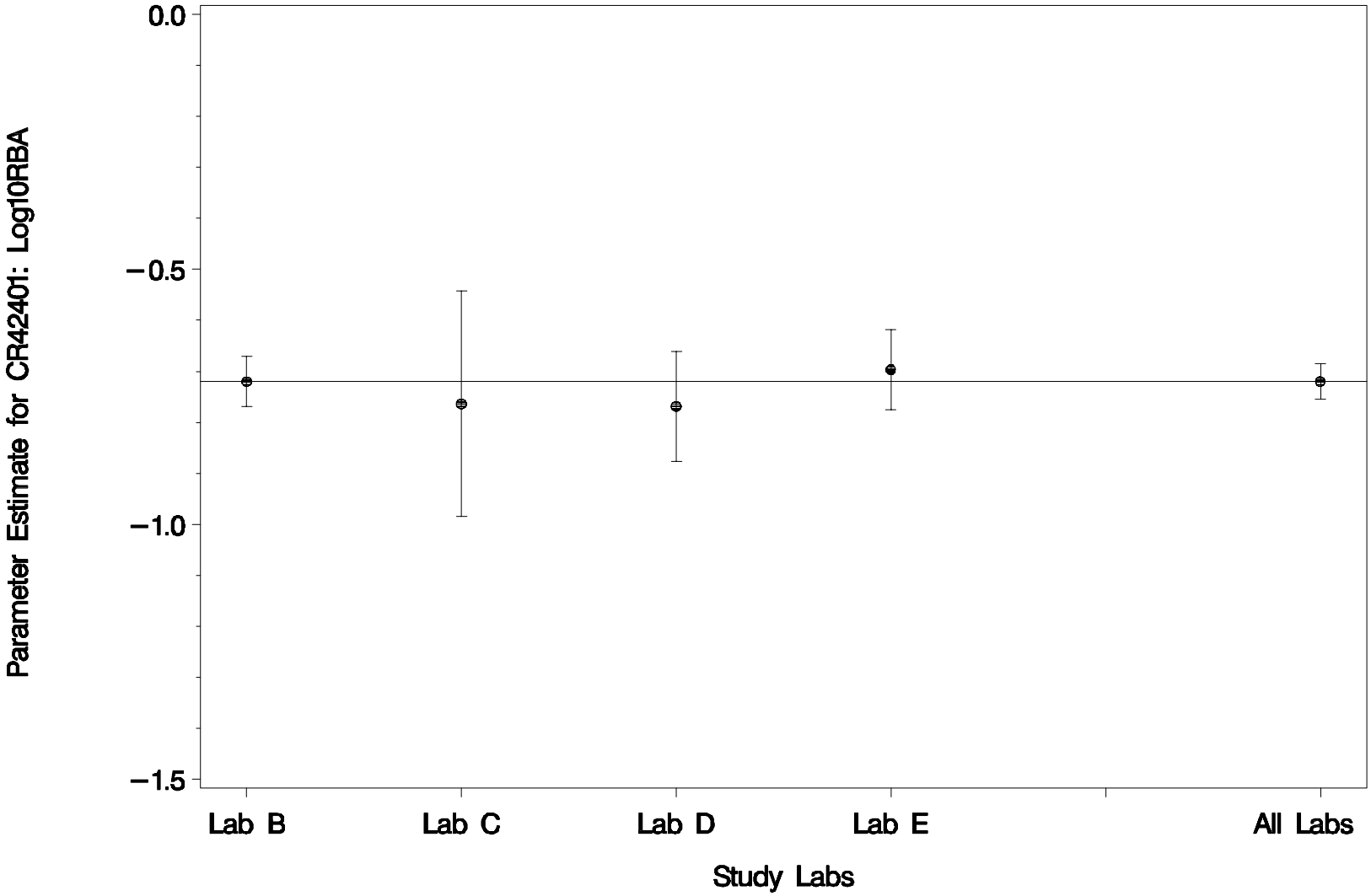

Parameter Estimate for CR42400: Log10RBA
1.0
0.5
0.0
−0.5
−1.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

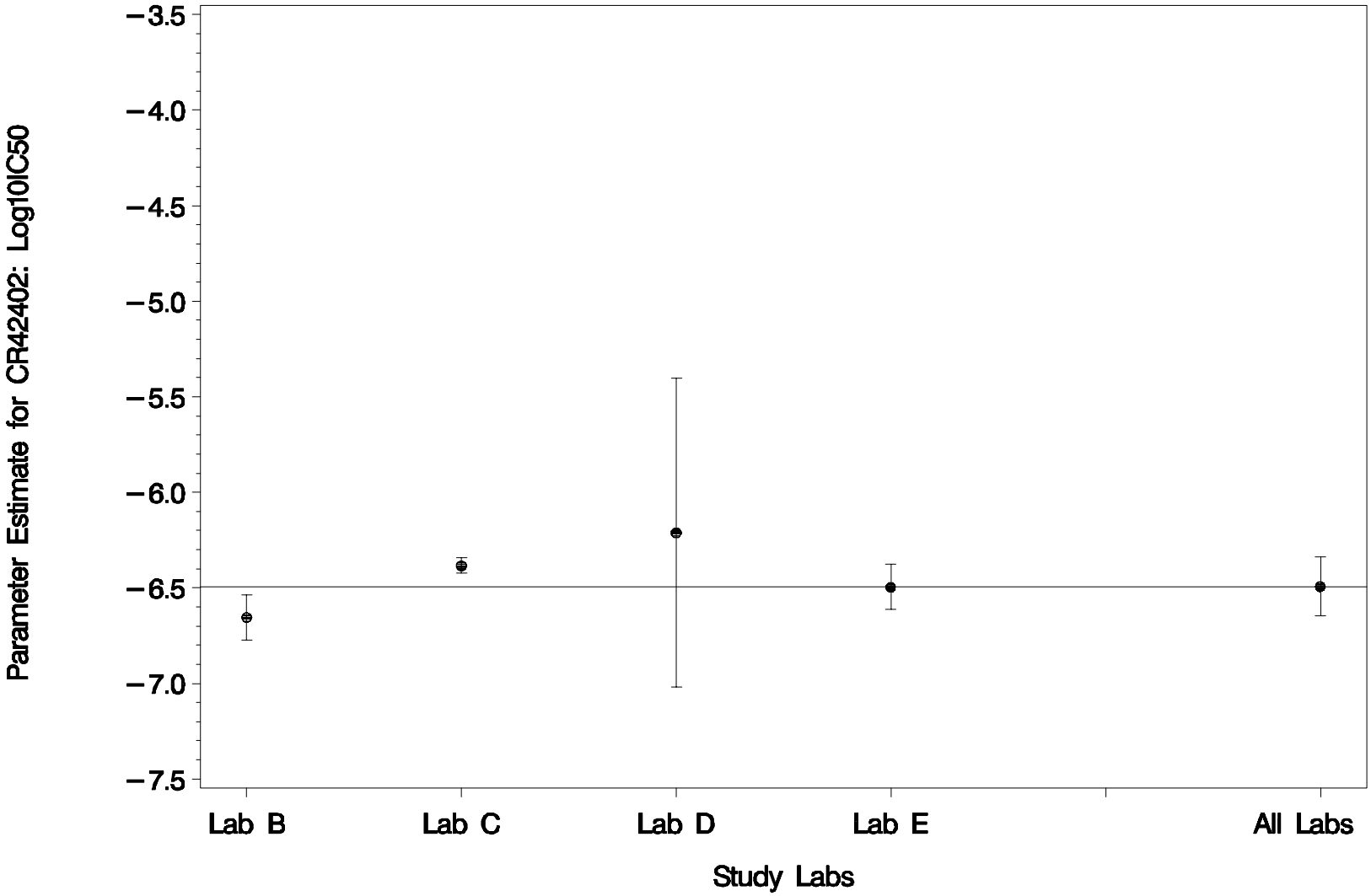

−7.0
−7.5
−8.0
−8.5
−9.0
Parameter Estimate for CR42401: Log10IC50
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

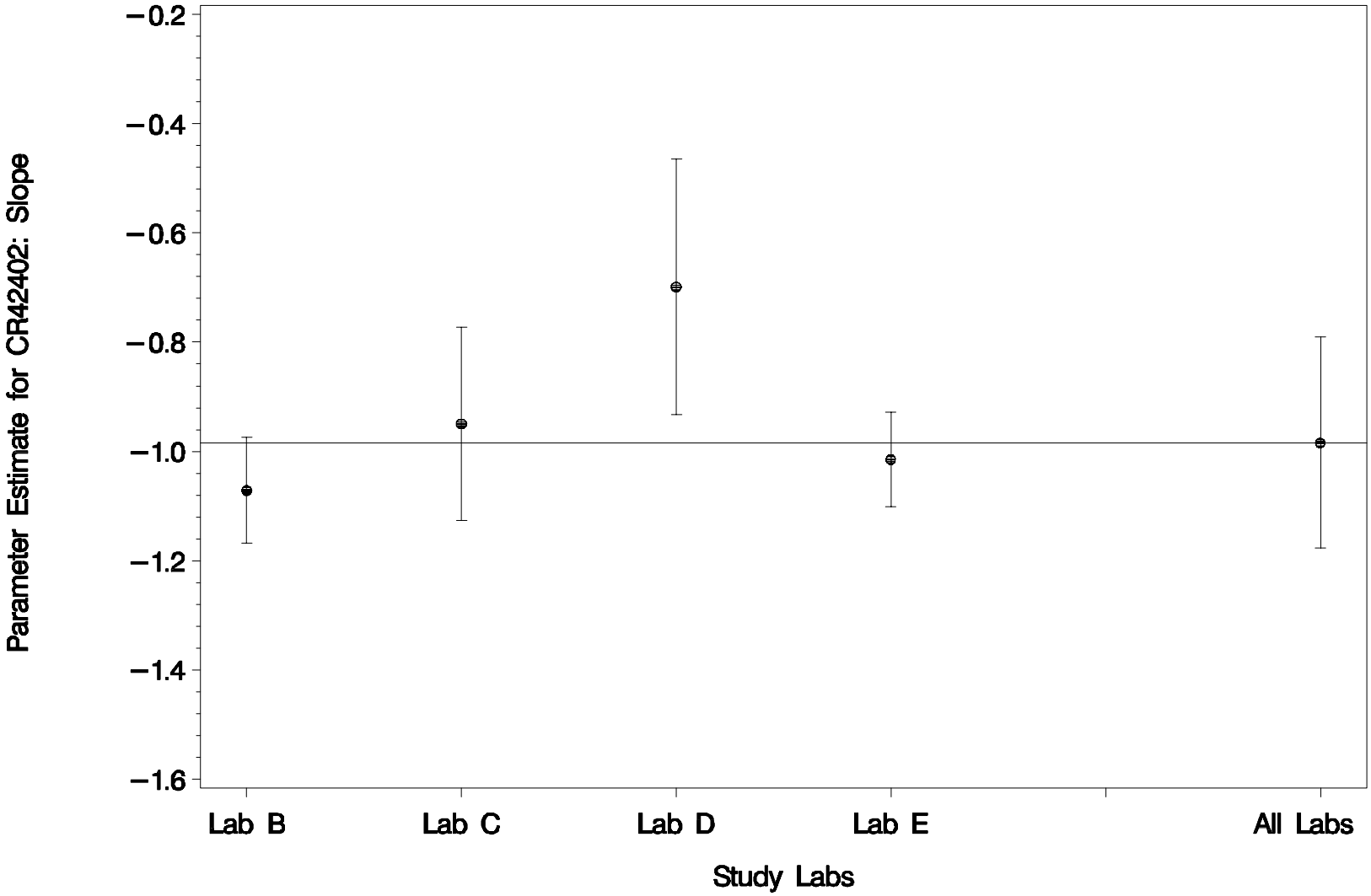

Parameter Estimate for CR42401: Slope
−0.2
−0.4
−0.6
−0.8
−1.0
−1.2
−1.4
−1.6
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

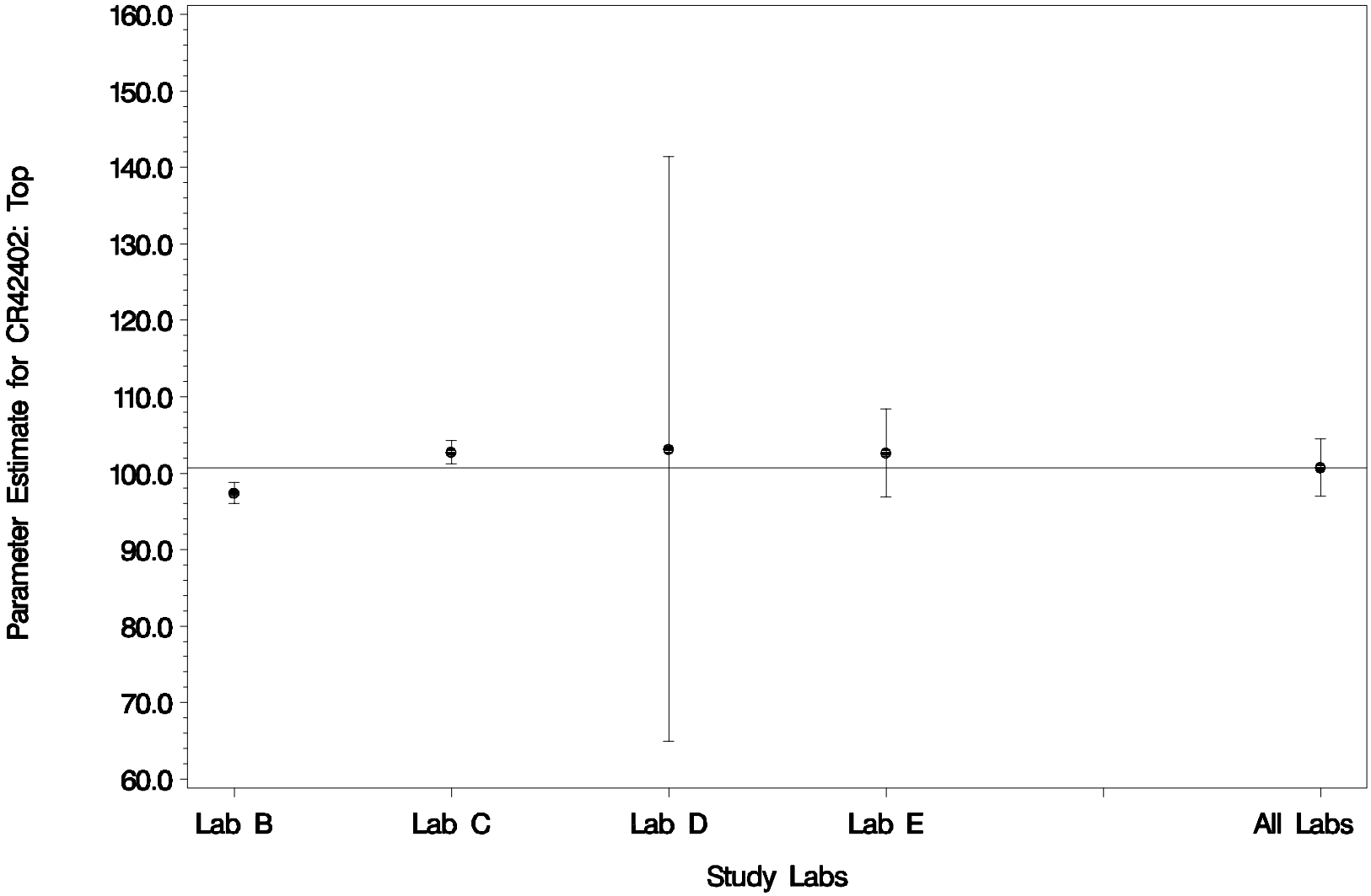

Parameter Estimate for CR42401: Top
160.0
150.0
140.0
130.0
120.0
110.0
100.0
90.0
80.0
70.0
60.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

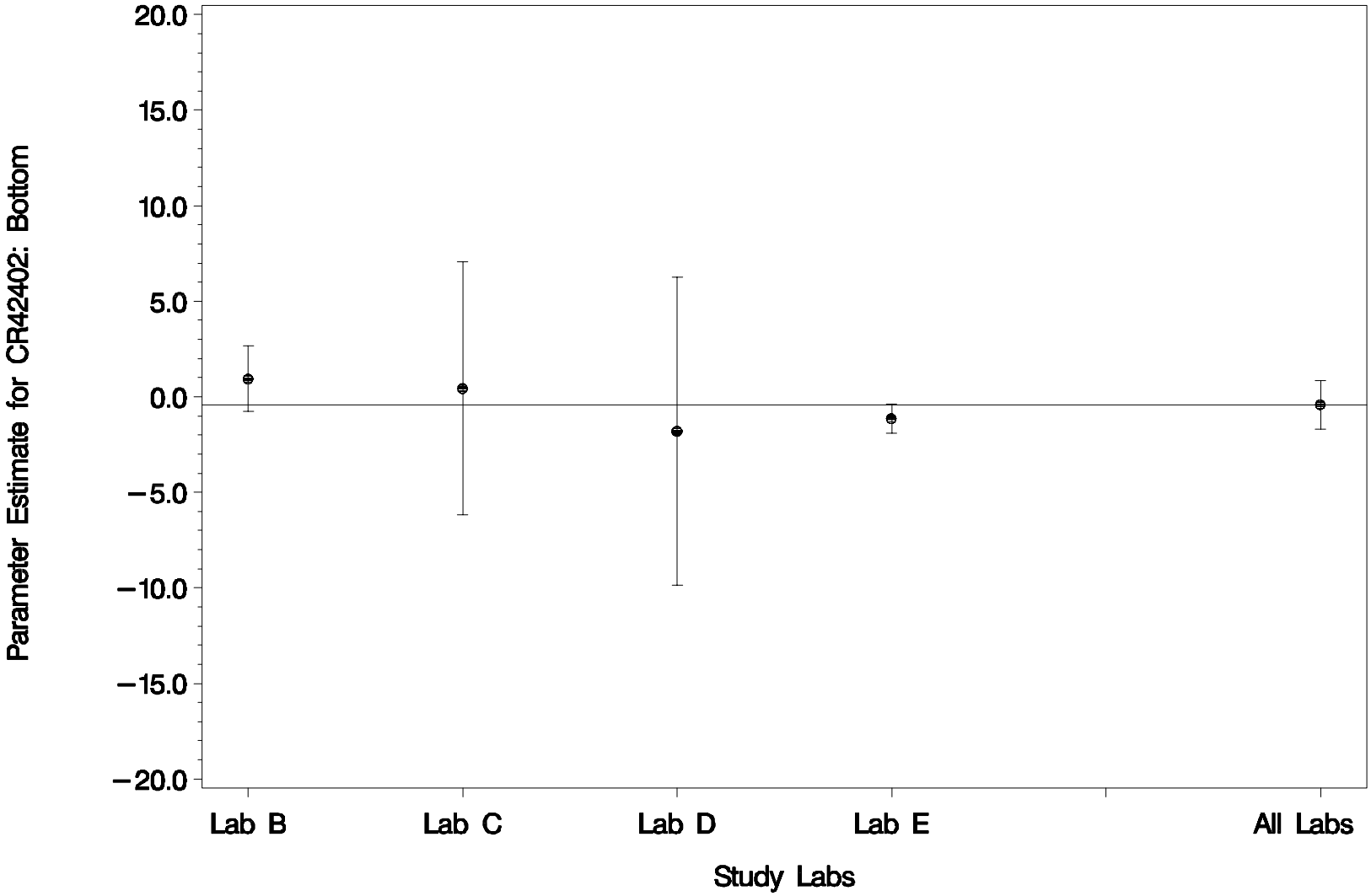

Parameter Estimate for CR42401: Bottom
20.0
15.0
10.0
5.0
0.0
−5.0
−10.0
−15.0
−20.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

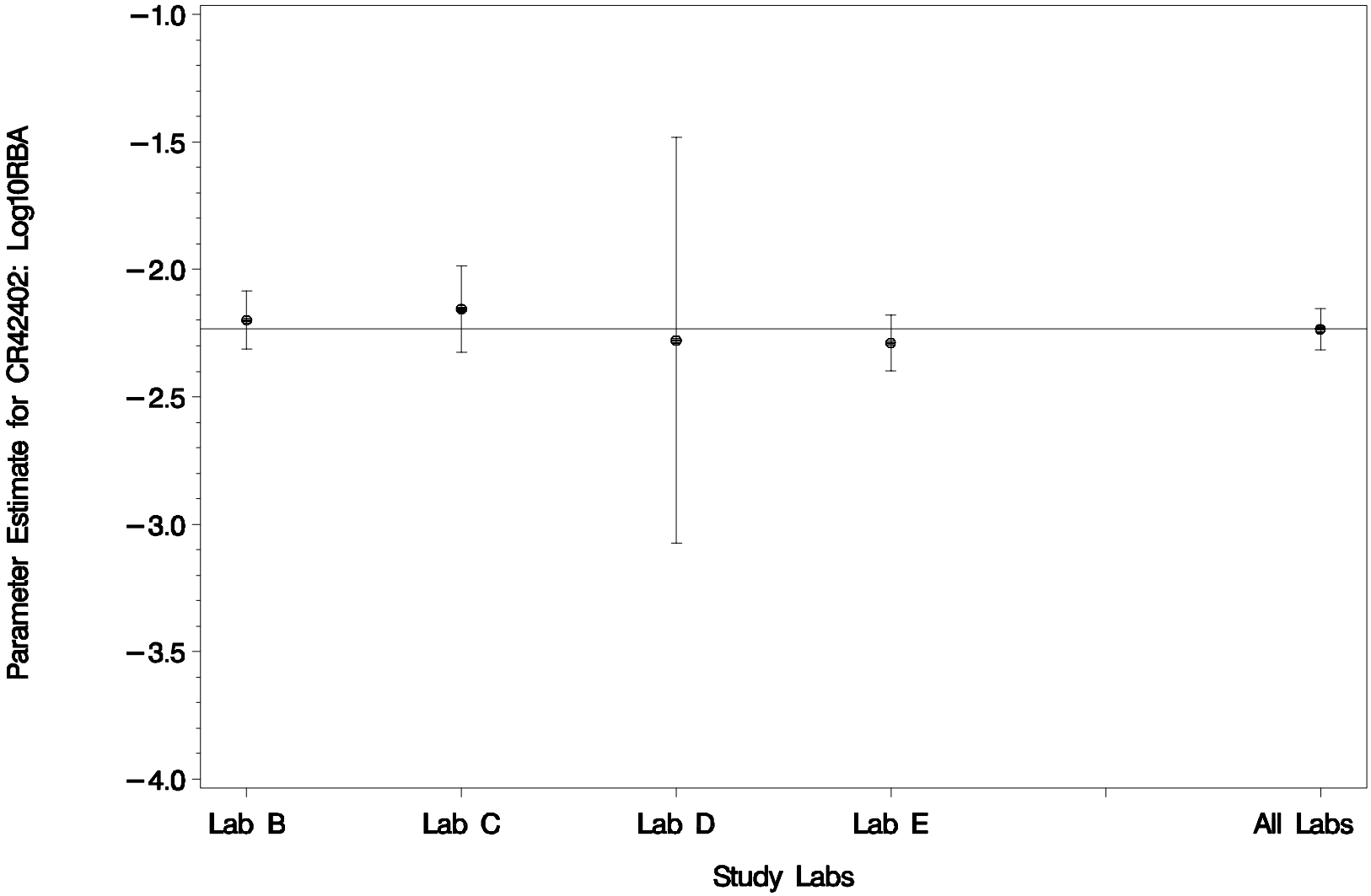

0.0
−0.5
−1.0
−1.5
Parameter Estimate for CR42401: Log10RBA
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

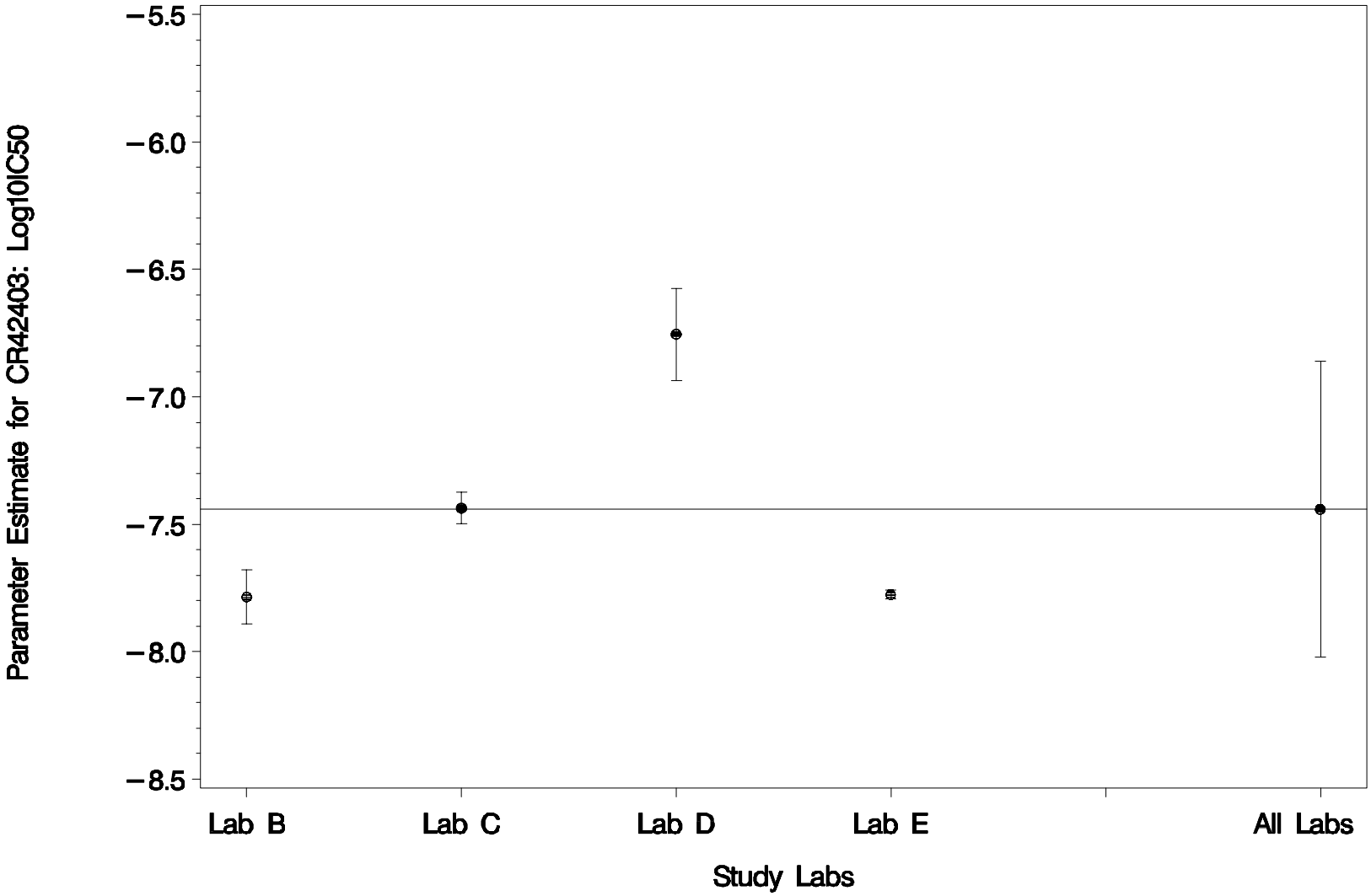

Parameter Estimate for CR42402: Log10IC50
−3.5
−4.0
−4.5
−5.0
−5.5
−6.0
−6.5
−7.0
−7.5
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

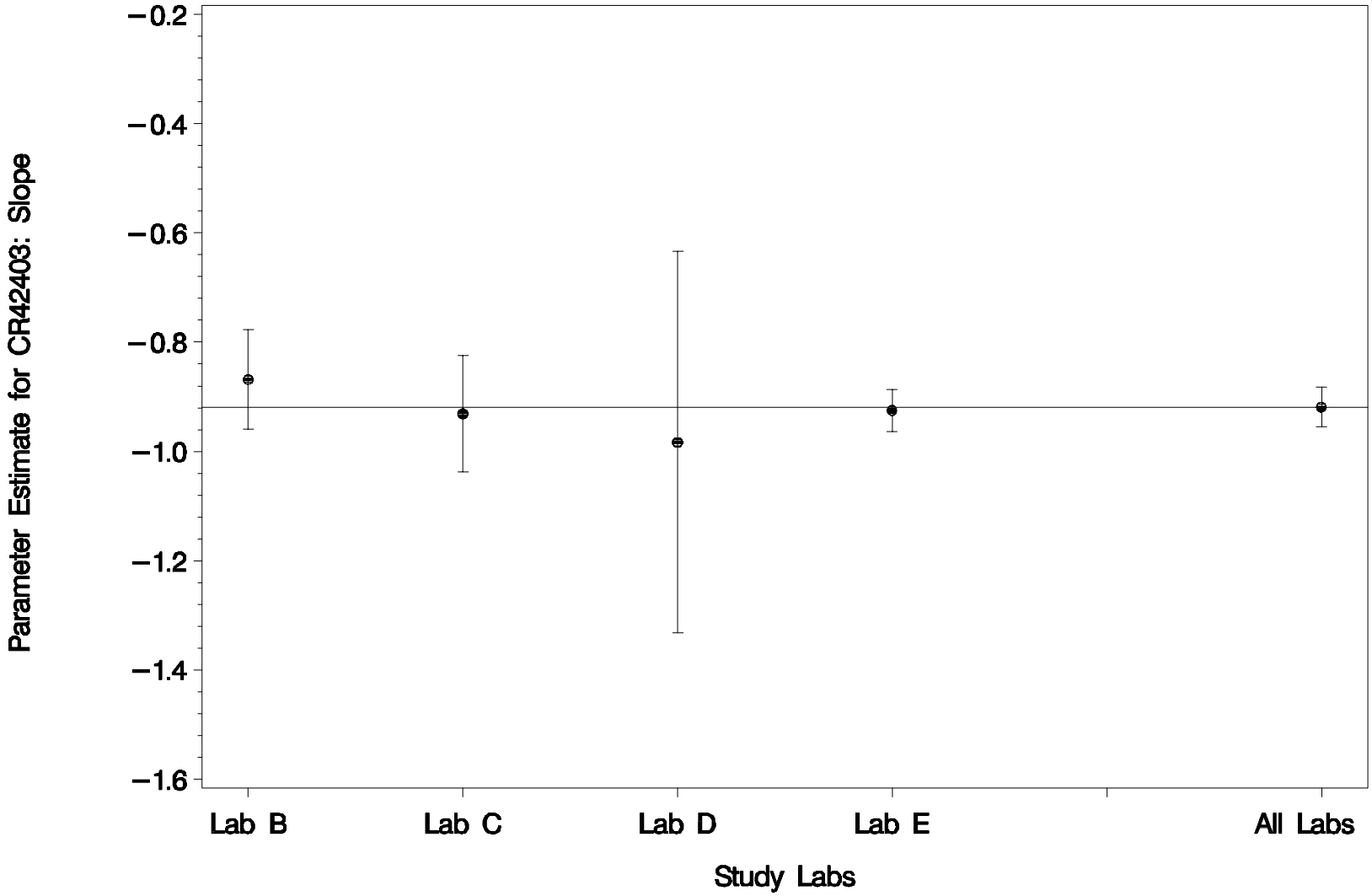

Parameter Estimate for CR42402: Slope
−0.2
−0.4
−0.6
−0.8
−1.0
−1.2
−1.4
−1.6
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

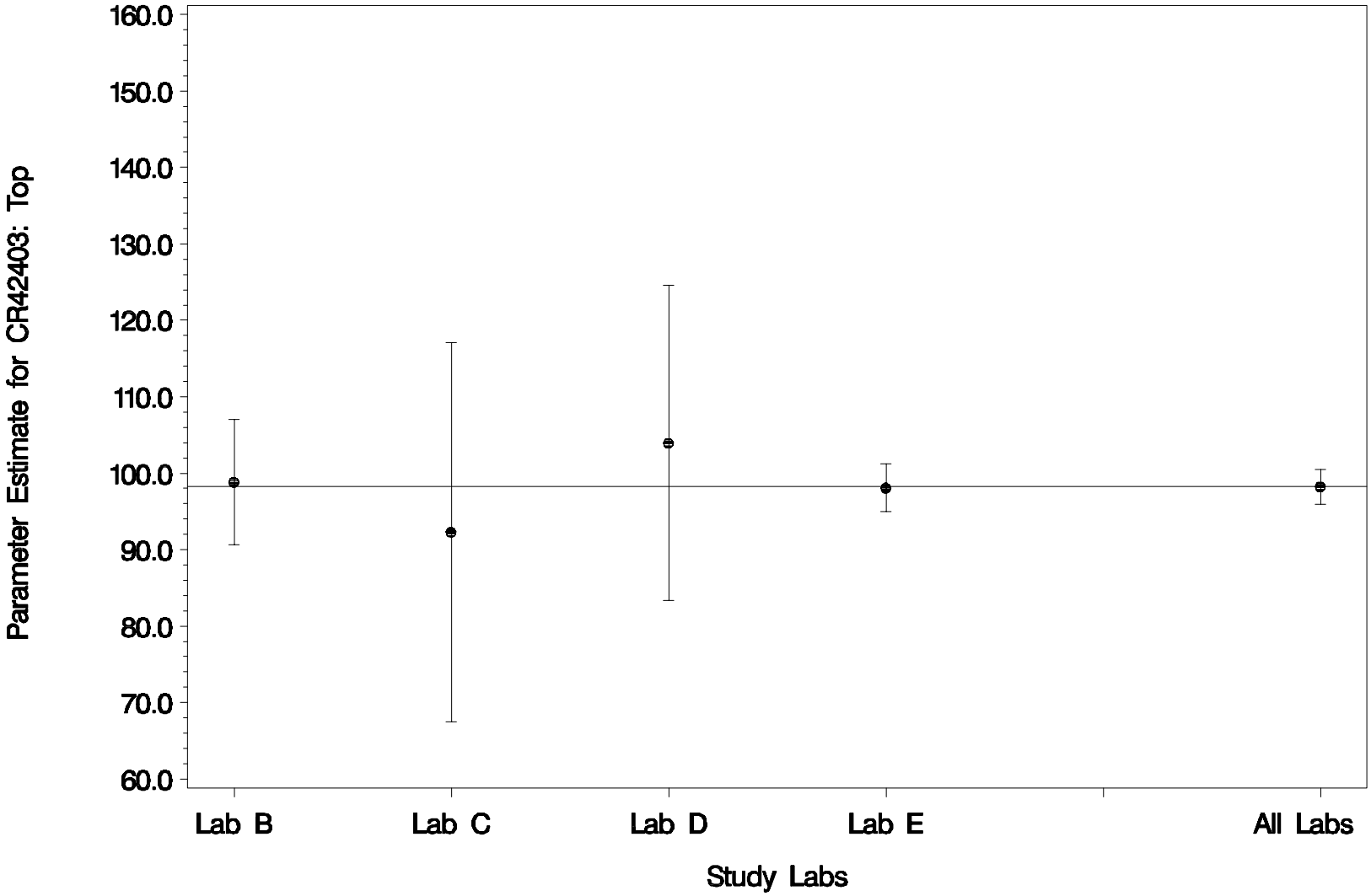

Parameter Estimate for CR42402: Top
160.0
150.0
140.0
130.0
120.0
110.0
100.0
90.0
80.0
70.0
60.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

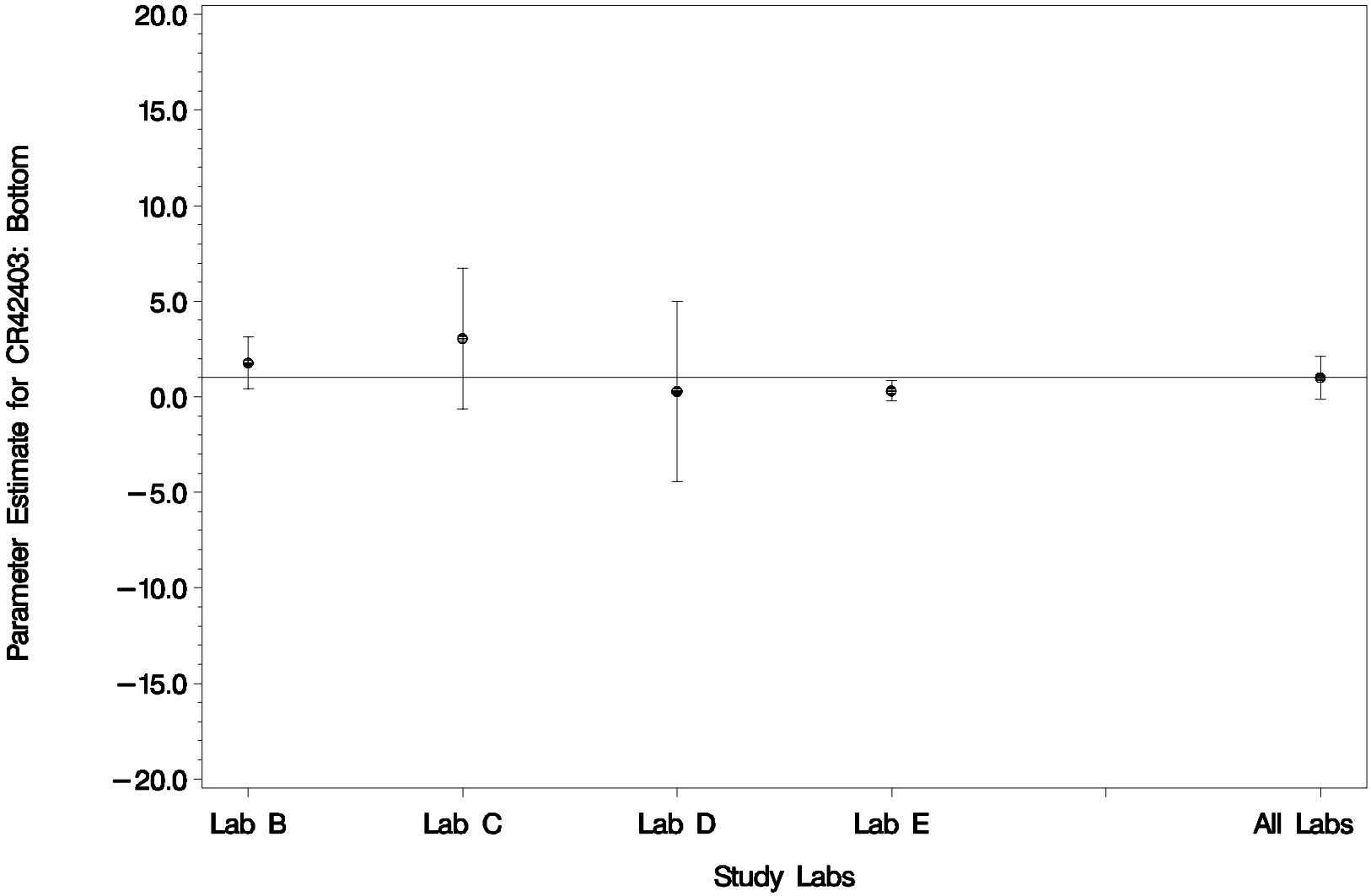

Parameter Estimate for CR42402: Bottom
20.0
15.0
10.0
5.0
0.0
−5.0
−10.0
−15.0
−20.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

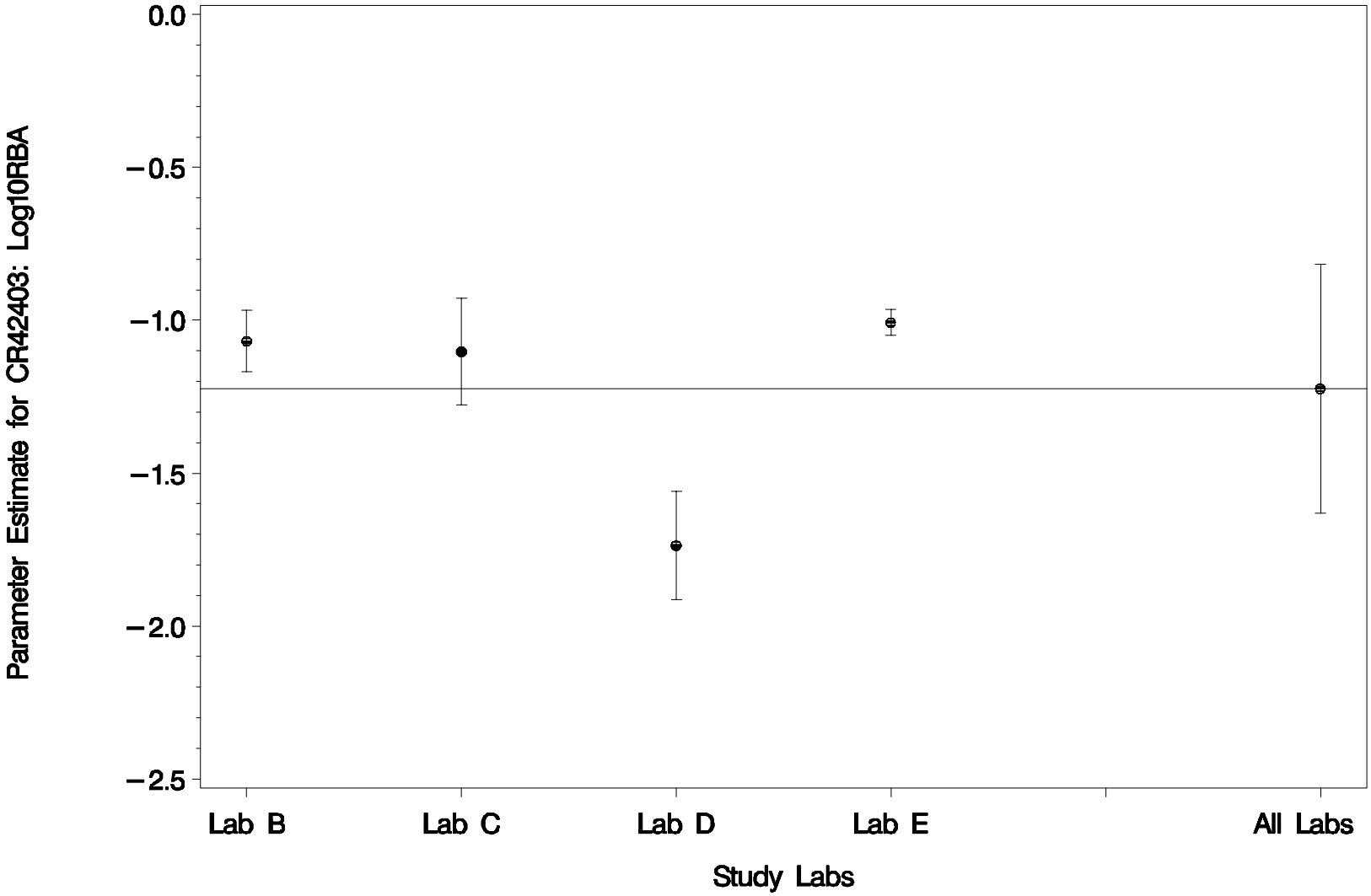

Parameter Estimate for CR42402: Log10RBA
−1.0
−1.5
−2.0
−2.5
−3.0
−3.5
−4.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

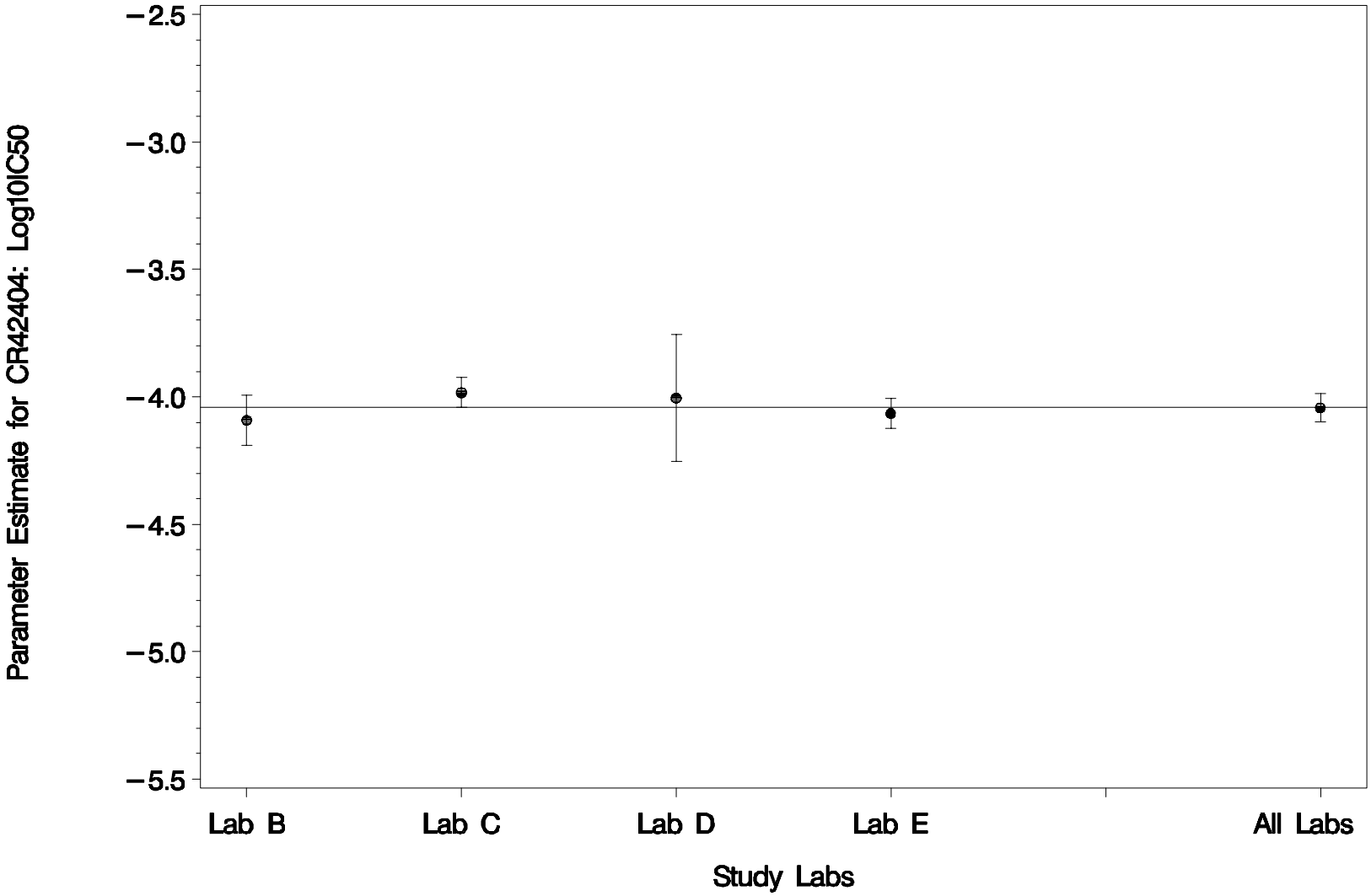

Parameter Estimate for CR42403: Log10IC50
−5.5
−6.0
−6.5
−7.0
−7.5
−8.0
−8.5
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

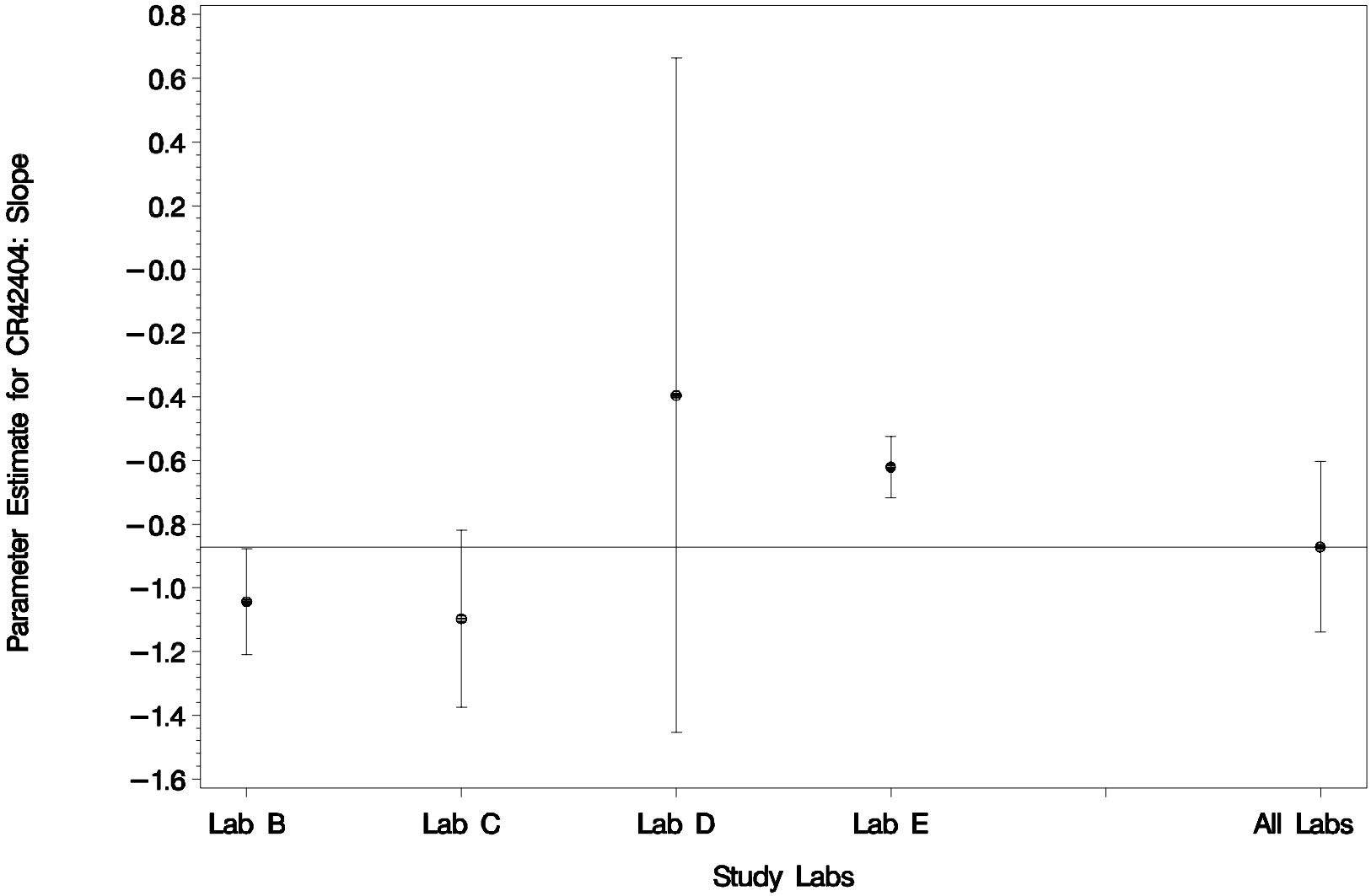

Parameter Estimate for CR42403: Slope
−0.2
−0.4
−0.6
−0.8
−1.0
−1.2
−1.4
−1.6
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

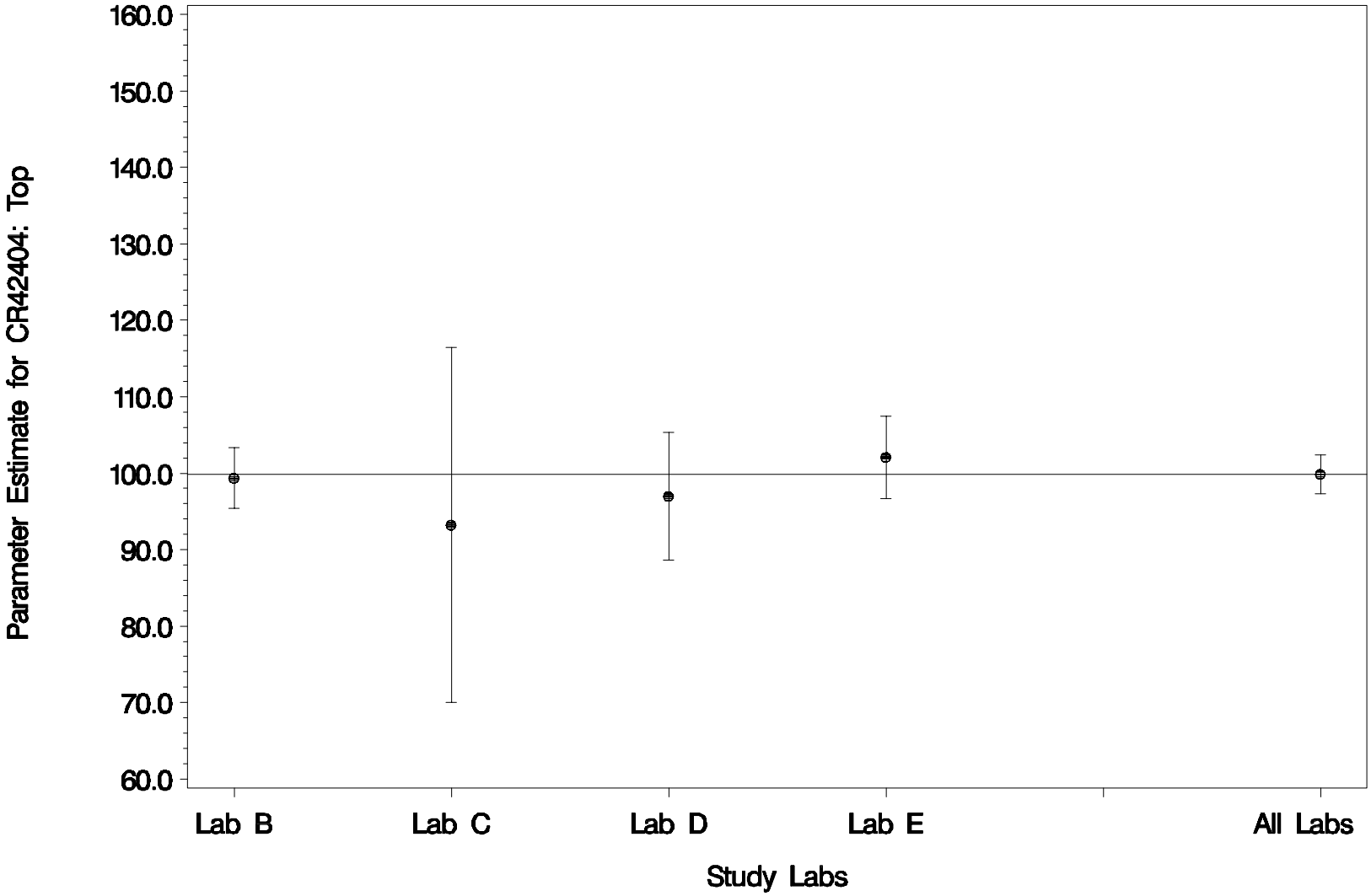

Parameter Estimate for CR42403: Top
160.0
150.0
140.0
130.0
120.0
110.0
100.0
90.0
80.0
70.0
60.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

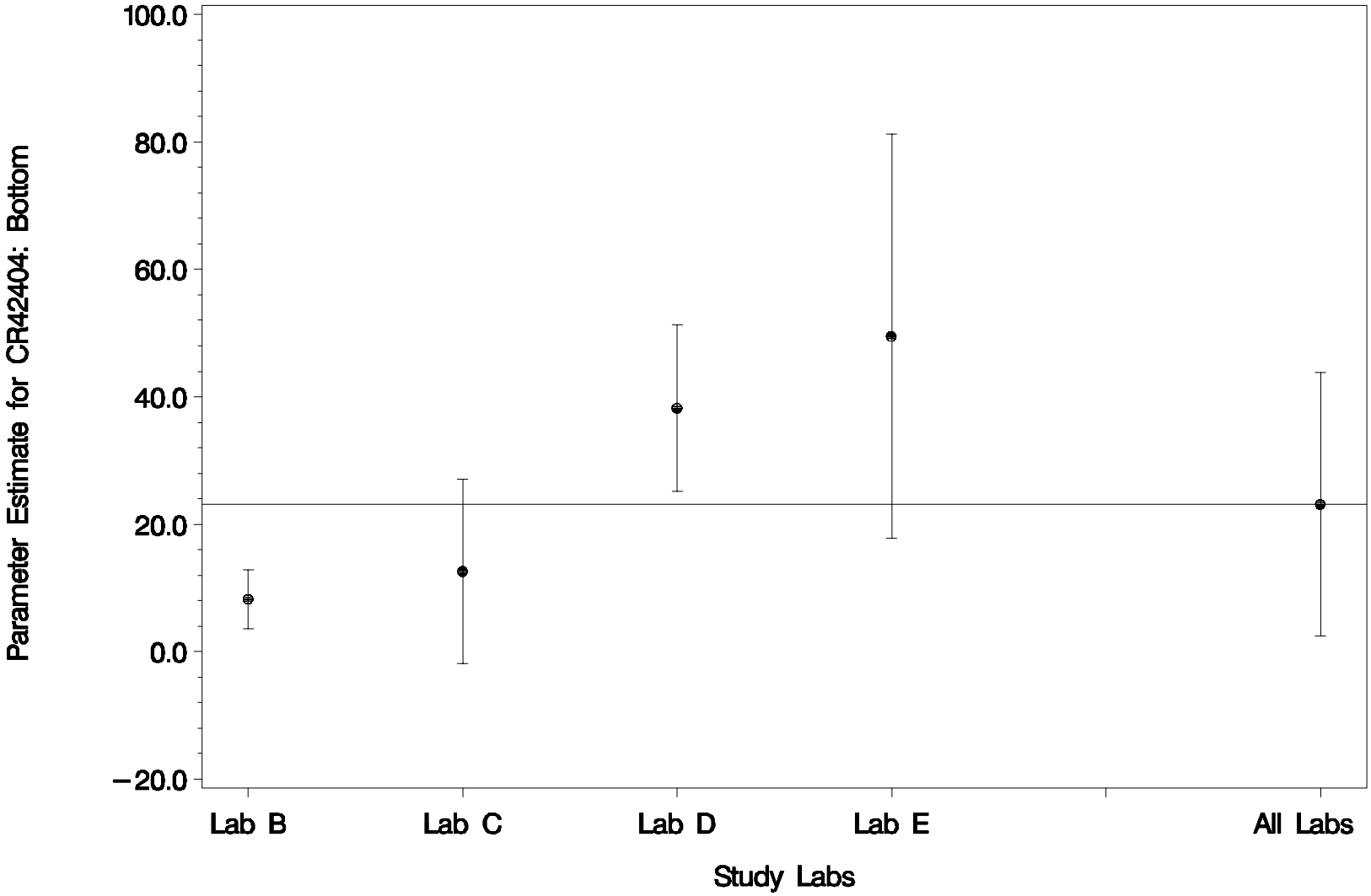

20.0
15.0
10.0
5.0
0.0
−5.0
−10.0
−15.0
−20.0
Parameter Estimate for CR42403: Bottom
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

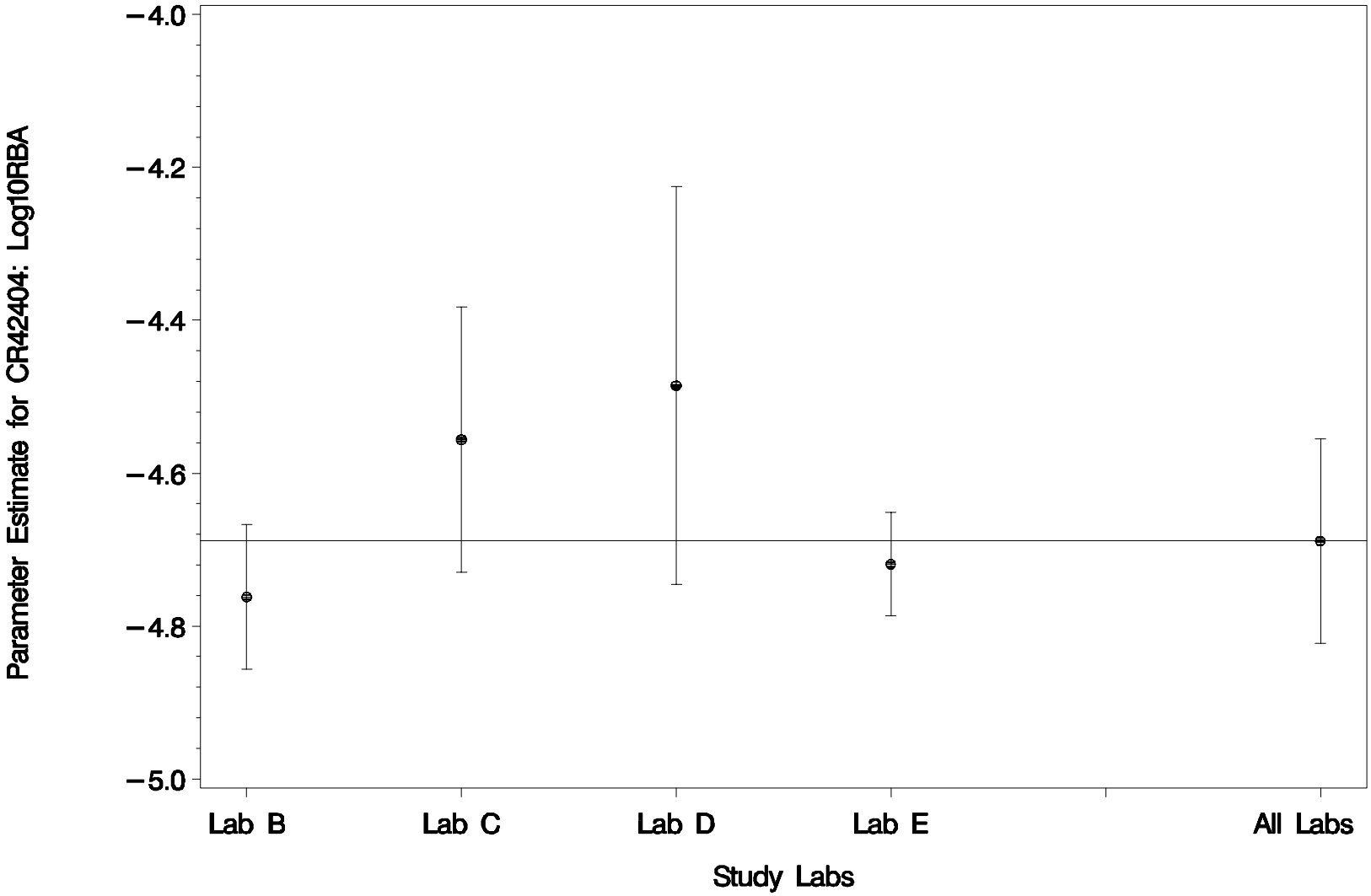

Parameter Estimate for CR42403: Log10RBA
0.0
−0.5
−1.0
−1.5
−2.0
−2.5
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

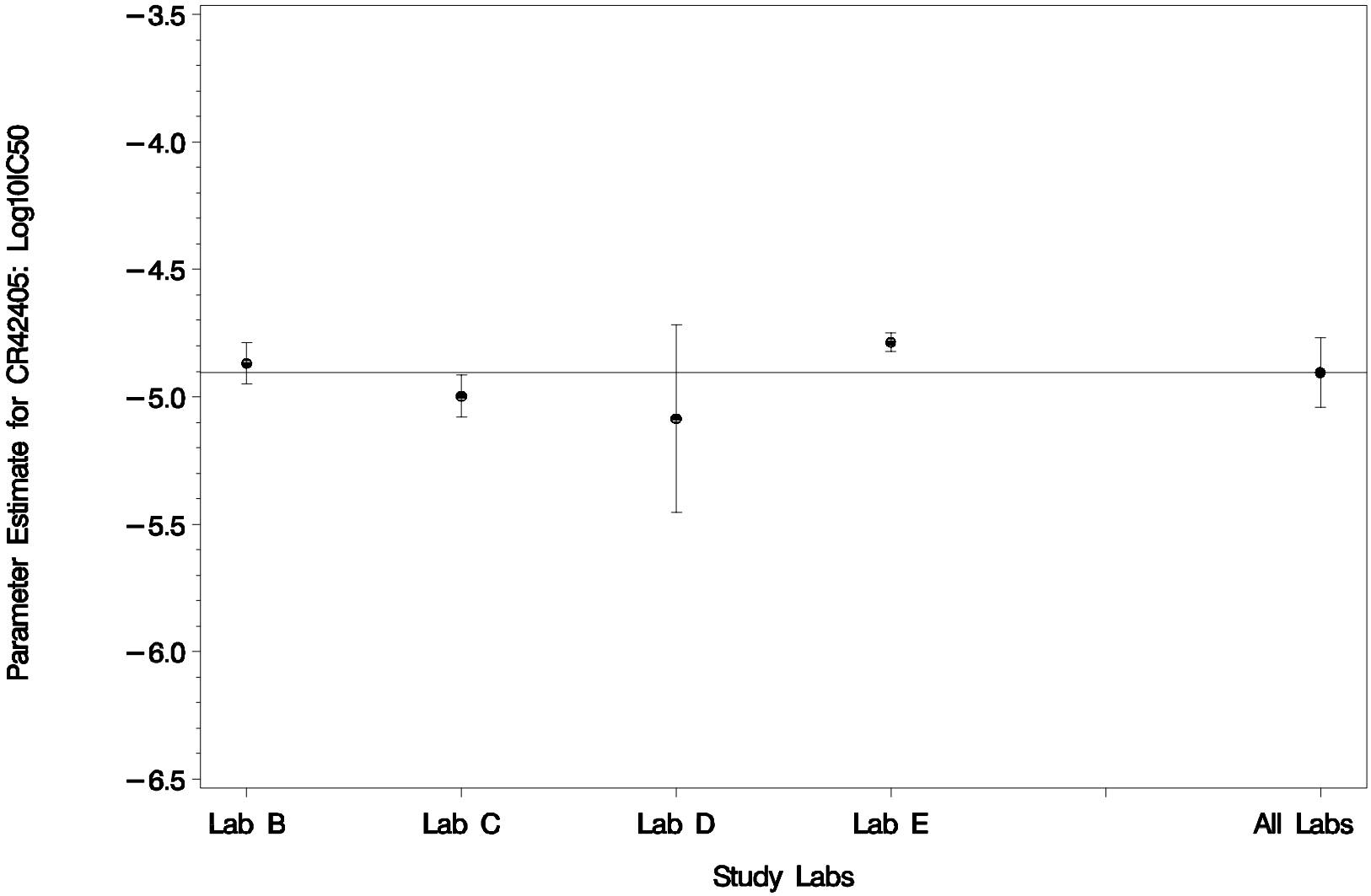

Parameter Estimate for CR42404: Log10IC50
−2.5
−3.0
−3.5
−4.0
−4.5
−5.0
−5.5
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

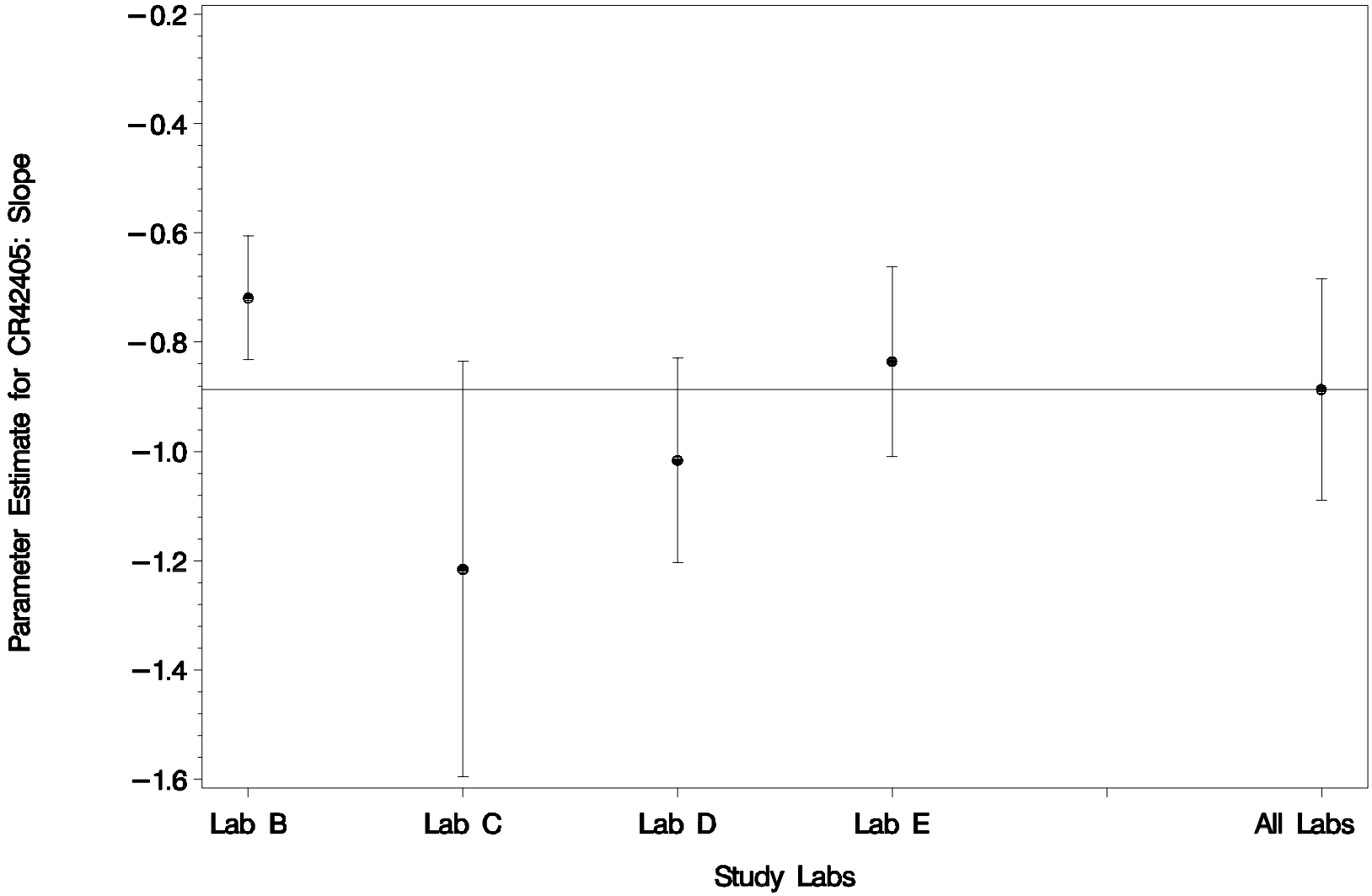

Parameter Estimate for CR42404: Slope
0.8
0.6
0.4
0.2
−0.0
−0.2
−0.4
−0.6
−0.8
−1.0
−1.2
−1.4
−1.6
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

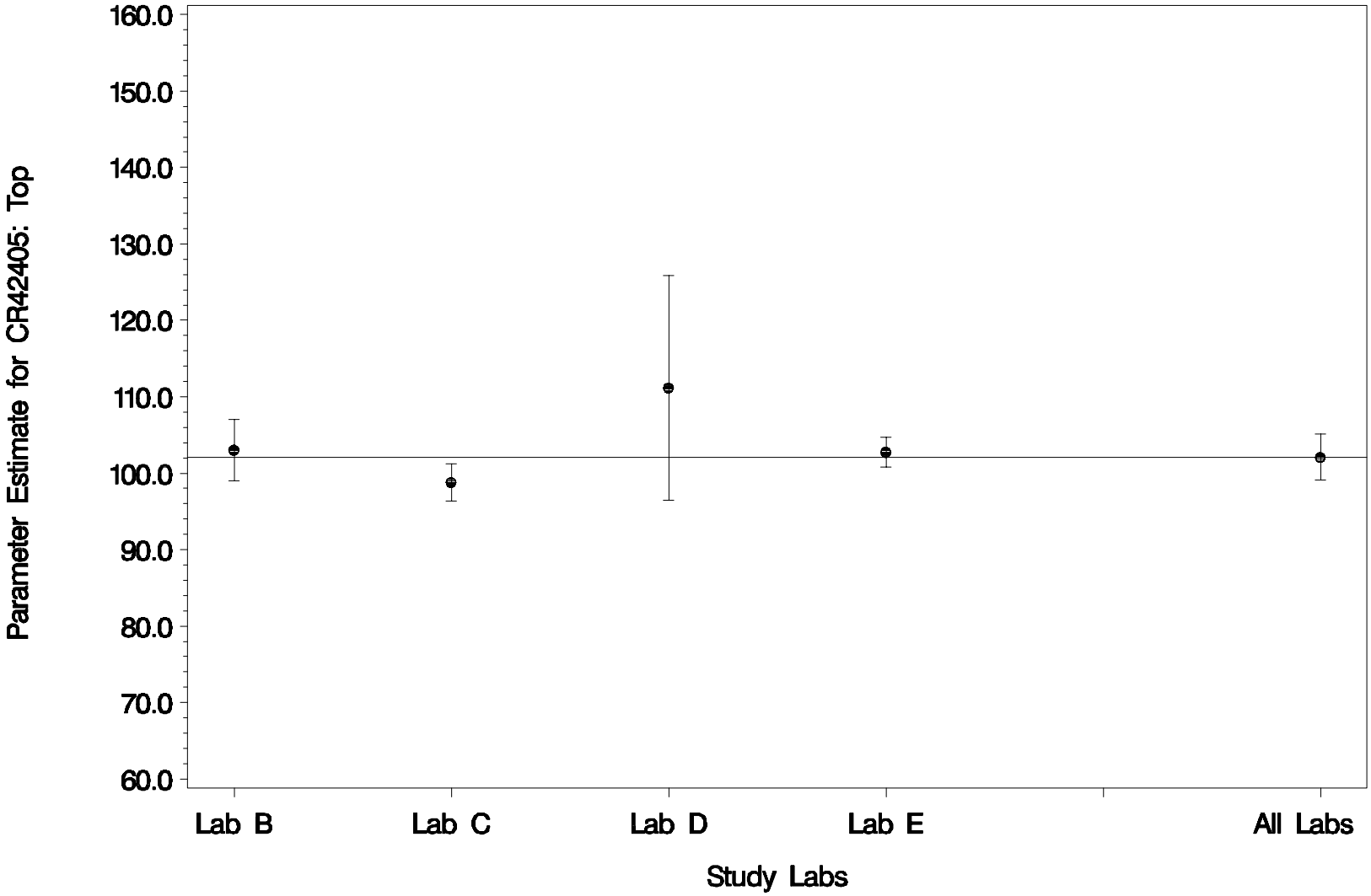

Parameter Estimate for CR42404: Top
160.0
150.0
140.0
130.0
120.0
110.0
100.0
90.0
80.0
70.0
60.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

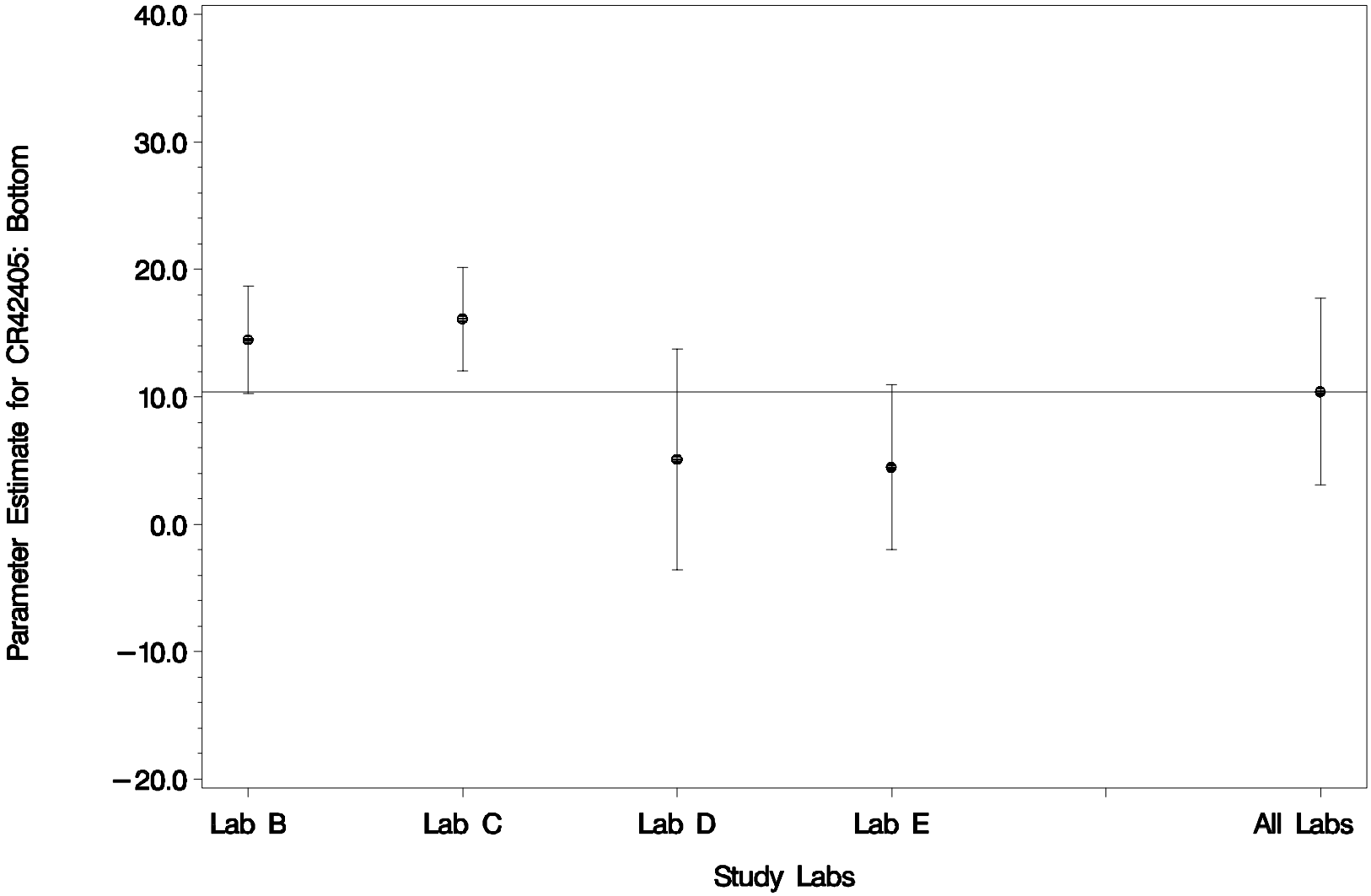

Parameter Estimate for CR42404: Bottom
100.0
80.0
60.0
40.0
20.0
0.0
−20.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

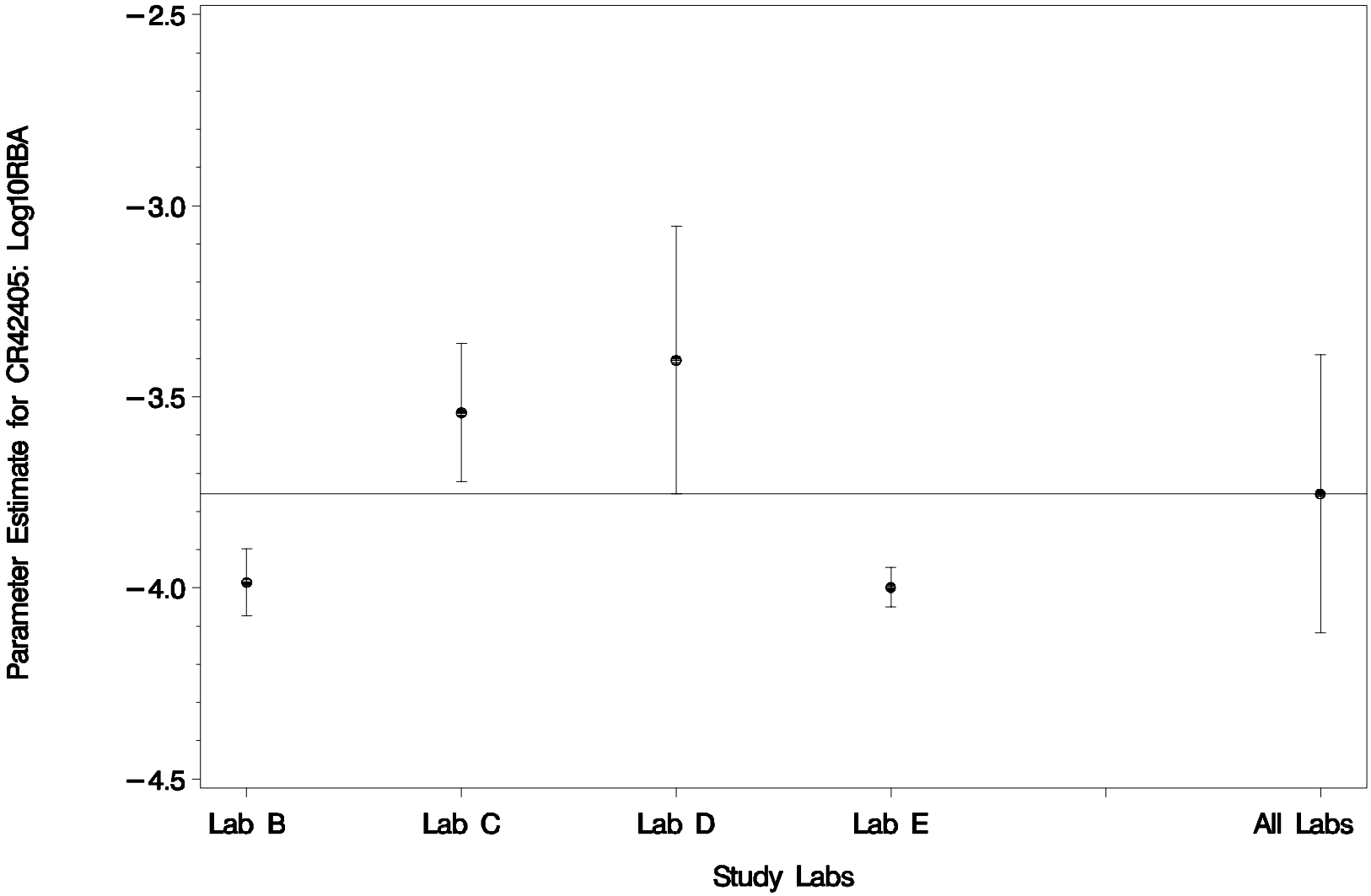

Parameter Estimate for CR42404: Log10RBA
−4.0
−4.2
−4.4
−4.6
−4.8
−5.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

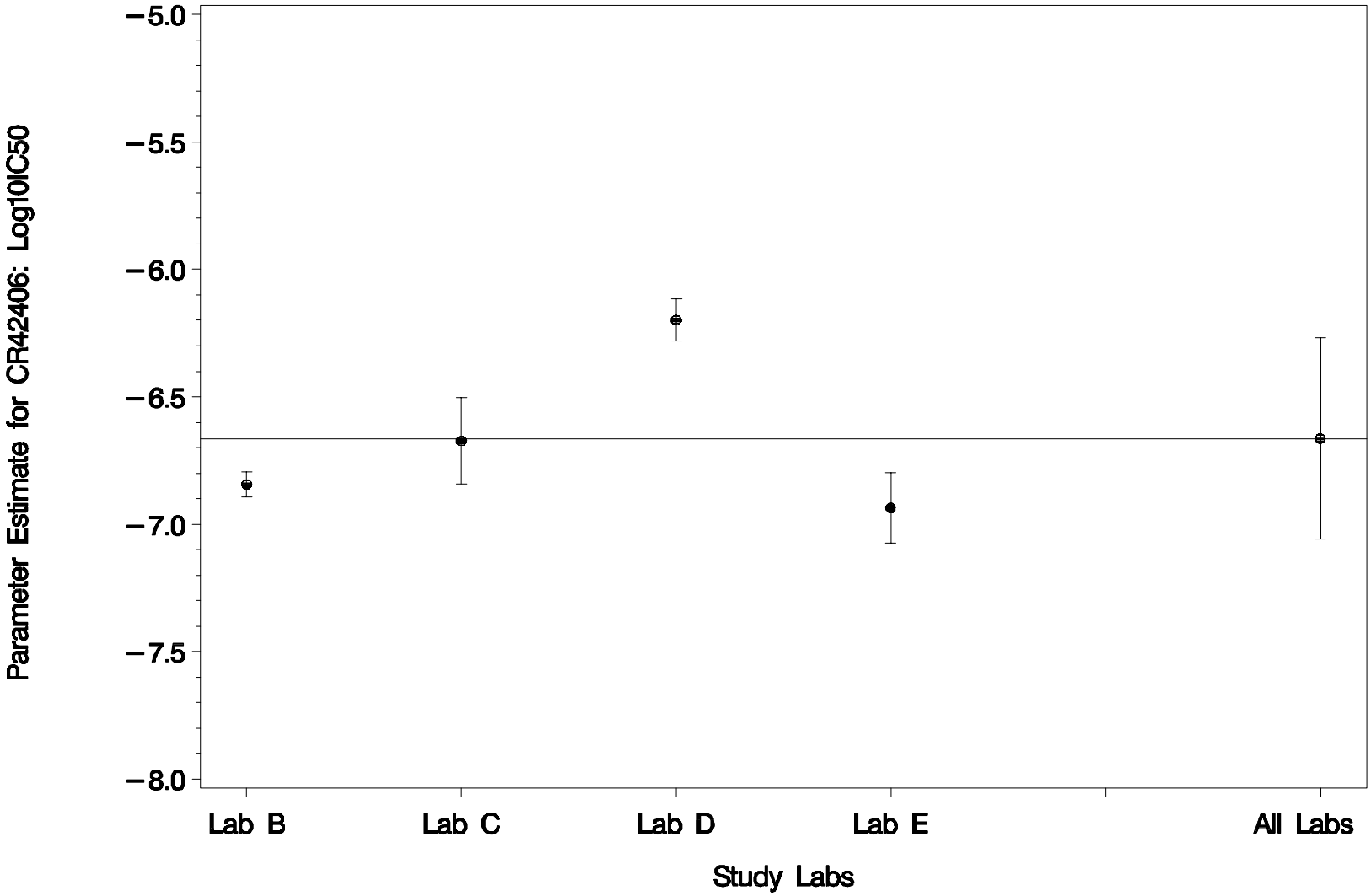

Parameter Estimate for CR42405: Log10IC50
−3.5
−4.0
−4.5
−5.0
−5.5
−6.0
−6.5
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

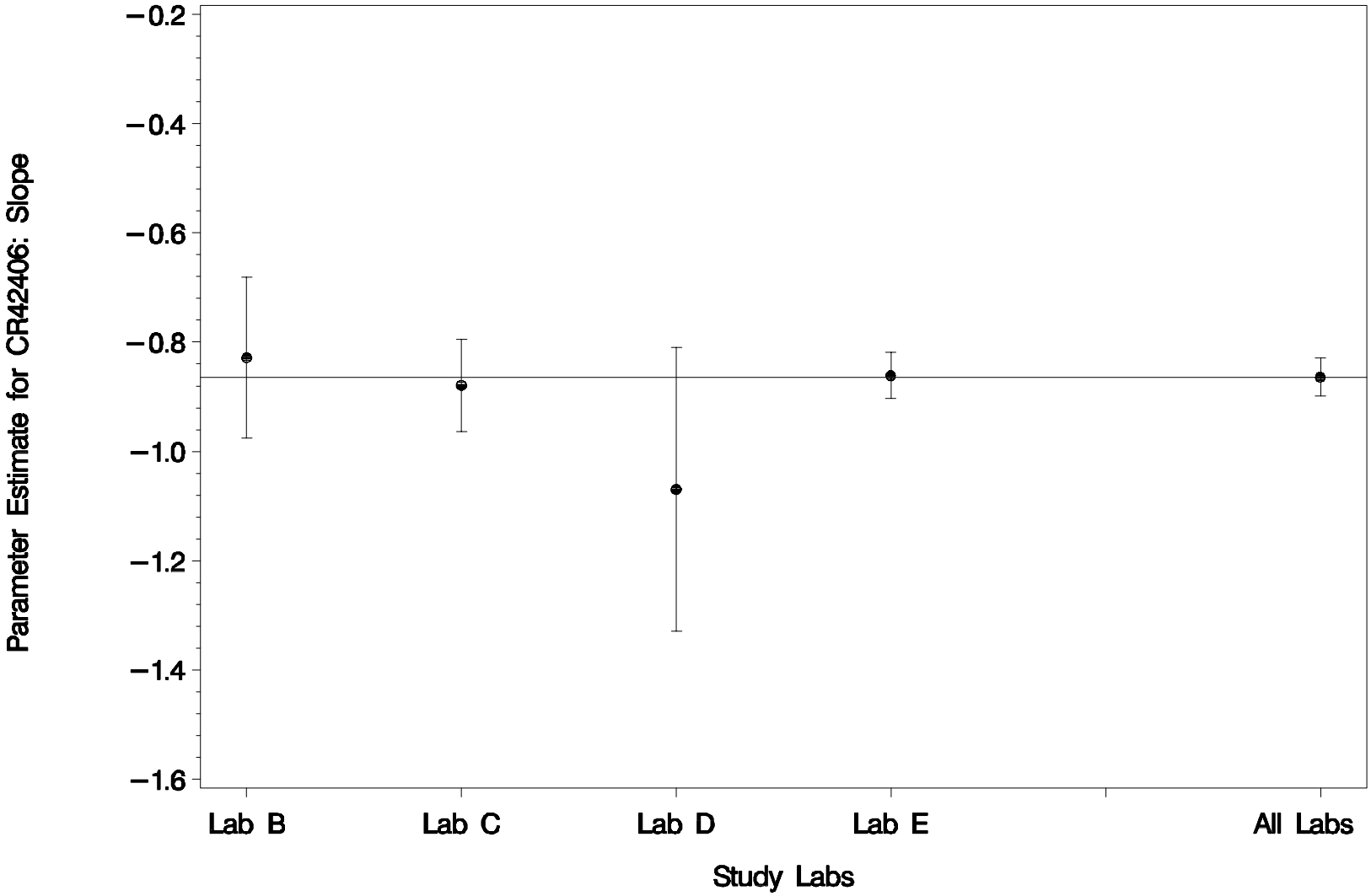

Parameter Estimate for CR42405: Slope
−0.2
−0.4
−0.6
−0.8
−1.0
−1.2
−1.4
−1.6
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

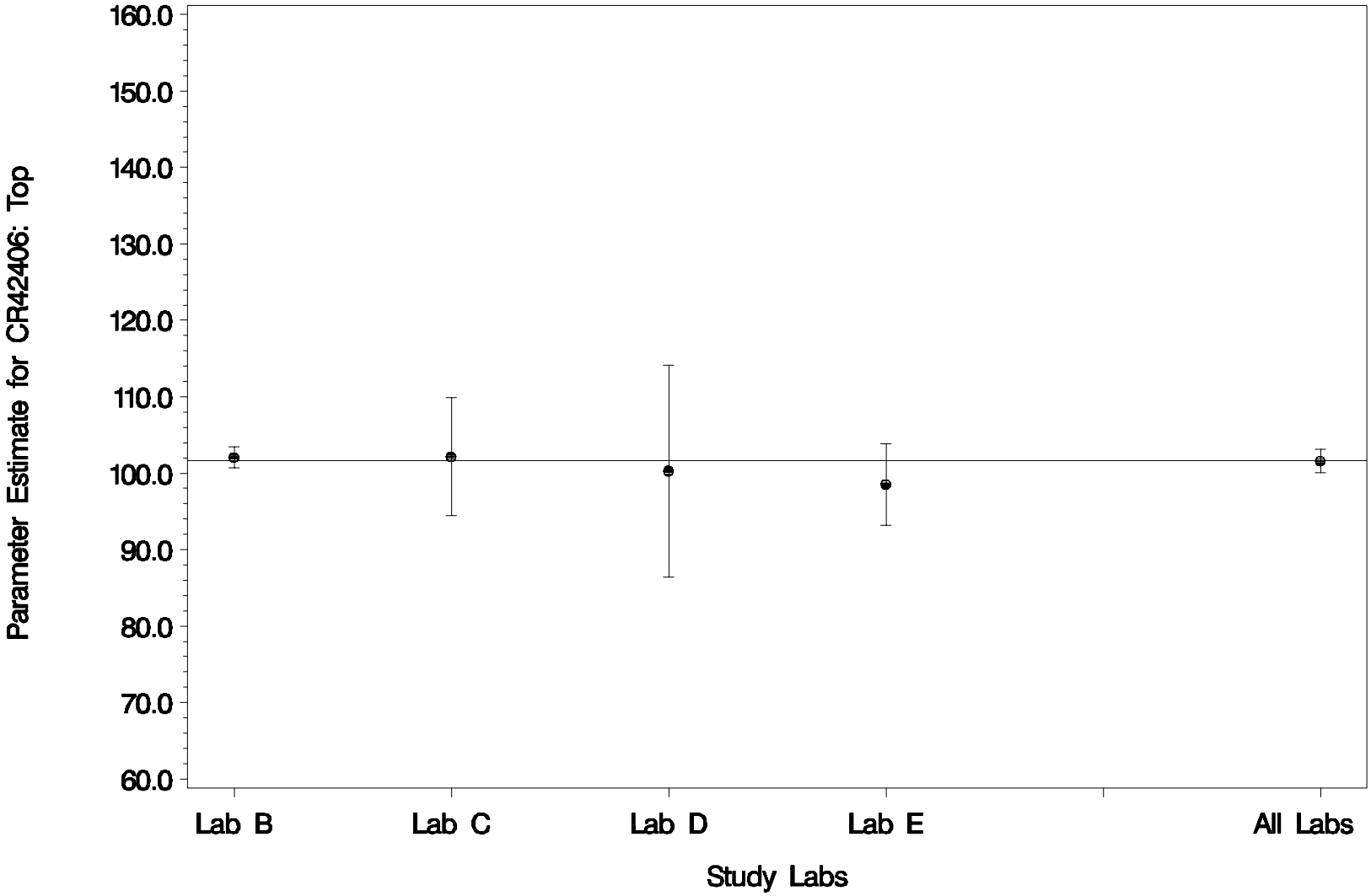

Parameter Estimate for CR42405: Top
160.0
150.0
140.0
130.0
120.0
110.0
100.0
90.0
80.0
70.0
60.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

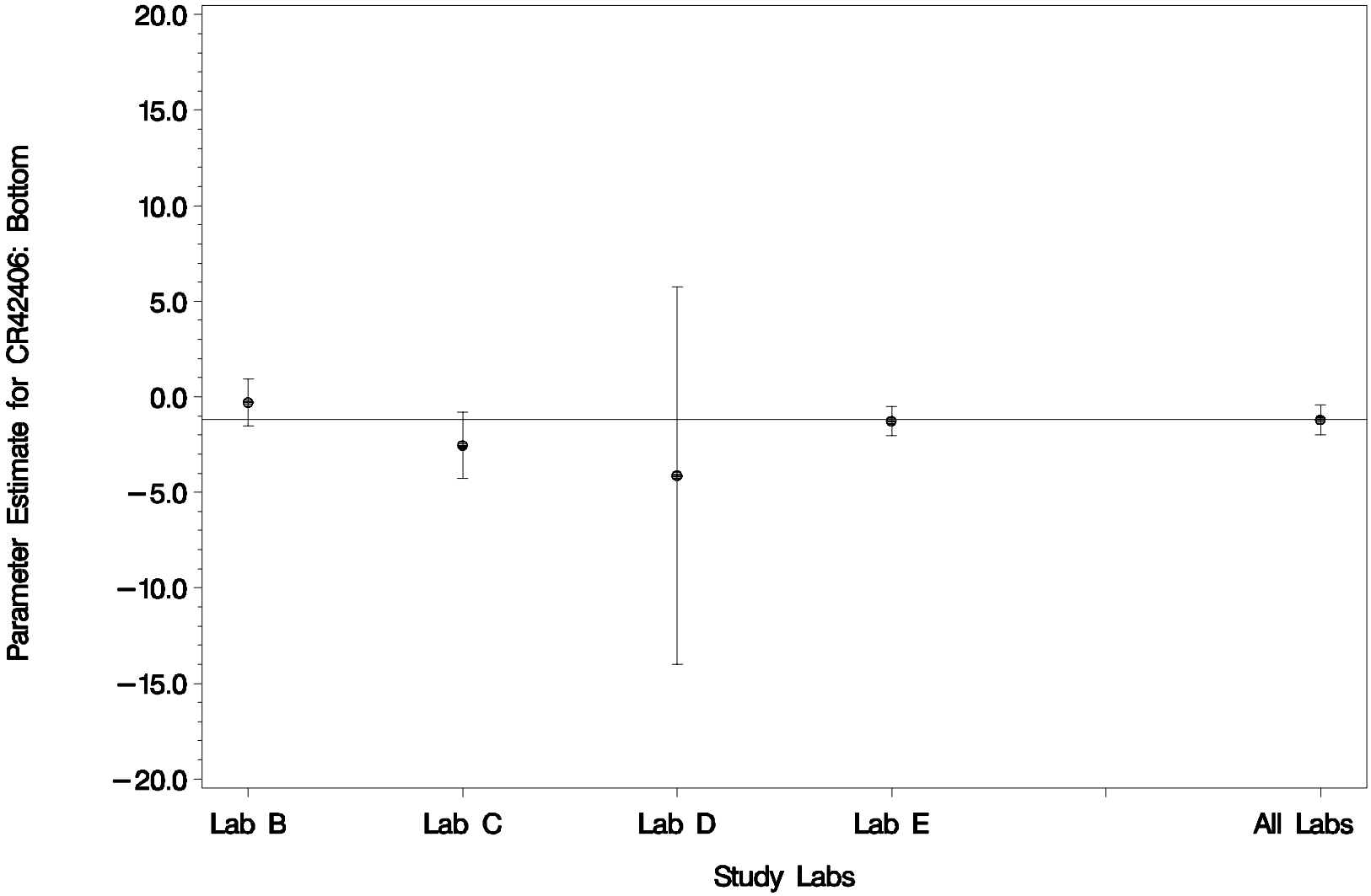

Parameter Estimate for CR42405: Bottom
40.0
30.0
20.0
10.0
0.0
−10.0
−20.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

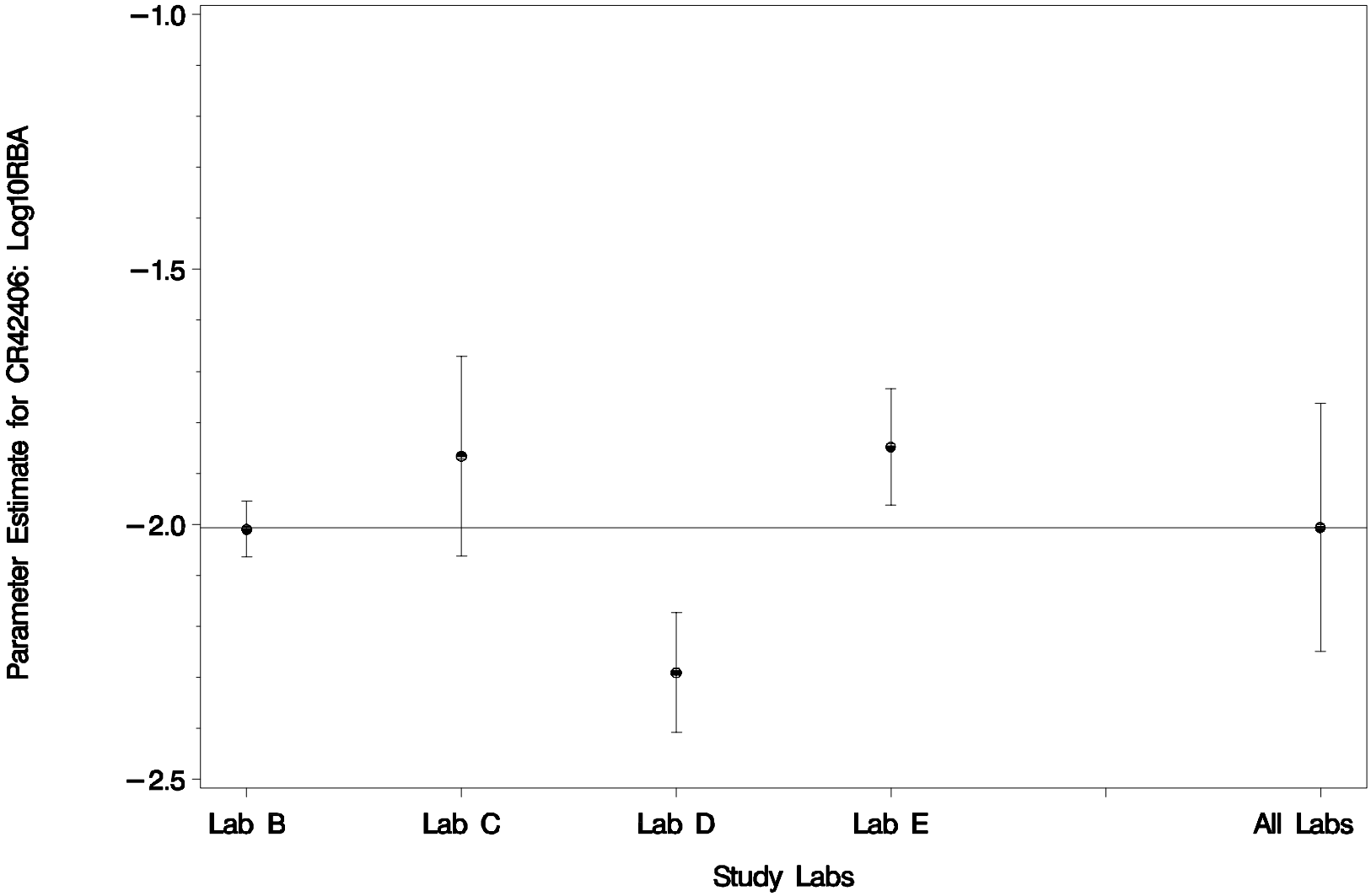

−2.5
−3.0
−3.5
−4.0
−4.5
Parameter Estimate for CR42405: Log10RBA
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

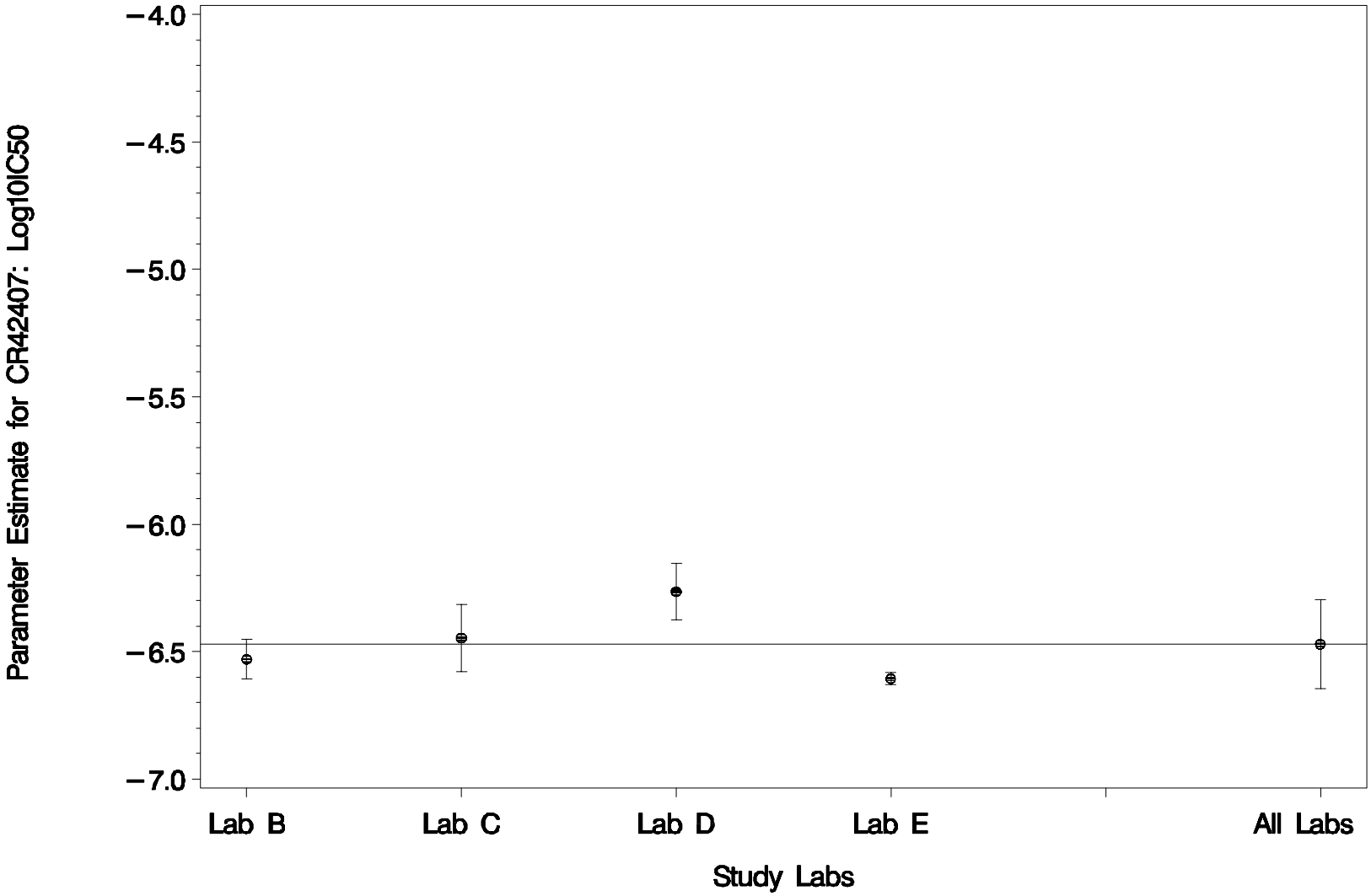

Parameter Estimate for CR42406: Log10IC50
−5.0
−5.5
−6.0
−6.5
−7.0
−7.5
−8.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

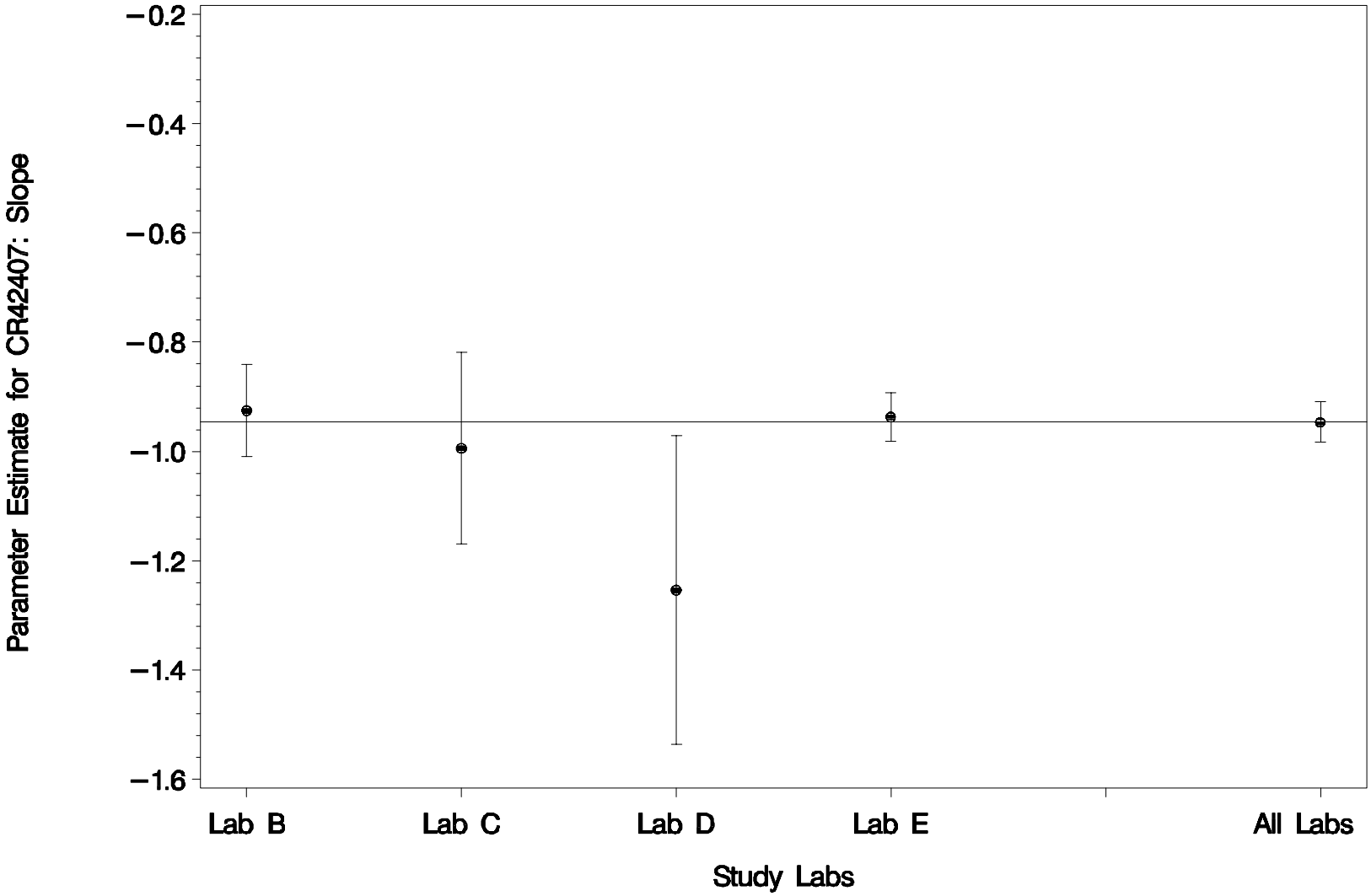

Parameter Estimate for CR42406: Slope
−0.2
−0.4
−0.6
−0.8
−1.0
−1.2
−1.4
−1.6
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

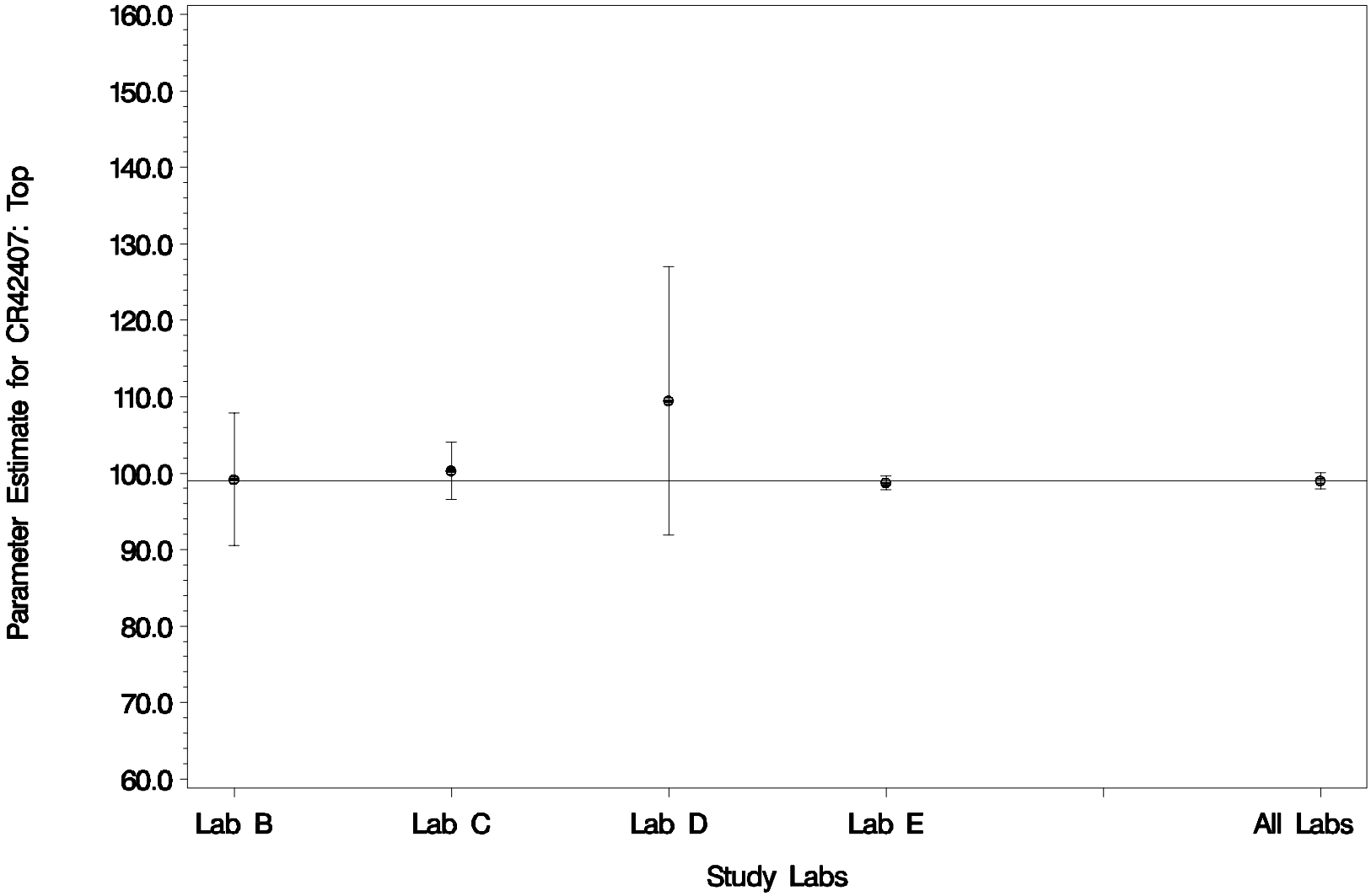

160.0
150.0
140.0
130.0
120.0
110.0
100.0
90.0
80.0
70.0
60.0
Parameter Estimate for CR42406: Top
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

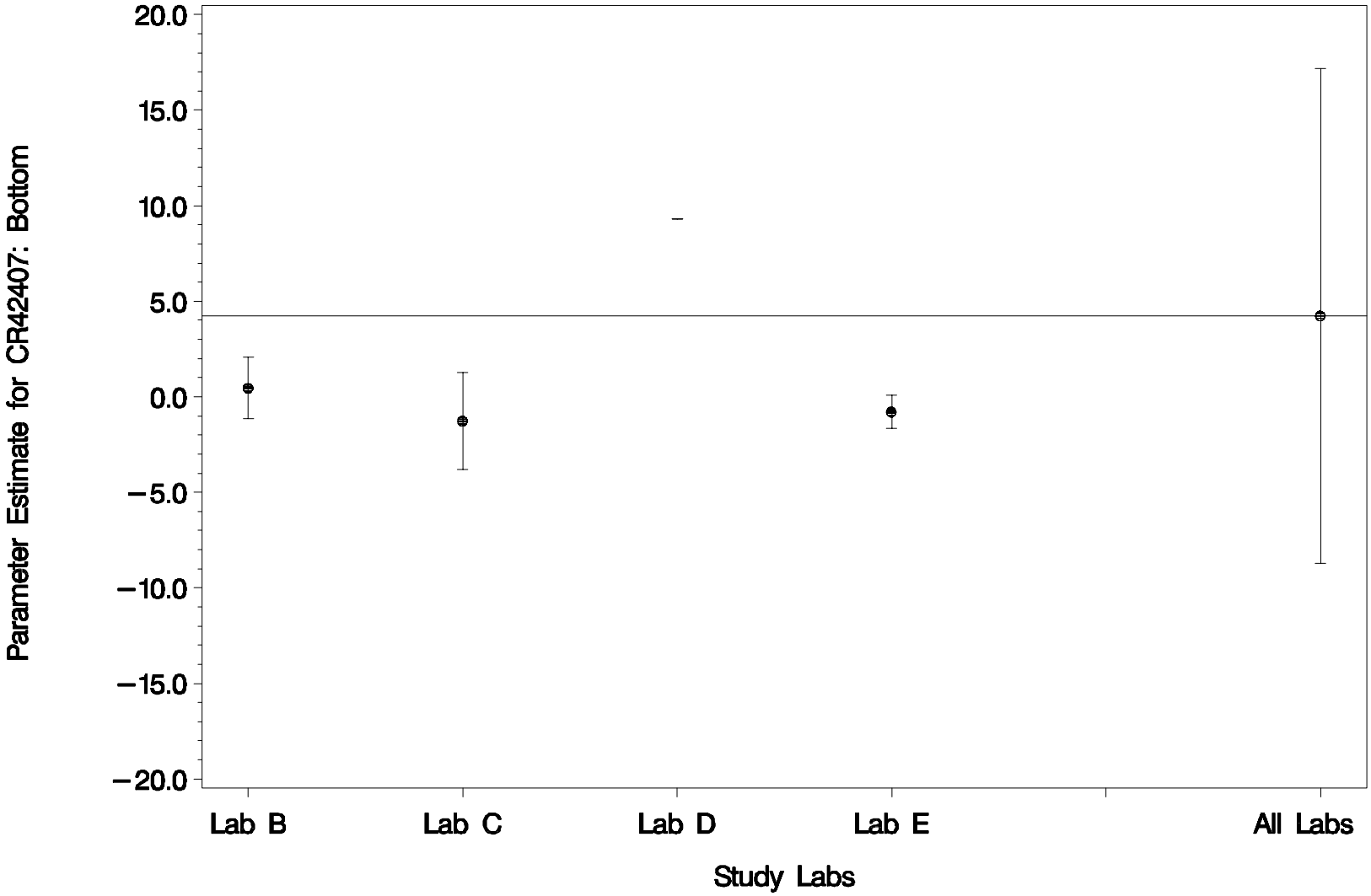

Parameter Estimate for CR42406: Bottom
20.0
15.0
10.0
5.0
0.0
−5.0
−10.0
−15.0
−20.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

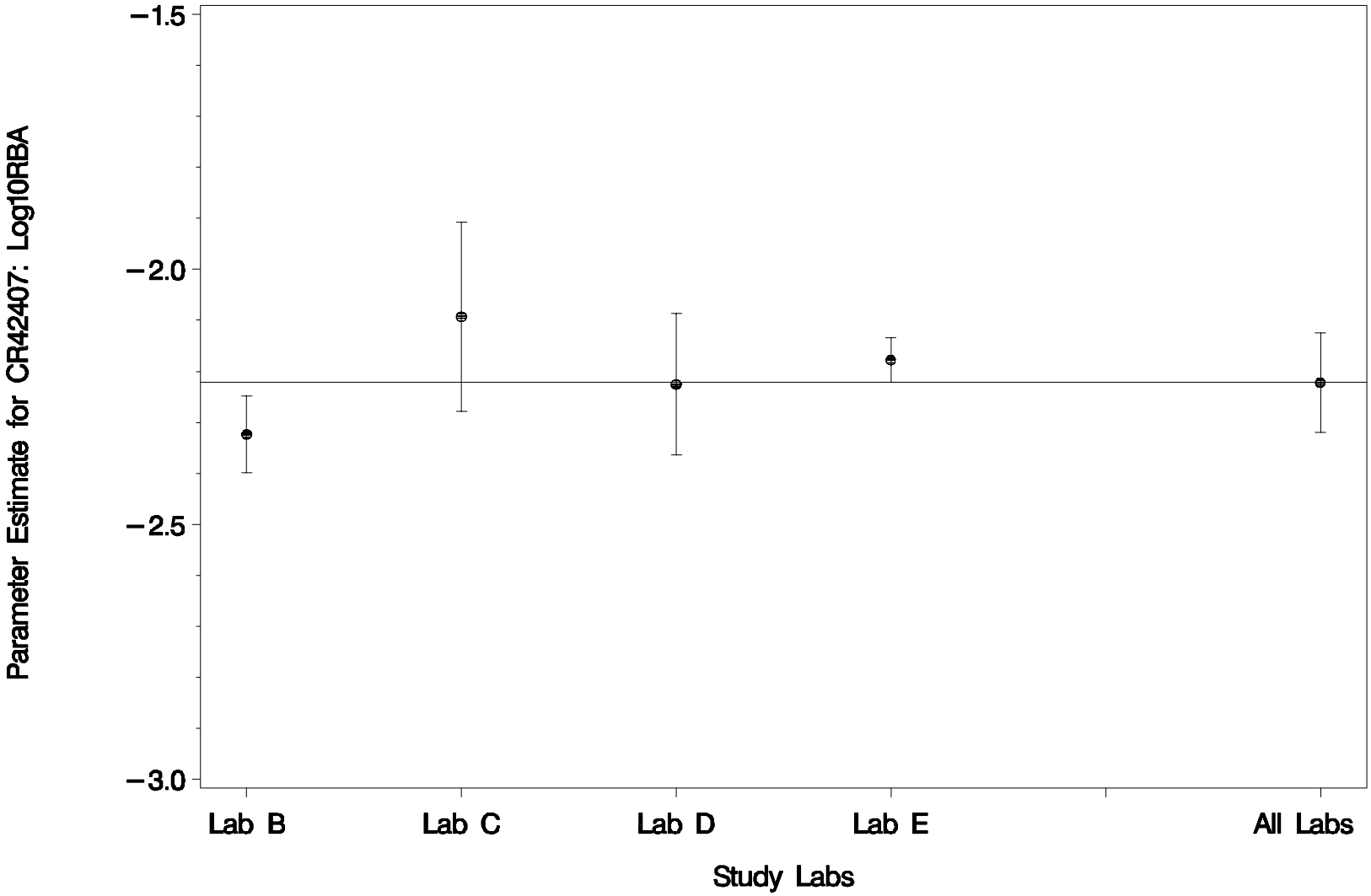

−1.0
−1.5
−2.0
−2.5
Parameter Estimate for CR42406: Log10RBA
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

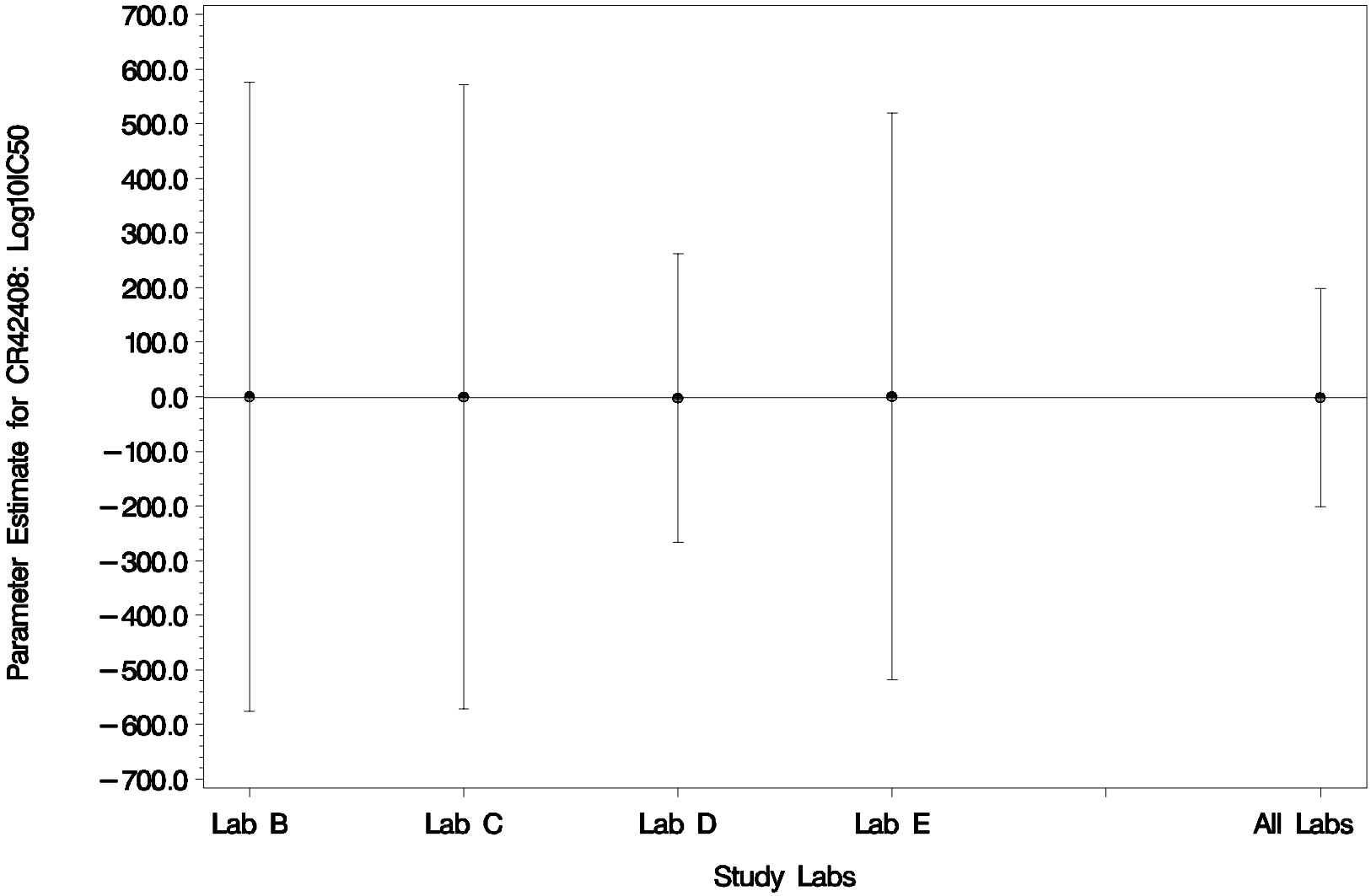

Parameter Estimate for CR42407: Log10IC50
−4.0
−4.5
−5.0
−5.5
−6.0
−6.5
−7.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

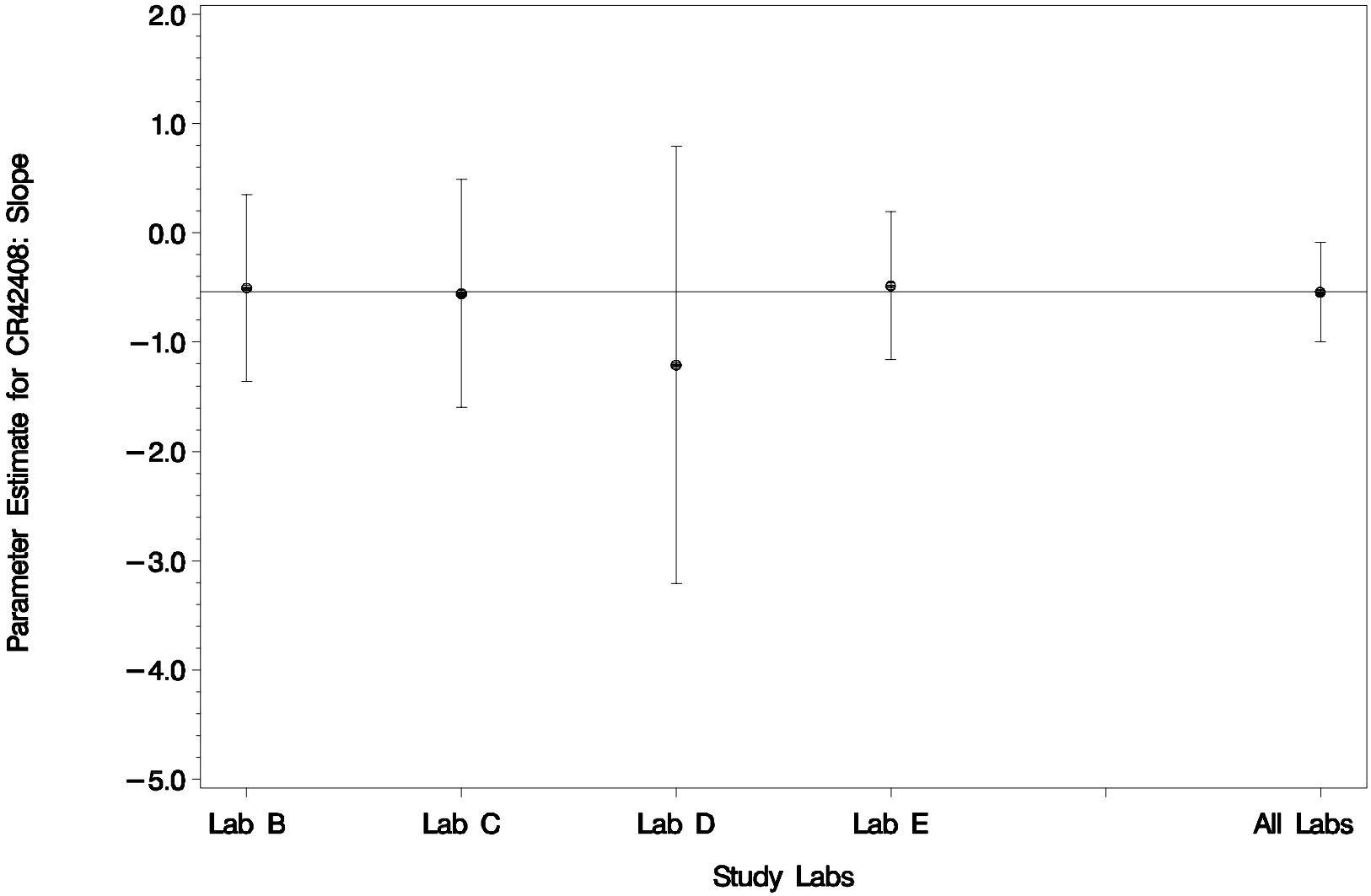

Parameter Estimate for CR42407: Slope
−0.2
−0.4
−0.6
−0.8
−1.0
−1.2
−1.4
−1.6
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

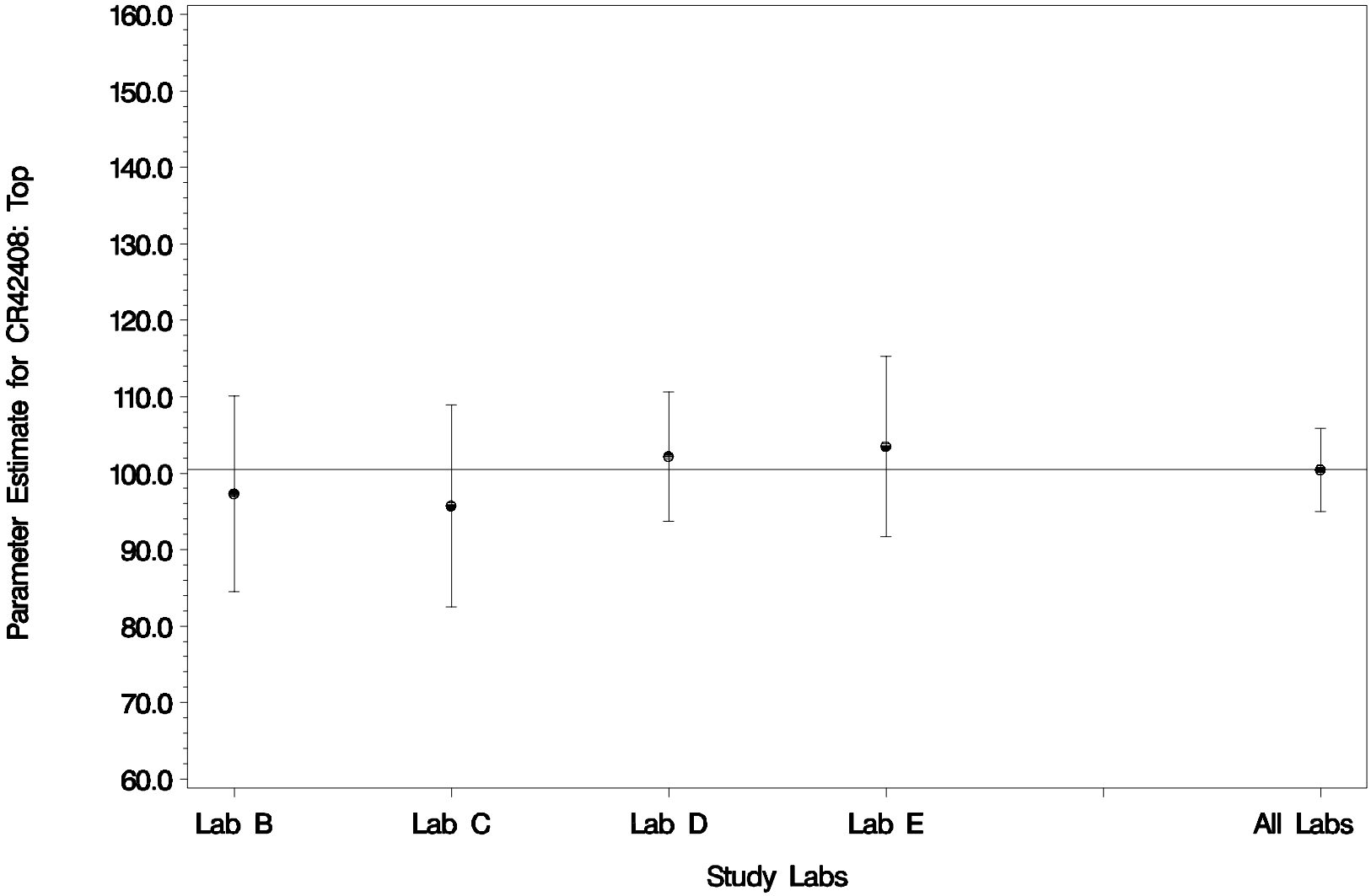

160.0
150.0
140.0
130.0
120.0
110.0
100.0
90.0
80.0
70.0
60.0
Parameter Estimate for CR42407: Top
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

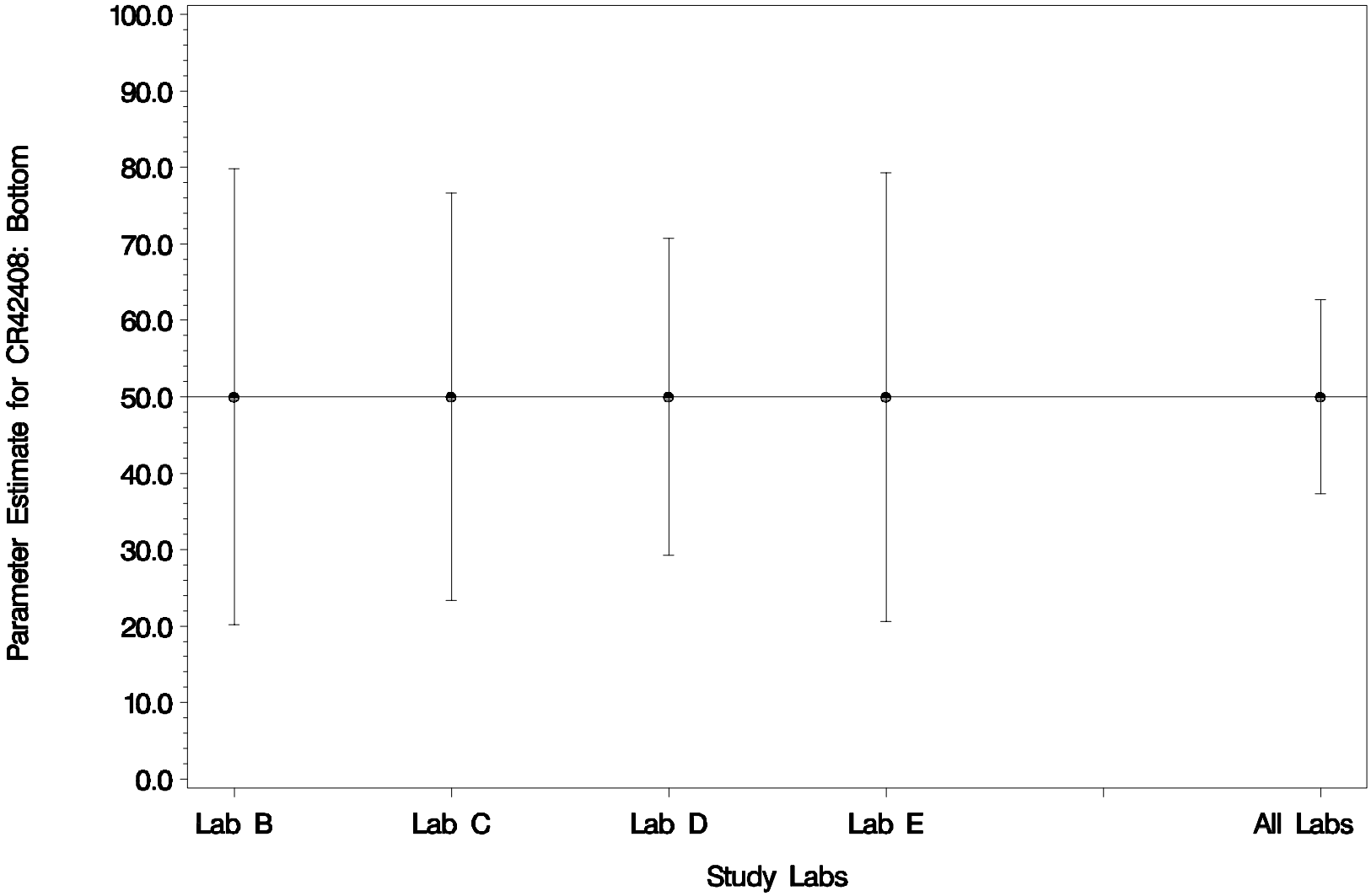

Parameter Estimate for CR42407: Bottom
20.0
15.0
10.0
5.0
0.0
−5.0
−10.0
−15.0
−20.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

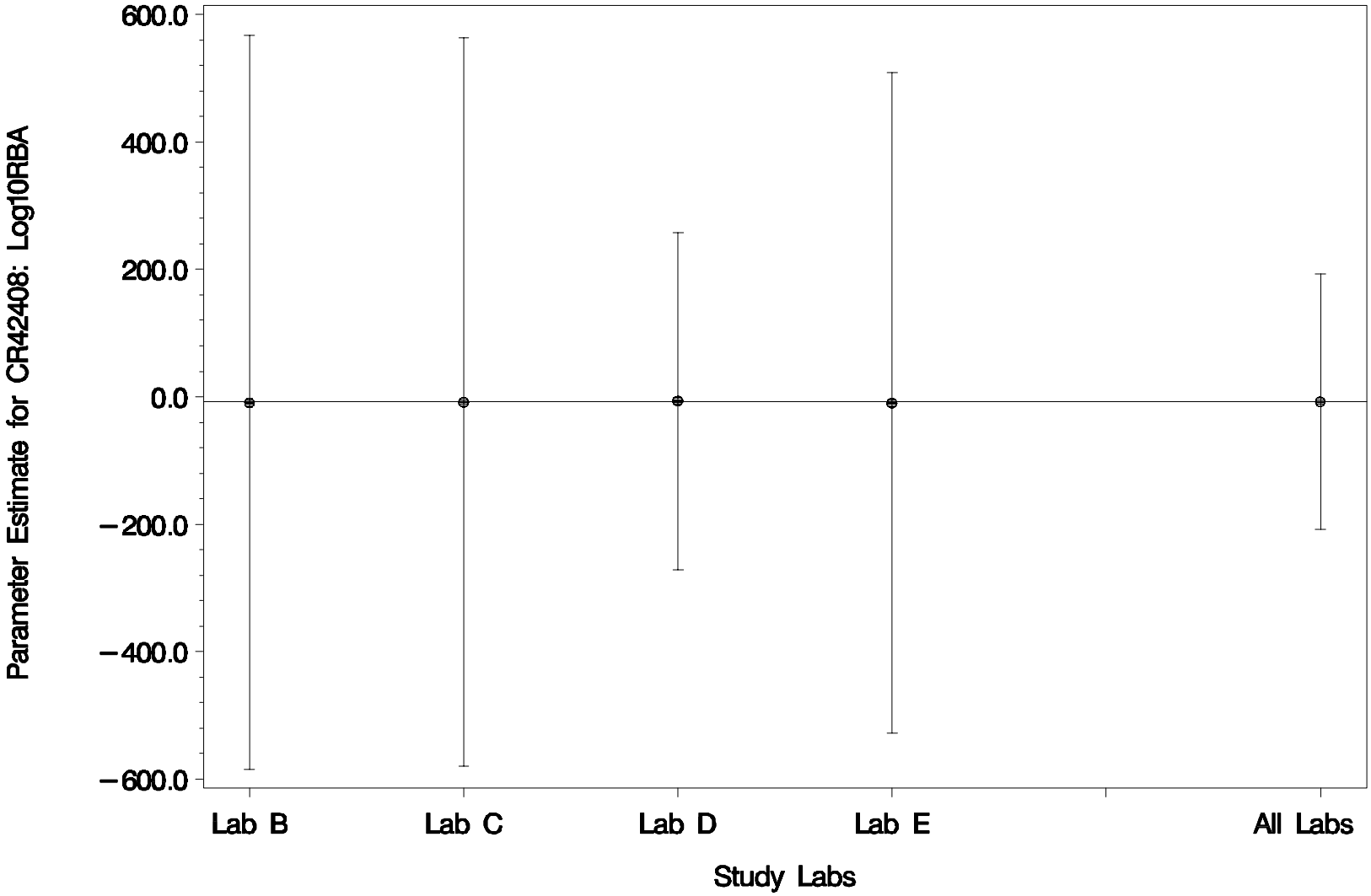

−1.5
−2.0
−2.5
−3.0
Parameter Estimate for CR42407: Log10RBA
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

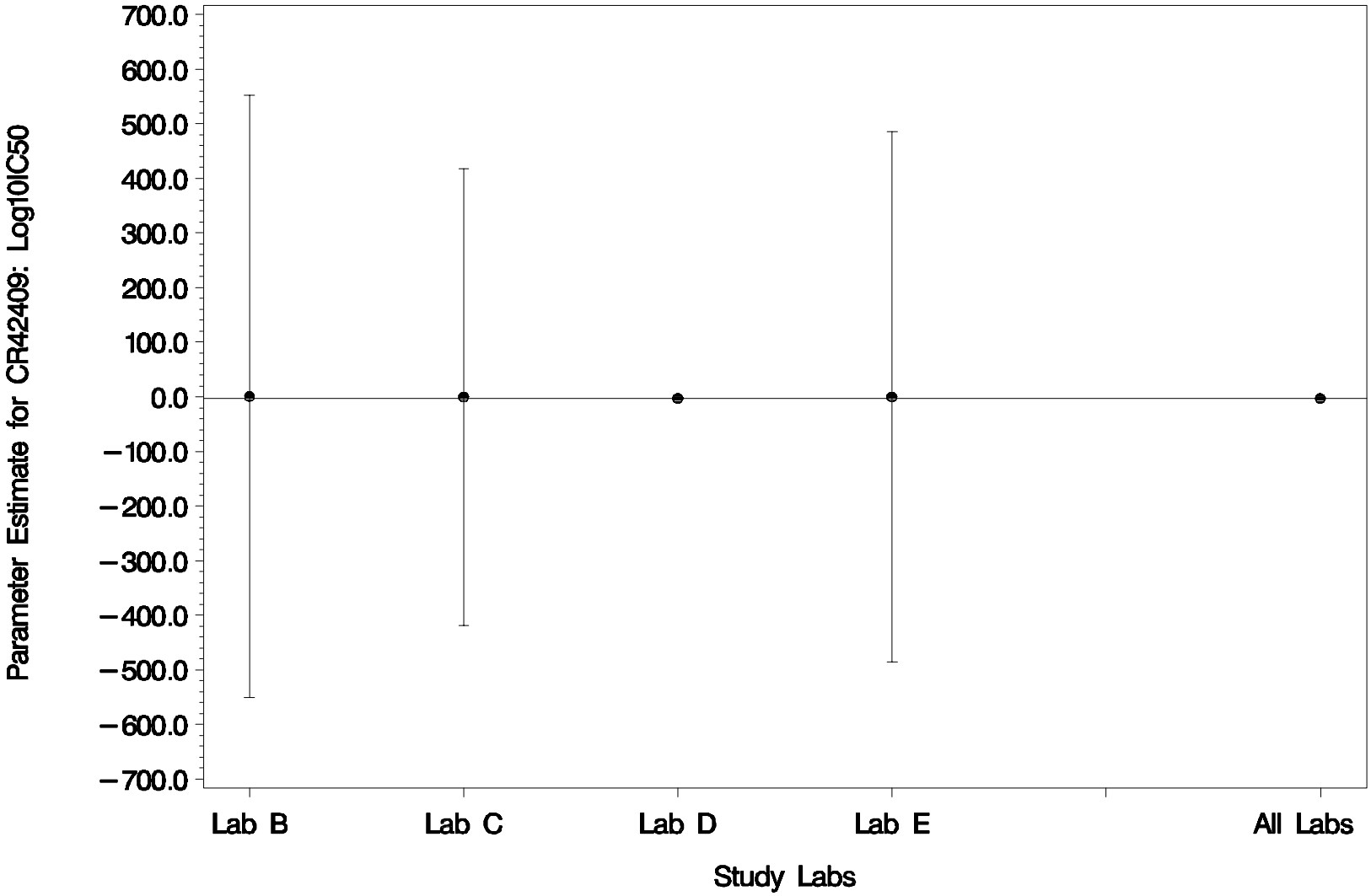

Parameter Estimate for CR42408: Log10IC50
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
−100.0
−200.0
−300.0
−400.0
−500.0
−600.0
−700.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

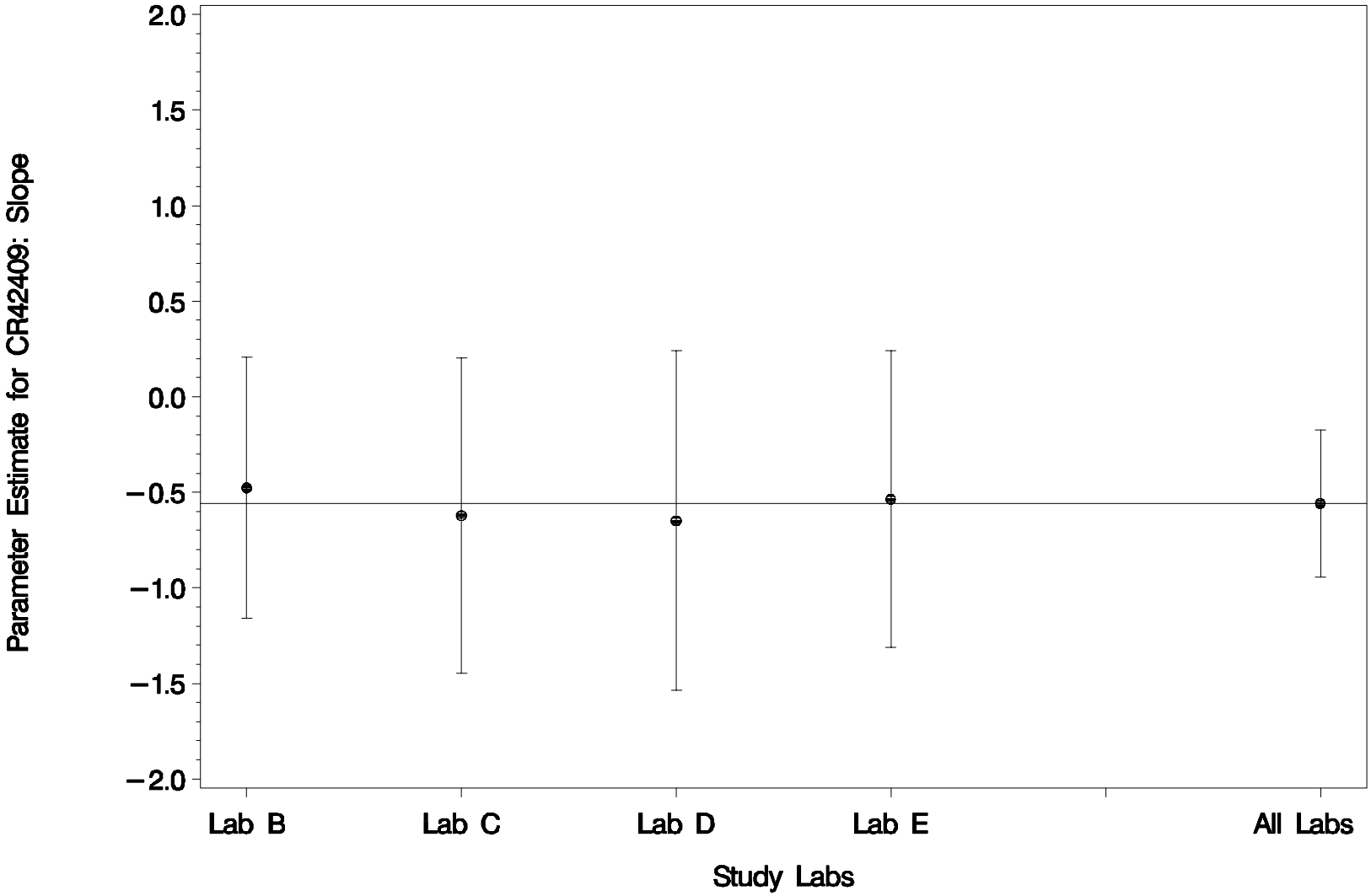

Parameter Estimate for CR42408: Slope
2.0
1.0
0.0
−1.0
−2.0
−3.0
−4.0
−5.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

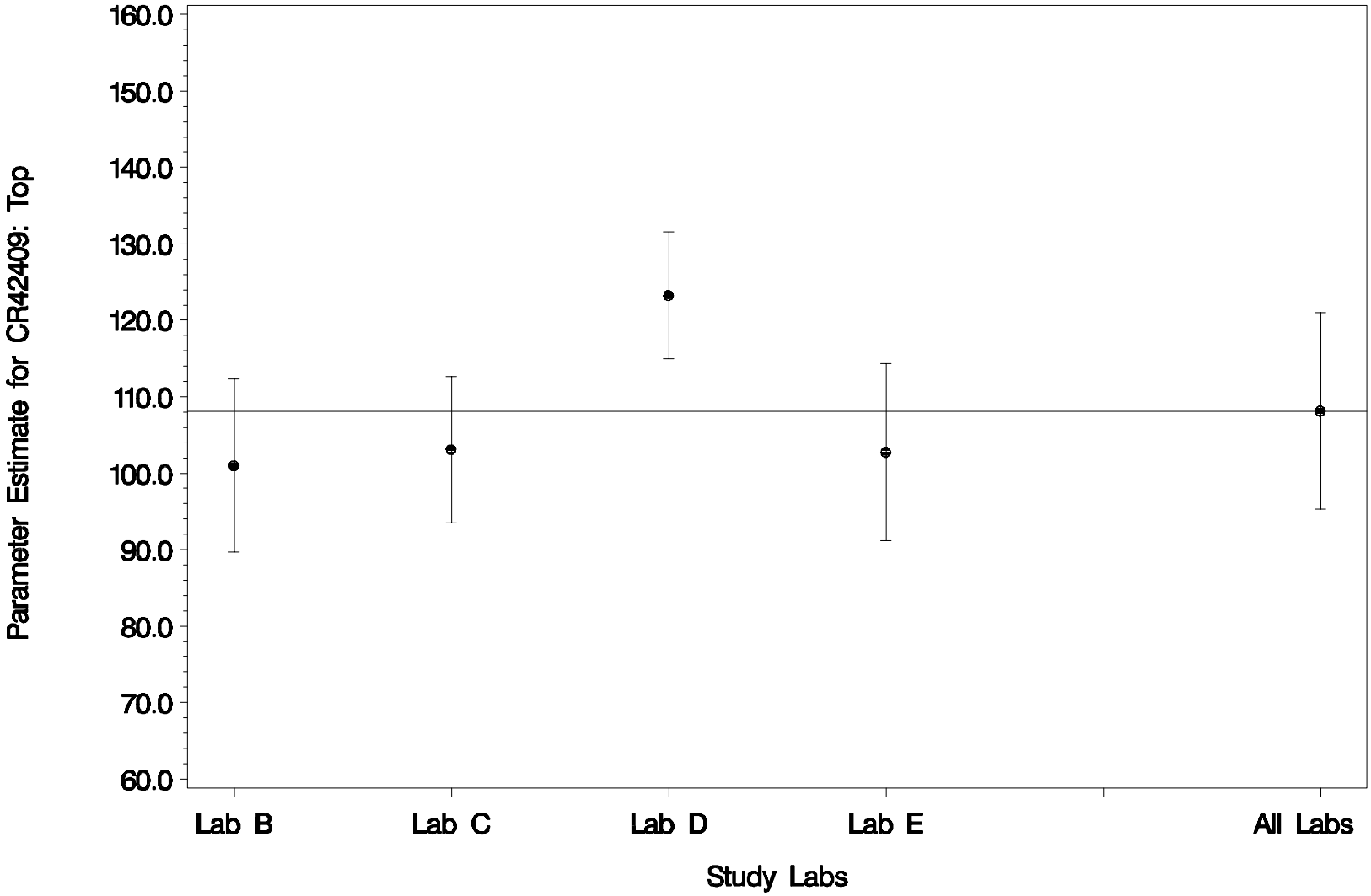

Parameter Estimate for CR42408: Top
160.0
150.0
140.0
130.0
120.0
110.0
100.0
90.0
80.0
70.0
60.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

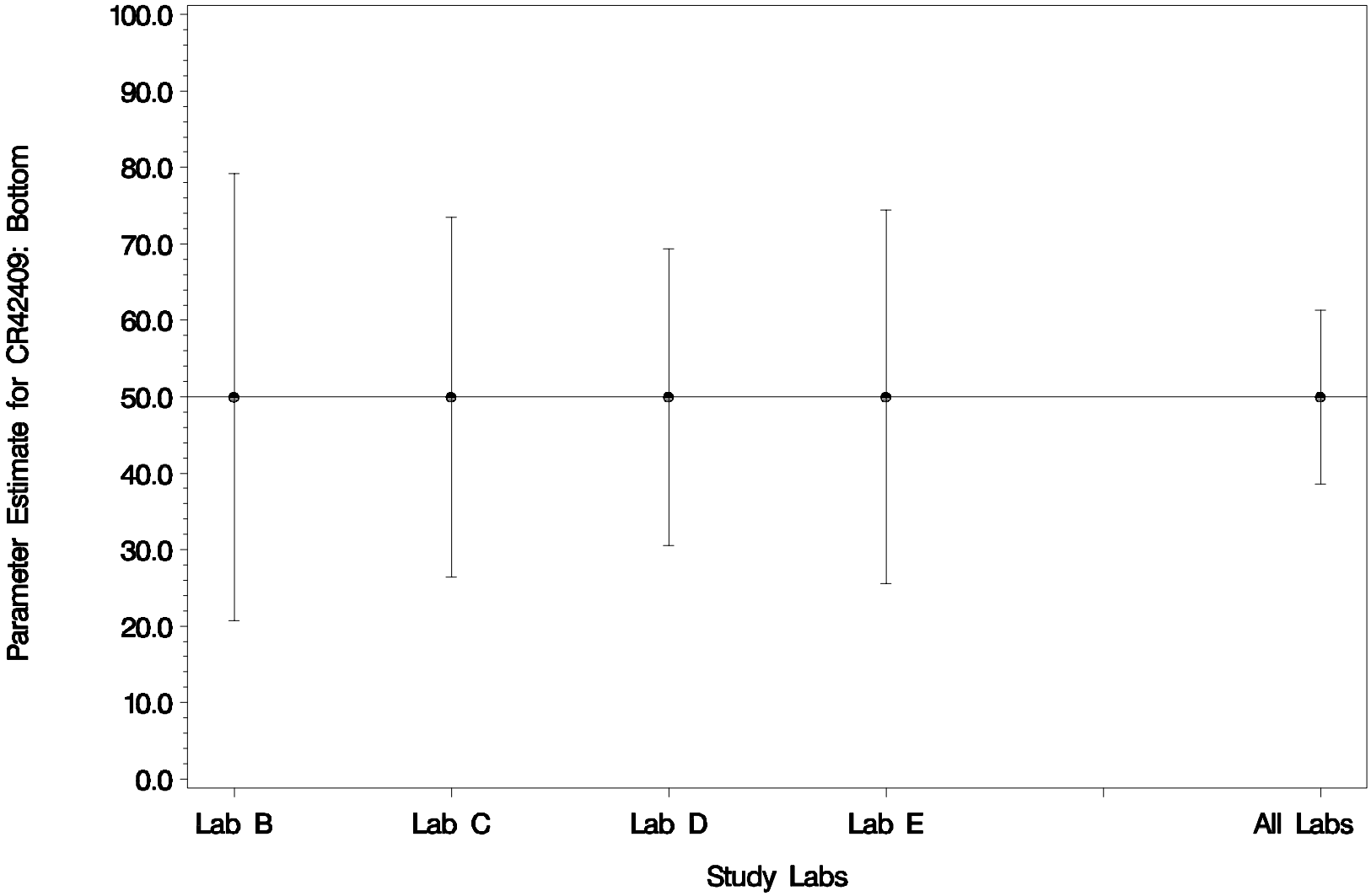

Parameter Estimate for CR42408: Bottom
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs

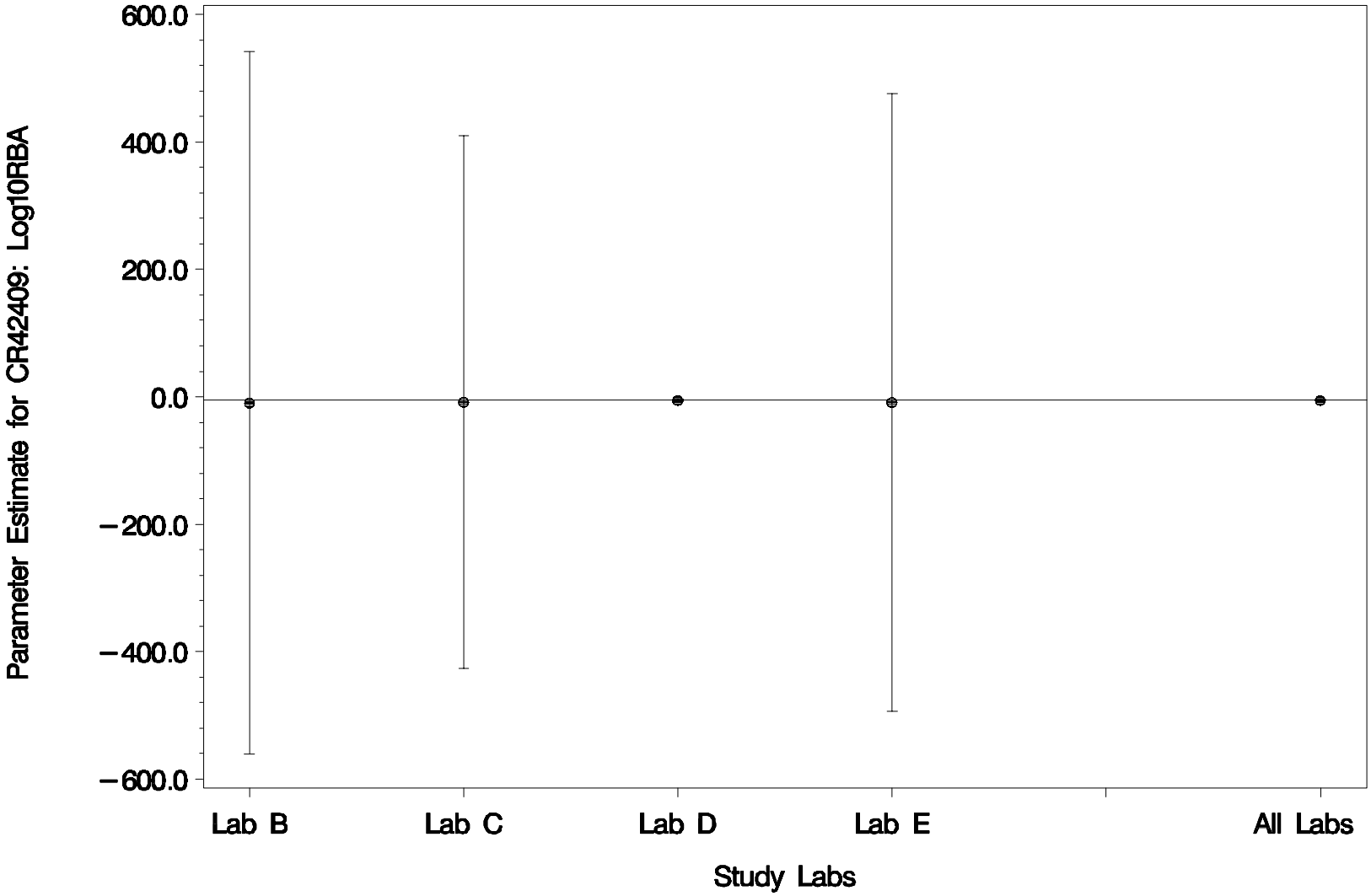

Parameter Estimate for CR42408: Log10RBA
600.0
400.0
200.0
0.0
−200.0
−400.0
−600.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs


Parameter Estimate for CR42409: Log10IC50
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
−100.0
−200.0
−300.0
−400.0
−500.0
−600.0
−700.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs


Parameter Estimate for CR42409: Slope
2.0
1.5
1.0
0.5
0.0
−0.5
−1.0
−1.5
−2.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs


Parameter Estimate for CR42409: Top
160.0
150.0
140.0
130.0
120.0
110.0
100.0
90.0
80.0
70.0
60.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs


Parameter Estimate for CR42409: Bottom
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs


600.0
400.0
200.0
0.0
−200.0
−400.0
−600.0
Parameter Estimate for CR42409: Log10RBA
Lab B
Lab C
Lab D
Lab E
All Labs
Study Labs