

A Practical Approach to Quantitatively Assessing Equilibrium-Constant Accuracy from a Single Binding Isotherm

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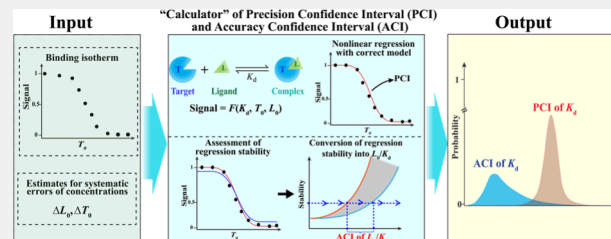
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ABSTRACT: Equilibrium constants are essential for understanding and predicting the behavior of chemical systems across various scientific disciplines. Traditionally, these constants are computed via nonlinear regression of reaction isotherms, which show the dependence of the unreacted fraction of one reactant on the total concentration of another reactant. However, while these equilibrium constants can be precise (with small random errors), they may also be grossly inaccurate (with large systematic errors), leading to potential misinterpretations. Although some statistical methods exist for assessing the accuracy of nonlinear regression, their limited practicality for molecular scientists has resulted in their neglect by this research community. The objective of this work is to develop a practical method for quantitatively assessing the accuracy of equilibrium constants that could be easily understood and immediately adopted by researchers routinely determining these constants. Our approach integrates error-propagation and regression-stability analyses to establish the accuracy confidence interval (ACI)—a range within which the true value of the computed parameter lies with a defined probability. In a proof-of-principle study, we applied this approach to develop a workflow for determining the ACI of the equilibrium dissociation constant (K_d) of affinity complexes from a single binding isotherm. We clearly explained how the input parameters for this workflow can be determined, and finally, we have implemented this workflow in a user-friendly web application (<https://aci.sci.yorku.ca>) to facilitate its immediate adoption by molecular scientists, regardless of their mathematical and computer proficiency. We further conducted three case studies exemplifying the use of the ACI in the context of simultaneous assessment of precision and accuracy of determined K_d values. By understanding the ACI of equilibrium constants and other parameters computed through nonlinear regression, researchers can avoid misconceptions that arise from relying solely on precision.

KEYWORDS: Accuracy of equilibrium constants, Equilibrium dissociation constant of affinity complexes, Accuracy Confidence Interval, Precision Confidence Interval, Regression stability, Error propagation

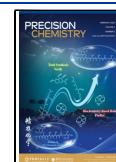


INTRODUCTION

Adequate quantitative description of natural phenomena requires the knowledge of accurate (i.e., free of unknown systematic errors) values of physicochemical parameters. Finding accurate values is only straightforward for parameters that can be directly measured against reference standards of length, mass, time, temperature, electric charge, etc.^{1–4} The majority of physicochemical parameters, however, do not have reference standards and, therefore, must be computed using formulas linking them to sets of dependent and independent variables.^{5–9} Nonlinear regression of experimental data is required for computing some of such parameters, e.g., various equilibrium constants (dissociation constant of biomolecular complexes, stability constant of metal–ligand complexes, Michaelis constant of enzymatic reactions, and Langmuir constant of adsorbent–adsorbate interactions).^{9–14} The regression models used to compute physicochemical parameters are usually correct, i.e., derived from “first principles” rather than “guessed” to approximate empirical data.

Nonlinear regression accounts only for random errors of variables and, accordingly, assesses only the random error of the computed parameter. This random error describes the precision of the parameter determined with varied independent variables.^{15,16} This error defines a range which we call a “precision confidence interval” (PCI) of the sought parameter. It is often presented as a mean value of the parameter plus/minus one or more standard deviations.¹⁷ Being an easily calculated descriptor of precision, PCI does not directly relate to the accurate value. In other words, the probability with which the accurate value lies within PCI is not only unknown

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but also can be very small. Basing conclusions solely on PCI may lead to serious misconceptions.^{18,19}

When finding an unknown parameter with nonlinear regression, we should aim to determine not only its PCI but also a range in which the accurate value of the sought parameter lies with a desired probability; we call this range an “accuracy confidence interval” (ACI). Accuracy and precision are unlinked (orthogonal) descriptors of the exactness (or trueness) of the determined value. Accordingly, ACI and PCI may differ significantly in position, width, and shape of probability distribution as schematically depicted in Figure 1.

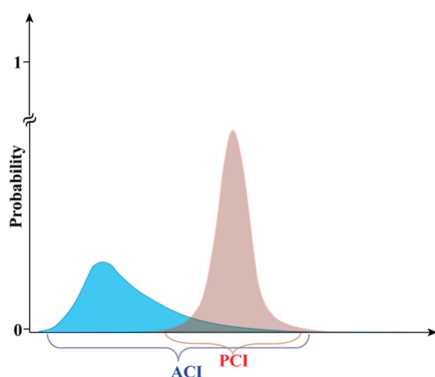


Figure 1. Schematic depiction of potentially large differences between the precision confidence interval (PCI) and accuracy confidence interval (ACI).

This difference makes it necessary to determine not only the PCI but also the ACI, raising a question of how the ACI of a parameter computed with nonlinear regression can be determined.

With the advent of computers, nonlinear regression has become a widely used tool for determining unknown parameters using mathematical models that connect these parameters to known variables via explicit formulas. Statistical methods based on Monte Carlo simulation have been developed to propagate the confidence intervals (uncertainties) of variables to the confidence intervals (uncertainties) of determined parameters.^{20–22} These methods were adopted for the determination of equilibrium constants, and both commercial statistical software and open-access codes are available for their implementation.^{21,23–25}

Despite these advancements, the quantitative assessment of the accuracy of equilibrium constants is grossly neglected by molecular scientists who routinely determine such constants using nonlinear regression. There are several reasons for this alarming oversight, with the major cause arguably being that the existing methods are not practical for researchers in this field. These methods may be difficult to understand for nonmathematicians, and they can require programming skills and/or intensive computational resources for implementation.^{21,23–25} Additionally, there is a significant conceptual barrier: the statistical methods do not clearly explain what should be used as input parameters for the confidence intervals of the variables, which is a very complex issue on its own.^{22,26}

In this study, we aim to address these knowledge gaps by (i) developing a practical approach to finding the ACI using language and concepts comprehensible to molecular scientists, (ii) creating a computationally efficient tool that is immediately accessible to all users regardless of their level of preparation, and (iii) explaining to experimentalists what

confidence intervals of variables (concentrations) should be used for the determination of ACI of the determined parameter (equilibrium constant).

Here, we introduce “ACI-Concept”, a methodology for finding ACI of parameters computed with correct nonlinear-regression models. The need for a correct model is essential for our approach which combines the analysis of regression sensitivity to errors of independent variables that are fixed in regression (e.g., concentration of a limiting component in the determination of equilibrium constants) with the standard propagation of errors of all (independent and dependent) variables or only independent variables (e.g., concentrations in the determination of equilibrium constants). We also propose a strategy to translate the theory underlying ACI-Concept into practical workflows for the determination of ACI.

Our ACI-determination strategy is designed to directly identify the boundaries of the ACI at a specified confidence level, focusing on determining the width of the ACI, which is arguably the most critical factor in assessing physicochemical parameters. This approach offers significantly greater computational efficiency compared to traditional Monte Carlo simulation-based methods, which require extensive resampling.^{20–22}

We chose the equilibrium dissociation constant (K_d) of affinity complexes as the first parameter for which a practical ACI-determination workflow was to be developed. K_d is determined through nonlinear regression of binding isotherms and is arguably the most frequently computed physicochemical parameter, reported in over 1,000 peer-reviewed papers per month. Many more K_d values are likely produced in high-throughput screening by drug-discovery companies for internal use. In the absence of a practical procedure for molecular scientists to find ACI of K_d , conclusions from binding experiments are based solely on PCI which may drastically differ from ACI. The failure to find ACI of equilibrium constants can lead to serious misinterpretations and misconceptions.^{8,18,19,27}

We used ACI-Concept to develop a practical workflow for the determination of ACI of K_d ; we term this workflow “ACI-Kd”. ACI-Kd was verified with computer-simulated binding isotherms resulting in a wide range of accuracy of determined values of K_d . The ACI-Kd workflow was initially exemplified by determining the ACI of K_d values in virtual binding experiments. It was then applied to real experiments involving two biomolecular complexes, where binding isotherms were obtained using methods based on different physical principles. To assist the scientific community with adopting ACI-Kd, we implement it as a user-friendly web app (<https://aci.sci.yorku.ca>) which computes both ACI and PCI and, thus, facilitates the assessment of both accuracy and precision of the determined K_d values. No math or programming skills are required from the users and the results, including graphs suitable for publication, are produced almost instantly. The consideration of both ACI and PCI while interpreting binding experiments will greatly improve the reliability of drawn conclusions.

It is important to emphasize that ACI-Kd is not a “panacea for all ills” as it considers only one potential source of inaccuracy: the transformation of small systematic errors of concentrations into large systematic errors of K_d . This source is arguably the most important as it is fundamental, but there are unique sources of inaccuracy for each experimental method and other imperfections that can bias ACI of K_d making it less

reliable.^{28–30} We emphasize the need for thoughtful experimentation, which should include (i) understanding and minimizing all method-dependent biases, (ii) excluding all known systematic errors, (iii) optimizing experimental conditions, (iv) ensuring equilibrium and saturation in binding reactions, etc.²⁶ At the end of the day, ACI of K_d is only as good as the data used to compute it.

We anticipate that ACI-Concept will be used to develop workflows for finding ACI of other physicochemical parameters computed through nonlinear regression. A key condition for the applicability of ACI-Concept is that the regression model is correct, which is typically not the case in areas other than natural sciences, and that the error is analytically propagated, which is always possible to do.^{31–33}

RESULTS AND DISCUSSION

Accuracy and ACI

We start with a theoretical consideration of the accuracy of parameters computed with nonlinear regression with a correct model. Accuracy indicates the closeness of the determined value to the true value of the parameter. A true value is never known but can be used here as a theoretical abstraction. The input values used in numerical simulations can be considered as true. When discussing accuracy in the experimental context, we will use “accurate” instead of “true” as a descriptor of value exactness. The accuracy can be expressed quantitatively in different ways, but its measures are always defined via the determined and true values. For example, a relative systematic error is a difference between the determined and true values divided by one of them, whereas an accuracy ratio is a ratio between them.^{34,35} Because a true value is fundamentally unknown, neither measure of accuracy can be calculated using its definition. Moreover, since a determined value is always uncertain due to experimental systematic errors, accuracy can only be assessed with the corresponding uncertainty. This uncertainty defines ACI of the determined value.

ACI-Concept: Combining Regression-Stability and Error-Propagation Analyses

In the determination of equilibrium constants with nonlinear regression, researchers usually assume that parameter p is a known function of a measurable independent variable x_c that is kept as a constant in a single nonlinear regression, a measurable independent variable x_v that is varied in a large range, and a measurable dependent variable, signal S :^{9–14}

$$p = F(x_c, x_v, S) \quad (1)$$

The case can be generalized, for example, to multiple independent variables that are kept constant (Note S1). The requirement that this function is known is equivalent to the requirement that the regression model is correct. In turn, the correct regression model is needed as it is a key reference in accuracy assessment with the accurate value being unknown. Assessing the accuracy of a determined value of p (p_{det}) requires the knowledge of systematic errors of all the variables, which are Δx_c , Δx_v , and ΔS , and we assume that they are also measurable (known). Here, we consider only the determination of p via a commonly used regression approach which assumes Δx_c , Δx_v , and ΔS are all null and allow only random errors in S . Our goal is to integrate the nonzero systematic errors of variables in this approach. In the simplest implementation of this approach, a theoretical dependence of

S on a single variable x_v is fitted into a corresponding experimental dependence with x_c kept constant:

$$S = f(x_c, x_v, p) \quad (2)$$

using p as a fitting parameter. A regression procedure can be done with various computer algorithms, and it typically reports p_{det} along with its standard deviation (i.e., random error), an indicator of the precision of p_{det} determined with varied x_v .¹⁶ The random error does not show how close p_{det} is to the true value of p (note, we use p without a subscript for both naming parameter p and indicating the true value of p). Here, we are developing an approach to assessing the accuracy of p_{det} assuming that the systematic errors of the variables are analytically propagated into the systematic error of the sought parameter.

The initiating point in this development is our insight that this approach can be built upon a link between the accuracy of p_{det} and the sensitivity of p_{det} to a change in x_c across different nonlinear-regression processes. As p is a constant, we can discuss instead a link between the accuracy of p_{det} and the sensitivity of p_{det} to a change in the x_c/p ratio. This link can be proven mathematically, and it implies that the accuracy of p_{det} can be improved by choosing x_c at which p_{det} is less sensitive to the change of x_c/p across different regressions.^{36,37} The underlying reason for this relationship between p_{det} accuracy and its sensitivity to changes in x_c/p is that when p_{det} is less sensitive to variations in x_c/p , the error in p_{det} —propagated from the systematic errors in x_c , x_v , and S (represented by the vector $\Delta = \{\Delta x_c, \Delta x_v, \Delta S\}$)—is minimized.³⁶

Experimentalists are aware of this relation between the accuracy and sensitivity and sometimes vary x_c across different p -determination experiments to identify the x_c (with an unknown value of x_c/p) where p_{det} is not sensitive to a change in x_c .^{38–40} However, experimentally varying x_c greatly increases the amount of required experimentation, which restricts the use of this approach. More importantly, this approach can only suggest if the error of p_{det} has been minimized for a fixed Δ but cannot produce a quantitative assessment of the accuracy of p_{det} . These two limitations led us to consider the known qualitative sensitivity of p_{det} to x_c (or x_c/p) for a fixed Δ from a different angle: the x_c/p ratio can be assessed by measuring the sensitivity of p_{det} to the changes in the vector of systematic errors $\Delta = \{\Delta x_c, \Delta x_v, \Delta S\}$. To simplify the analysis, we reduce the change of the vector $\Delta = \{\Delta x_c, \Delta x_v, \Delta S\}$ to a change in a single element, Δx_c .

The sensitivity of the determined value p_{det} to changes in Δx_c can be quantified by the ratio $p'_{\text{det}}/p_{\text{det}}$ where p_{det} and p'_{det} represent the values of parameter p determined at a specific value of x_c/p for two different vectors of systematic errors in variables, such as $\Delta = \{\Delta x_c, \Delta x_v, \Delta S\}$ and $\Delta' = \{\Delta x'_c, \Delta x_v, \Delta S\}$, respectively. We refer to this sensitivity of the determined parameter to systematic errors of variables in nonlinear regression as “regression stability”. We postulate that if the theoretical relation between $p'_{\text{det}}/p_{\text{det}}$ and x_c/p can be solved analytically or numerically when expressed explicitly, the accurate value of the studied parameter can be computed by evaluating the regression stability and assessing the value of x_c . The following is the logic developed upon this postulate.

Theoretically, when the range of x_v (and corresponding dependent variable S) adequately covers the critical region for determining p , and data is densely sampled, we can assume the accuracy of p is weakly dependent on the specific choices of x_v .⁴¹ Under this readily achievable condition, the systematic

error of p_{det} (Δp) will primarily depend on the chosen value of the variable kept constant (x_c) and the errors of all variables (eq 3):

$$\Delta p = G(x_c, \Delta) \quad (3)$$

where $\Delta = \{\Delta x_c, \Delta x_v, \Delta S\}$. Also, since a systematic error is *per se* the deviation of the determined value from the accurate value, eq 3 can be rewritten as

$$\begin{aligned} \Delta p &= p_{\text{det}} - p = G(x_c, \Delta) \\ \Rightarrow p_{\text{det}} &= G(x_c, \Delta) + p \\ \Rightarrow p_{\text{det}} &= g(x_c, \Delta) \end{aligned} \quad (4)$$

A transformation of eq 4 into an expression linking a unitless accuracy ratio p_{det}/p to unitless ratio x_c/p and unitless relative errors of variables has been previously demonstrated for a specific type of eq 1.³⁶ A similar transformation strategy can be applied to other types of eq 1. The transformed dimensionless expression can be presented as

$$\frac{p_{\text{det}}}{p} = H\left(\frac{x_c}{p}, \Delta^*\right), \quad \text{where } \Delta^* = \left\{\frac{\Delta x_c}{x_c}, \frac{\Delta x_v}{x_v}, \frac{\Delta S}{S}\right\} \quad (5)$$

To study the sensitivity of p_{det}/p to the unitless relative error of the variable kept constant ($\Delta x_c/x_c$), we need to have at least two points, corresponding to errors $\Delta^* = \{\Delta x_c/x_c, \Delta x_v/x_v, \Delta S/S\}$ and $\Delta^{*'} = \{\Delta x_c/x_c + \delta, \Delta x_v/x_v, \Delta S/S\}$, where δ should be large to ensure that the values of p_{det} obtained with these two errors are distinguishable. eq 5 can be written for $\Delta^{*'}$ as

$$\frac{p'_{\text{det}}}{p} = H\left(\frac{x_c}{p}, \Delta^{*'}\right) \quad (6)$$

To investigate the sensitivity of the determined parameter to the systematic errors of variables at different x_c/p , we divide eq 6 over eq 5 to obtain

$$\frac{p'_{\text{det}}}{p_{\text{det}}} = \frac{H\left(\frac{x_c}{p}, \Delta^{*'}\right)}{H\left(\frac{x_c}{p}, \Delta^*\right)} = h\left(\frac{x_c}{p}, \Delta^*, \Delta^{*'}\right) \quad (7)$$

The theoretical relation shown in eq 7 can be numerically tabulated by fitting the simulated data sets (involving relative systematic errors of $\Delta x_v/x_v$ and $\Delta S/S$) with eq 2, using $(1 + \Delta x_c/x_c)x_c/p$ and $(1 + \Delta x_c/x_c + \delta)x_c/p$ as the “nominal” values of the variable kept constant. The relationship between these two nominal values is given by $(1 + \Delta x_c/x_c + \delta)x_c/p = \alpha[(1 + \Delta x_c/x_c)x_c/p]$, where $\alpha = 1 + \delta/(1 + \Delta x_c/x_c)$. To determine the x_c/p ratio for fixed Δ^* and $\Delta^{*'}$, one can consider the inverse relationship between $p'_{\text{det}}/p_{\text{det}}$ and x_c/p , as follows:

$$\frac{x_c}{p} = h^{-1}\left(\frac{p'_{\text{det}}}{p_{\text{det}}}\right) \quad \text{at } \Delta^* \text{ and } \Delta^{*'} \quad (8)$$

In practice, p_{det} and p'_{det} can be determined by using the nominal value of x_c (involving relative systematic error of $\Delta x_c/x_c$) and αx_c , respectively, in a regression procedure with a correct regression model (fitting eq 2 into experimental data that includes experimental $\Delta x_v/x_v$ and $\Delta S/S$). Note that, the value of α is controllable in the data fitting process, and the confidence interval of Δ^* , i.e., the combination of $\Delta x_c/x_c$,

$\Delta x_v/x_v$, and $\Delta S/S$, with a desired confidence level, can be potentially determined.

We presume that the ratio $p'_{\text{det}}/p_{\text{det}}$ for a given value of α can be experimentally determined. By substituting this ratio into the numerically established eq 8, which involves the confidence interval of Δ^* and utilizes the same value of α , the confidence interval of x_c/p can be determined. Finally, since the nominal x_c is known, we can, in principle, determine the confidence interval of p , i.e., ACI of p . To determine ACI, it is essential to first establish the confidence interval of relative systematic errors of the variables (i.e., the components of vector Δ^*). This aspect is studied in the next section.

Systematic Errors of Variables Arising from Random Errors

The above theoretical consideration assumes that the confidence interval of systematic errors of variable Δ^* is known. Let us define what kind of systematic errors ACI-Concept is applicable to. As we explained in our previous work,²⁶ only if the systematic errors arise from normally distributed random errors of variables, the confidence intervals of systematic errors can be assessed.²⁶

Although normally distributed systematic errors are not commonly discussed in statistics, they likely play an important role in the determination of physicochemical parameters with nonlinear regression.²⁶ We would like to use an example of concentrations as variables. If we assume that the concentration is determined without a true systematic error, it always has a normally distributed random error. Such an error is necessarily present when preparing a stock solution and if the same stock solution is used to prepare all other solutions in the experiment, then the random error translates into a normally distributed systematic error.²⁶ Such normally distributed systematic errors establish the confidence interval for the vector of systematic errors in variables, Δ^* , which, in turn, defines the ACI within the ACI-Concept framework through error propagation (Figure 2). Thus, ACI-Concept is meant to be used for normally distributed systematic errors of variables. It is important to emphasize that normally distributed systematic errors represent a lower bound of systematic errors

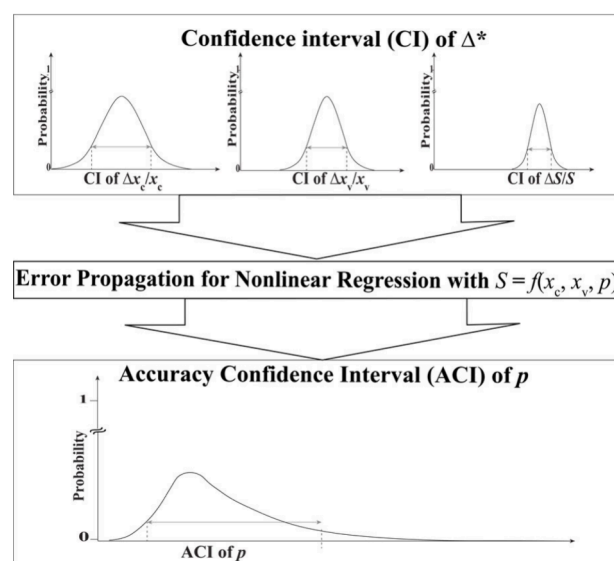


Figure 2. Schematic depiction of how the ACI of parameter p is defined by the confidence interval (CI) of Δ^* , which is the combination of CIs for the relative systematic errors of the variables.

as we assume that all identifiable and correctable sources of systematic errors have already been addressed and eliminated.²⁶ Therefore, ACI is a narrower limit of the range in which the accurate value of the sought parameter lies.

Graphical Strategy for Development of ACI-Determination Workflows

To be instructive, the above general theory needs to be converted into a specific strategy. There may be more than one strategy for assessing the accuracy of computed values based on the described combination of regression-stability analysis and error-propagation analysis. Here we propose a strategy that we nicknamed “graphical”. It aims at finding ACI, i.e., a range in which a true value of p lies with a desired probability.

The essence of the graphical strategy is the following. The required experimental data include (i) the dependence of S on x_v for a given x_c and (ii) the confidence intervals of (experimentally determinable) relative systematic errors of all variables, which are used to calculate the confidence interval of Δ^* (i.e., combinations of $\Delta x_c/x_c$, $\Delta x_v/x_v$, and $\Delta S/S$). To find the confidence interval of Δ^* , we can first analytically solve eq 1 for a specific case using the rules of error propagation to obtain a specific form of eq 5. Then, the experimentally determined confidence intervals of relative systematic errors of variables are plugged into eq 5 to find the confidence interval of Δ^* that is bordered with two boundary conditions: $\Delta^*_{\min}$ and $\Delta^*_{\max}$, which cause the smallest and largest accuracy ratio p_{det}/p , respectively. Equation 7 is used to compute $p'_{\text{det}}/p_{\text{det}}$ as functions of x_c/p for these two values of Δ^* (i.e., $\Delta^*_{\min}$ and $\Delta^*_{\max}$). The two lines along with the gray shadow between them in a graph schematically depicted in Figure 3, which we

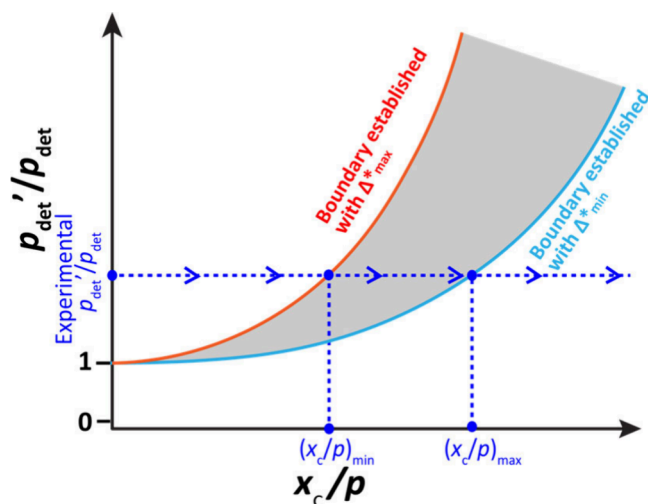


Figure 3. Schematic of an ACI graph.

call an ACI graph, border the ranges within which x_c/p is positioned with a probability defined by those at which Δ^* was obtained. The numerically derived ACI graph (Figure 3) is equivalent to the explicit expression presented in eq 7. A known value of $p'_{\text{det}}/p_{\text{det}}$ is placed on the graph as a horizontal line, and the intersections of this line/surface with the two border lines define the boundary values of $(x_c/p)_{\min}$ and $(x_c/p)_{\max}$. This step corresponds to solving eq 8 for x_c/p . Finally, the nominal value of x_c is used to calculate the confidence interval in which p lies: between $p_{\min} = x_c/(x_c/p)_{\max}$ and $p_{\max} = x_c/(x_c/p)_{\min}$. The presented graphical strategy can be presumably applied to any parameter computed with the

regression approach, but proving this is not a subject of our current work. Note that our graphical strategy is designed to directly identify the boundaries of the ACI at a specified confidence level without relying on random resampling, a core element of traditional Monte Carlo simulation-based methods.^{20–22} Below, we use the graphical strategy to develop a workflow for finding ACI of K_d .

Focus on K_d

Drugs and diagnostic probes (collectively called ligands in this work) are developed to form stable affinity complexes with their molecular targets (usually proteins).^{42–45} The stability of a target–ligand complex (C) is characterized by the equilibrium dissociation constant (K_d) of the binding reaction between a target (T) and a ligand (L):



K_d is defined as

$$K_d = \frac{TL}{C} \quad (10)$$

where T , L , and C are equilibrium concentrations of the target, ligand, and complex, respectively, in the reversible one-to-one binding process described by eq 9. Lower K_d values correspond to the higher thermodynamic stability of the complex and are usually desired in the development of drugs and diagnostic probes.

The values of K_d define therapeutic concentrations of drugs and analytical parameters of diagnostic methods; they must be known accurately for important therapeutic and diagnostic applications of ligands.^{46–48} K_d values are a key decision-influencing parameter in early stage drug discovery where the most potent candidates are chosen for further development. However, the discrepancies between K_d values obtained even within the same laboratory for the same complex and with the same method often reach many folds or even orders of magnitude.^{19,40,49} Such discrepancies may lead to misconceptions and wrong conclusions about ligand potency with far-going negative consequences.^{18,19,49}

There are no reference standards for K_d values; they are classically computed using the following procedure based on nonlinear regression (or a variation of this procedure). A series of equilibrium mixtures of the target and ligand are prepared in which the total concentration of ligand $L_0 = L + C$ is kept constant (limiting component) while the total concentration of target $T_0 = T + C$ is varied in a wide range (2 or 3 orders of magnitude). A fraction of unbound ligand R ($R = L/L_0$) is measured for each equilibrium mixture and plotted as a function of T_0 ; the dependence of R on T_0 is called a binding isotherm (Figure 4a).^{50–53} Finally, K_d is determined by nonlinear regression of the binding isotherm with the following theoretical dependence of R on T_0 (red line in Figure 4a) using K_d as the only unknown parameter:^{36,37,50,54}

$$R = -\frac{K_d + T_0 - L_0}{2L_0} + \sqrt{\left(\frac{K_d + T_0 - L_0}{2L_0}\right)^2 + \frac{K_d}{L_0}} \quad (11)$$

It is important to note that eq 11 is obtained without simplifying assumptions from the definition of K_d (eq 10), the definition of R ($R = L/L_0$), and the mass balance for ligand and target ($L_0 = L + C$ and $T_0 = T + C$).

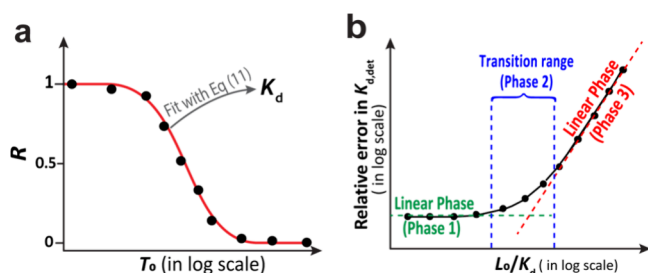


Figure 4. Schematic illustration of K_d determination. (a) A binding isotherm (dots) (i.e., the experimental dependence of the fraction of unbound ligand (R) on the total target concentration (T_0)) and the result of fitting the isotherm with eq 11 (red line), which is the theoretical dependence of R on T_0 . (b) Triphasic dependence of relative error in the determined value of $K_{d,det}$ on L_0/K_d with constant errors in T_0 , L_0 , and R , where L_0 and K_d represent true values of the total ligand concentration and the equilibrium constant, respectively. In Phase 1, the relative error of $K_{d,det}$ is virtually insensitive to change in L_0/K_d , while in Phase 3 it is sensitive. Adapted from ref 36. Copyright 2023 American Chemical Society.

The computation of K_d would be straightforward and error-free if R , L_0 , and T_0 were known precisely and accurately. However, the values of R , L_0 , and T_0 always have errors making experimental determination of K_d error-prone, especially for highly stable complexes that need to be studied at low concentrations of both ligand and target.^{36,37,40,55,56} It is important to stress that nonlinear regression of a binding isotherm produces only PCI.

It has been demonstrated numerically and proven analytically that the translation of the systematic errors of R , L_0 , and T_0 into the systematic error of the determined value of K_d ($K_{d,det}$) is triphasic when presented as a function of the ratio between true values of L_0 and K_d : L_0/K_d (Figure 4b).^{36,37} Note, we use “ K_d ” to name accurate K_d values which correspond to an ideal case of no errors in L_0 and T_0 . (Accurate K_d is fundamentally unknown; we can only aim to find a confidence interval in which the accurate value of K_d lies with a known probability). “ $K_{d,det}$ ” is used to name determined K_d values which are subject to systematic errors. These errors are due to nonlinear regression of binding isotherms under an assumption of no systematic errors in L_0 and T_0 while such errors are always present. This triphasic dependence suggests that with constant systematic errors of R , L_0 , and T_0 , the sensitivity of $K_{d,det}$ to the change in L_0 increases with increasing L_0/K_d (Figure 4b). We can also interpret Figure 4b in a different way: the sensitivity of $K_{d,det}$ to the systematic errors of R , L_0 , and T_0 increases with the increase of L_0/K_d . This relationship can serve as a foundation for determining L_0/K_d , which subsequently aids in evaluating the accuracy of the determined K_d .

The triphasic dependence in Figure 4b suggests the use of the lowest possible L_0 for decreasing errors of $K_{d,det}$. However, L_0 cannot be lower than the limit of quantitation (LOQ) of an instrument utilized for measuring the values of R .^{36,57,58} Thus, for very stable complexes (small K_d values), it is likely that the lowest L_0 that can be used for a given LOQ is much greater than K_d . In such cases, L_0/K_d is large (Phase 3 in Figure 4b) and unknown. Accordingly, small errors of R , L_0 , and T_0 are greatly magnified when propagated into the error of $K_{d,det}$. This type of error in K_d is systematic and fundamental, i.e., it cannot be reduced by repeating experiments with the same solutions (or even with different solutions but the same mass-measuring

and volumetric equipment) and does not depend on an instrumental platform used to measure R .

We are aware of only one way to confirm that the error of $K_{d,det}$ is not greatly magnified by using too high L_0 . It requires experimentally obtaining multiple binding isotherms for different values of L_0 and, essentially, plotting $K_{d,det}$ as a function of L_0 .^{38–40} If $K_{d,det}$ is found to not depend significantly on L_0 , i.e., $K_{d,det}$ is insensitive to changing L_0 , then it is assumed that the value of $K_{d,det}$ is accurate, i.e., $K_{d,det} \approx K_d$. This approach has two major limitations. First, building multiple binding isotherms is much more resource-intensive than building one. This limitation is likely the main reason for this approach not being used in the vast majority of peer-reviewed works reporting K_d determination. The second limitation is that this approach cannot provide any quantitative information on the accuracy of $K_{d,det}$, i.e., on ACI. These limitations motivated us to apply ACI-Concept to the development of ACI-Kd, a practical workflow for finding ACI of K_d .

ACI-Kd: A Workflow for Finding ACI of K_d

The relation between the terms used in the theory section and the specific case of K_d determination is explicit: p corresponds to K_d , S corresponds to R , x_c corresponds to L_0 , and x_v corresponds to T_0 . Equation 1 corresponds to⁵⁹

$$K_d = \frac{T_0 - L_0(1 - R)}{(1/R - 1)} \quad (12)$$

To determine ACI of K_d , we should apply error propagation rules to eq 12 and thus obtain an equation similar to eq 5 that suggests how the confidence interval of Δ^* can be defined based on the confidence intervals of variables that have normally distributed systematic errors.

To obtain an analytical solution of error propagation for eq 12, it is necessary to focus on a single data point by fixing R . In a representative case of $R = 0.5$, which corresponds to the least error in K_d determination,³⁷ the general eq 5 can be specified as follows (Note S2):

$$\frac{K_{d,det}}{K_d} = \left[\frac{0.5(\Delta T_0/T_0 - \Delta L_0/L_0 - \Delta R/R)}{1 + \Delta R/R} + \frac{0.5\Delta R/R}{0.5 + \Delta R/R} \right] \frac{L_0}{K_d} + \left[\Delta T_0/T_0 + \frac{\Delta R/R(1 + \Delta R/R)}{0.5 + \Delta R/R} + 1 \right] \quad (13)$$

In the experimental determination of K_d with nonlinear regression, careful experimental design and method optimization typically result in normally distributed systematic errors in the concentrations of stock solutions. These solutions are used to prepare other solutions, thus, making T_0 and L_0 the independent variables with normally distributed systematic errors.²⁶ In contrast, at this stage, we have not identified a quantitative approach for assessing the confidence interval of ΔR .²⁶ Therefore, due to the lack of inputting information for ΔR , this study will only consider the systematic errors of T_0 and L_0 . Consequently, the determined ACI of K_d will be the narrower limit of the confidence interval for K_d with the specified confidence levels (probabilities).

Without considering ΔR , eq 13 can be simplified to eq 14 (Note S2):

$$\frac{K_{d,det}}{K_d} = 0.5 \left(\frac{\Delta T_0}{T_0} - \frac{\Delta L_0}{L_0} \right) \frac{L_0}{K_d} + \frac{\Delta T_0}{T_0} + 1 \quad (14)$$

With this simplification, the vector of relative systematic errors Δ^* corresponds to $\{\Delta L_0/L_0, \Delta T_0/T_0\}$, i.e., the combination of relative systematic errors of L_0 and T_0 . In the derivations of eqs 13 and 14, we assumed the error sources of the concentrations are strongly correlated, which is a valid assumption for the vast majority of experimental cases.^{36,60} The case for weakly correlated errors is conceptually and quantitatively similar but not considered here.³⁶

Based on the above specifications, the dependence of $p'_{\text{det}}/p_{\text{det}}$ on x_c/p shown in eq 7 becomes the dependence of $K'_{\text{d,det}}/K_{\text{d,det}}$ on L_0/K_d . Here, $K_{\text{d,det}}$ and $K'_{\text{d,det}}$ are the K_d values determined by fitting eq 11 into a single experimental binding isotherm twice, with two different ligand concentrations (used in fitting): $L_{0,\text{fit}}$ and $L'_{0,\text{fit}}$. $K_{\text{d,det}}$ is found with $L_{0,\text{fit}} = L_0$ (nominal value), and $K'_{\text{d,det}}$ is found with $L'_{0,\text{fit}} = \alpha L_0$. As discussed in the theory section, multiplying the nominal L_0 by a factor α introduces a large “artificial” systematic error to L_0 in order to test the sensitivity of $K_{\text{d,det}}$ to changes in $\Delta L_0/L_0$. To ensure that $K'_{\text{d,det}}$ is distinguishable from $K_{\text{d,det}}$ —especially in the range of low L_0/K_d values— α must be significantly greater or smaller than unity. We choose α to be much less than 1 (e.g., $\alpha = 0.005$) to create a substantial difference between $L_{0,\text{fit}}$ and $L'_{0,\text{fit}}$ while also ensuring that $K'_{\text{d,det}}$ remains positive (Note S3 and Table S1).

According to the general theory and graphical strategy schematized in Figure 3, if the confidence intervals of $\Delta L_0/L_0$ and $\Delta T_0/T_0$ can be experimentally estimated and the boundary conditions of combinations of $\Delta L_0/L_0$ and $\Delta T_0/T_0$ (i.e., Δ^*_{min} and Δ^*_{max}) can be determined, then we can numerically establish the ACI graph of “ $K'_{\text{d,det}}/K_{\text{d,det}}$ vs L_0/K_d ” and use it to find ACI of K_d . Based on these considerations, we propose a step-by-step ACI-Kd workflow for finding ACI of K_d value (Figure 5). ACI-Kd requires two procedures to be specified/developed: (i) a method for estimating the confidence intervals of the relative systematic errors of L_0 and T_0 and (ii) a method of establishing the ACI graph of “ $K'_{\text{d,det}}/K_{\text{d,det}}$ vs L_0/K_d ”.

In K_d -determination experiments, all predictable systematic errors of variables must be eliminated with method optimizations including calibration of instruments, elimination

of material adsorption to surface, the necessary mathematical corrections based on the purities of reagents, etc.^{26,61–63} When the systematic errors of concentrations are minimized, the unpredictable systematic errors of L_0 and T_0 , which inevitably exist in each experiment, are from the normally distributed systematic errors in the concentrations of their stock solutions.²⁶ Specifically, for each single preparation of a stock solution in the ACI-Kd workflow, the normal distribution of concentrations of stock solutions collapses to one single concentration, i.e., the random error converts into the systematic error. With these considerations, the confidence intervals of $\Delta L_0/L_0$ and $\Delta T_0/T_0$ can be estimated from the relative random errors of stock L_0 and T_0 (i.e., $\delta L_0/L_0$ and $\delta T_0/T_0$ for stock solutions). The values of $\delta L_0/L_0$ and $\delta T_0/T_0$ can be experimentally determined based on the spectroscopic signals (e.g., light absorption or fluorescence intensity) from ligand and target in multiple preparations of stock solutions (examples are shown in the section describing applications of ACI-Kd to real binding experiments).

Based on the confidence intervals of $\Delta L_0/L_0$ and $\Delta T_0/T_0$, we can calculate the confidence level of ACI (Figure 5). In this work, the confidence levels of ACI and PCI will be harmonized, i.e., presented with the same value. The confidence level of PCI can be varied by choosing different numbers of standard deviation (SD) as the uncertainty of determined value $K_{\text{d,det}}$. Conventionally, PCI is presented as “mean value $\pm$ SD” with a confidence of 68.3%. To harmonize the confidence levels of PCI and ACI, our goal in this work was to use ACI-Kd to find ACI with 68.3% confidence. Since the probability distribution of ACI may not be normal, and therefore, cannot be presented as “mean value $\pm$ SD”, we will present both PCI and ACI as ranges between the boundaries found with 68.3% confidence instead of the format of “mean value $\pm$ SD”.¹⁷

To achieve a confidence level of 68.3% for ACI, by consulting the standard normal table (Z-score table) (Figure S1), we choose $\Delta L_0/L_0 \in [-1.36\delta L_0/L_0, 1.36\delta L_0/L_0]$ and $\Delta T_0/T_0 \in [-1.36\delta T_0/T_0, 1.36\delta T_0/T_0]$ as the confidence intervals of relative systematic errors of L_0 and T_0 , respectively (i.e., each concentration interval with a confidence level of 82.63%, and the combination of the concentration intervals with a confidence level of $82.63\% \times 82.63\% \approx 68.3\%$) (Note S4). Evidently, we can vary the confidence level of ACI by choosing different numbers of $\delta L_0/L_0$ and $\delta T_0/T_0$ in the confidence intervals of $\Delta L_0/L_0$ and $\Delta T_0/T_0$. Note that, to reduce the amount of experimental work required by the ACI-Kd workflow, we can estimate the confidence intervals of relative systematic errors of concentrations once and use these intervals for experiments conducted with similar samples prepared in the same way.

To establish the ACI graph of “ $K'_{\text{d,det}}/K_{\text{d,det}}$ vs L_0/K_d ”, we need to determine the combinations of $\Delta L_0/L_0$ and $\Delta T_0/T_0$ that correspond to Δ^*_{min} and Δ^*_{max} , respectively. According to eq 14 and the chosen confidence intervals of $\Delta L_0/L_0$ and $\Delta T_0/T_0$, we find that $\Delta^*_{\text{min}} = \{\Delta L_0/L_0 = 1.36\delta L_0/L_0, \Delta T_0/T_0 = -1.36\delta T_0/T_0\}$, and $\Delta^*_{\text{max}} = \{\Delta L_0/L_0 = -1.36\delta L_0/L_0, \Delta T_0/T_0 = 1.36\delta T_0/T_0\}$ (Note S5), with which we can establish the two boundary curves in the ACI graph. To numerically build the two boundary curves, we first need to generate two simulated data sets of “R vs T_0 ” for a large range of L_0/K_d and for two systematic errors: Δ^*_{min} and Δ^*_{max} . Then, we need to fit the generated data sets with eq 11 using $L_{0,\text{fit}} = L_0$ and $L'_{0,\text{fit}} = \alpha L_0$ ($\alpha \ll 1$, e.g., $\alpha = 0.005$) to obtain $K_{\text{d,det}}$ and $K'_{\text{d,det}}$ at each L_0/K_d

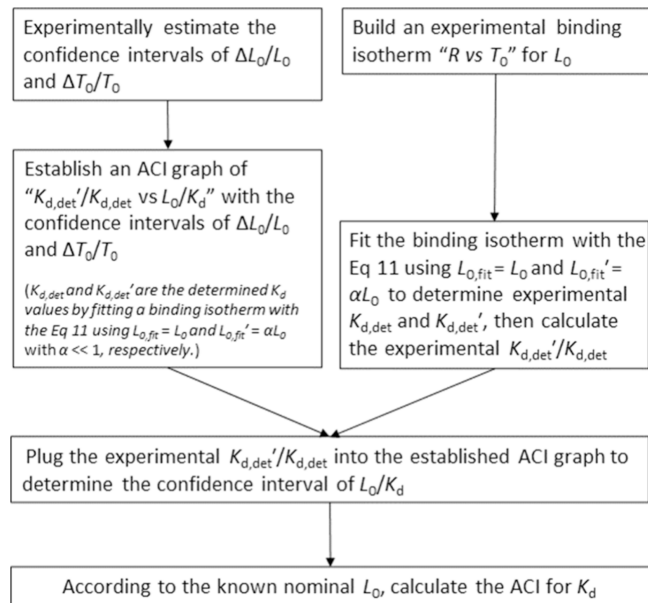


Figure 5. Step-by-step ACI-Kd workflow for finding ACI of K_d .

K_d , respectively. As a result, the theoretical value of $K'_{d,det}/K_{d,det}$ at each value of L_0/K_d can be calculated and the two boundary curves of “ $K'_{d,det}/K_{d,det}$ vs L_0/K_d ” in the ACI graph can be established (Figure 3). To be noted, we should use the nominal (instead of accurate) ligand concentration as L_0 for both fitting and calculating L_0/K_d in establishing the ACI graph since the accurate L_0 is unknown in real experiments.

By now, we have both procedures required for the proposed ACI-Kd workflow (Figure 5) and the rest of the workflow is straightforward. When an experimental binding isotherm is obtained, it is fitted with eq 11 twice, using $L_{0,fit} = L_0$ and $L'_{0,fit} = \alpha L_0$, to obtain experimental $K_{d,det}$ and $K'_{d,det}$, respectively. By plugging the experimental $K'_{d,det}/K_{d,det}$ into the established ACI graph, the confidence interval of L_0/K_d (with 68.3% confidence) is determined as illustrated in Figure 3 (and eq 8). According to the computed confidence interval of the ratio of nominal L_0 to accurate K_d (L_0/K_d) and the known nominal L_0 , ACI of K_d is calculated. Finally, an experimentalist can decide whether ACI is satisfactory or not based on the intended application of the ligand. If ACI is too large, the experiment must be performed with a lower L_0 , which may require an instrument with a lower LOQ (an example is shown in the section describing applications of ACI-Kd to real binding experiments).

Considering the computational complexity of the ACI-Kd workflow, we developed a dedicated program implemented as a user-friendly web app (<https://aci.sci.yorku.ca>) to compute both ACI and PCI of K_d from a single binding isotherm. The detailed computational workflow (Figure S2) and algorithm (Figure S3) for the ACI-Kd web app are shown in Note S6. The ACI-Kd web app produces a report including the input and output data, a publication-quality binding isotherm and the ACI graph (an example of the graphs is shown in Figure 6).

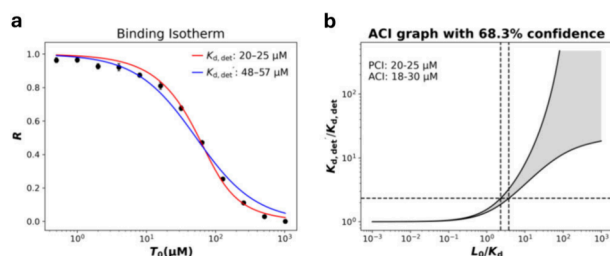


Figure 6. Example of binding isotherm (a) and ACI graph (b) produced by ACI-Kd web app (<http://aci.sci.yorku.ca>). The binding isotherm was obtained from an ACTIS experiment using BAS–fluorescein as the binding pair, with a nominal ligand concentration (L_0) of 70 μM . Refer to the experimental demonstration section and Notes S8 and S9 for more experimental details.

This report can be downloaded in a PDF format ready for saving and distributing (e.g., publishing) the results of the ACI analysis. For added flexibility in data interpretation, both ACI and PCI are computed for three confidence levels: 68.3, 95.5, and 99.7%.

Since the ACI-Kd workflow was developed to calculate the confidence interval of an accurate K_d by accounting for its fundamental sources of the inaccuracy, specifically the systematic errors in variables, this workflow can be applied to experimental data obtained using any K_d -determination method that employs nonlinear regression of binding isotherms. However, the current version of the ACI-Kd workflow is only applicable to one-to-one binding processes

(eq 9) where K_d is the sole unknown parameter in the nonlinear regression (eq 11).

Verification of the ACI-Kd Workflow

Verification of ACI-Kd requires showing that ACI-Kd is capable of producing theoretical results, which can be arranged without leaving the realm of solid theory. Since the accurate values (e.g., K_d , L_0 , T_0 and their systematic errors) in real experiments are fundamentally unknown and uncontrollable, the most reliable data source for verifying ACI-Kd is the simulated data with controllable variables and parameters. Thus, we simulated binding isotherms with eq 11; they are available as a supporting spreadsheet (Data S1). In the simulations, the input values of K_d , L_0 , and T_0 can be considered as true values with no uncertainty. Simulating all the cases of accurate (true) K_d locating in a confidence interval with a known probability (i.e., true ACI) was impractical because it required infinite or a very large number of simulated binding isotherms. Thus, to simplify the verification process, we classified the infinite binding isotherms for true ACI into three scenarios: (i) true K_d was theoretically located inside the computed ACI, (ii) true K_d was theoretically close to one of the boundaries of computed ACI, and (iii) true K_d was theoretically outside the computed ACI.

First, we investigated if ACI-Kd can correctly produce the theoretically predicted results when the accurate K_d was theoretically located inside ACI. To conduct this investigation, with the spreadsheet (Data S1), we set $K_d = 1$ (unitless) and simulated the binding isotherms “ R vs T_0 ” for a large range of L_0/K_d without introducing any error. Then, we input the simulated binding isotherms, corresponding L_0 and reasonable relative random errors (e.g., $\delta L_0/L_0 = \delta T_0/T_0 = 5\%$ in this study) of concentrations into ACI-Kd to compute ACI and PCI for each L_0/K_d . Since a zero error (used in the simulations) was at the center of the assumed interval of systematic errors in concentrations (used in ACI-Kd), theoretically true $K_d = 1$ should always be located inside and close to the center of computed ACI. Because all the input concentrations in ACI-Kd were identical to the values used for simulating binding isotherms, the computed PCI should be very narrow and close to the true K_d value. Our results (Figure 7a) produced by ACI-Kd agreed with all the theoretical predictions.

Second, we studied if ACI-Kd could correctly determine the ACI of K_d when the combinations of true systematic errors of L_0 and T_0 (i.e., $\Delta L_0/L_0$ and $\Delta T_0/T_0$) were the boundary conditions, for which true K_d was theoretically located on the boundary of computed ACI. To study the two boundary conditions, we simulated the binding isotherms using the spreadsheet (Data S1) by adding error combinations of “ $\Delta L_0/L_0 = 1.36\delta L_0/L_0$ and $\Delta T_0/T_0 = -1.36\delta T_0/T_0$ ” ($\Delta^*_{\min}$) and “ $\Delta L_0/L_0 = -1.36\delta L_0/L_0$ and $\Delta T_0/T_0 = 1.36\delta T_0/T_0$ ” ($\Delta^*_{\max}$) for a large range of L_0/K_d . Here, $\Delta L_0/L_0$ and $\Delta T_0/T_0$ were the true relative systematic errors added in the simulated binding isotherms while $\delta L_0/L_0$ and $\delta T_0/T_0$ were the assumed relative random errors of L_0 and T_0 that would be input in ACI-Kd. By inputting the simulated binding isotherms, corresponding L_0 , and the assumed relative random errors into ACI-Kd, we obtained that true $K_d = 1$ was close to the upper or lower limit of the computed ACI (depending on the introduced error combination in simulations) for the whole range of studied L_0/K_d (Figure 7b1,b2) as theoretically expected. According to Figure 7b1,b2, the computed PCI consistently shifted away

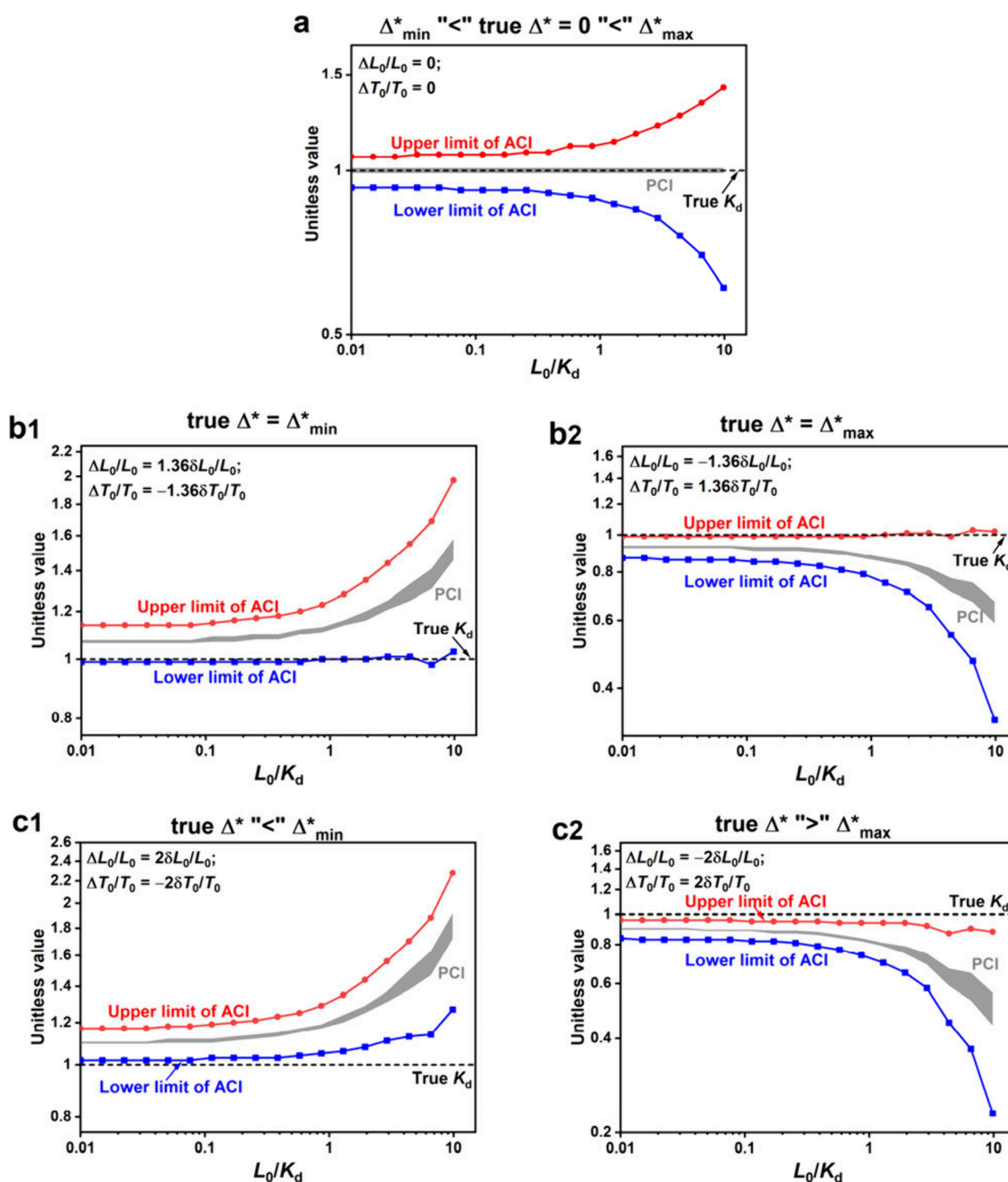


Figure 7. Verification of ACI-K_d with simulated binding isotherms. Shown are comparisons of true K_d to computed ACI and PCI for (a) true $\Delta^* = 0$, which was between $\Delta^*_{\min}$ (i.e., $\{\Delta L_0/L_0 = 1.36\delta L_0/L_0, \Delta T_0/T_0 = -1.36\delta T_0/T_0\}$) and $\Delta^*_{\max}$ (i.e., $\{\Delta L_0/L_0 = -1.36\delta L_0/L_0, \Delta T_0/T_0 = 1.36\delta T_0/T_0\}$); (b1) true $\Delta^* = \Delta^*_{\min}$; (b2) true $\Delta^* = \Delta^*_{\max}$; (c1) true $\Delta^* < \Delta^*_{\min}$ (i.e., true $\Delta^* = \{\Delta L_0/L_0 = 2\delta L_0/L_0, \Delta T_0/T_0 = -2\delta T_0/T_0\}$); and (c2) true $\Delta^* > \Delta^*_{\max}$ (i.e., true $\Delta^* = \{\Delta L_0/L_0 = -2\delta L_0/L_0, \Delta T_0/T_0 = 2\delta T_0/T_0\}$). For all verification steps, the input $\delta L_0/L_0 = \delta T_0/T_0 = 0.05$ in ACI-K_d, which was a reasonable value in experiments. To clearly show the detailed value change in large ranges of L_0/K_d , the results are presented on log–log scales.

from the accurate (true) K_d as L_0/K_d increased, as suggested by Figure 4b and eq 14. Additionally, the width of the PCI expanded with the increase in L_0/K_d (Figure 7b1,b2) due to the decreased precision of K_{d,det}, which resulted from increasing systematic errors in K_{d,det} values calculated using different data points in a binding isotherm. Note that, in real experiments, there might be no specific trends for the dependence of the PCI width on L_0/K_d or the relationship between the widths of PCI and ACI. This is because either experimental random errors or experimental systematic errors can dominate the other type of errors for any given L_0/K_d .

Finally, we tested the cases of true K_d theoretically locating outside computed ACI. True K_d being outside the computed ACI meant that the combination of true systematic errors of L₀ and T₀ was beyond the interval of Δ^* defined by $\Delta^*_{\min}$ and $\Delta^*_{\max}$. To conduct this test, we simulated the binding isotherms across a large range of L_0/K_d , introducing two types of systematic errors. In the first scenario, we applied the error combination " $\Delta L_0/L_0 = 2\delta L_0/L_0 > 1.36\delta L_0/L_0$ and $\Delta T_0/T_0 = -2\delta T_0/T_0 < -1.36\delta T_0/T_0$ " (i.e., true $\Delta^* < \Delta^*_{\min}$) which is theoretically expected to cause the true K_d to fall below the lower limit of calculated ACI. In the second

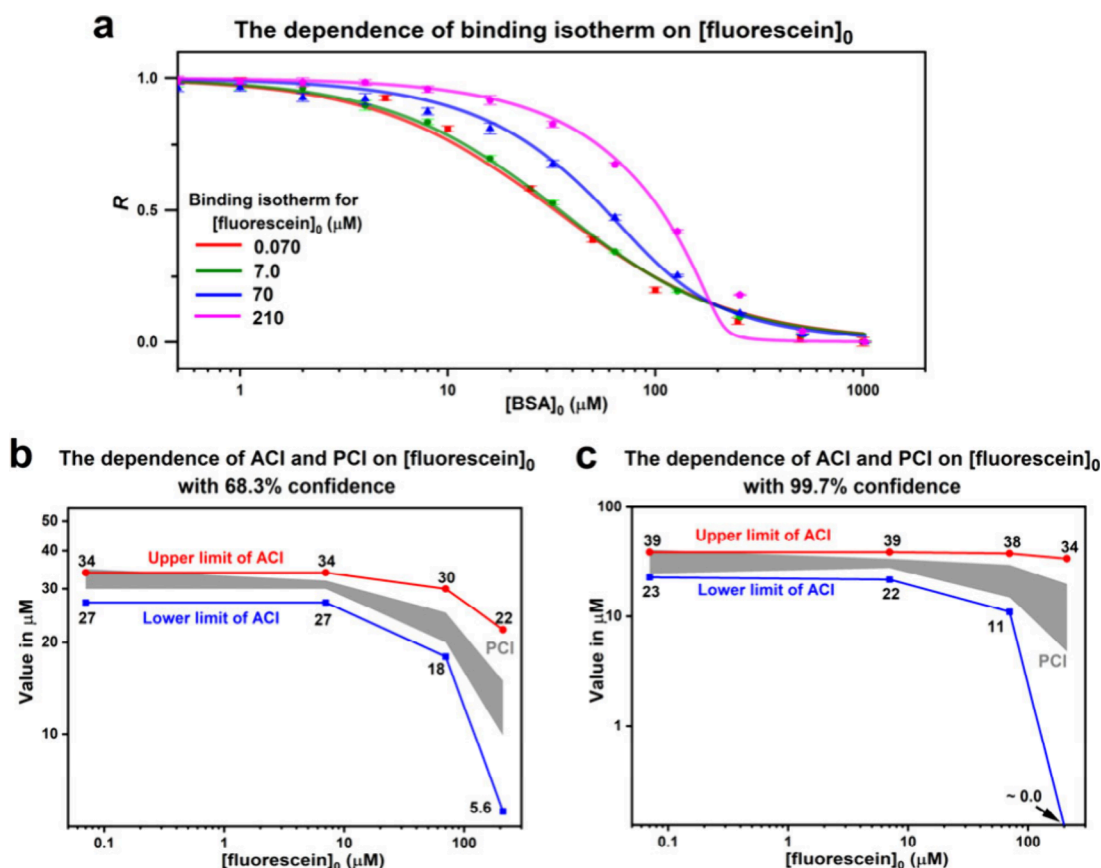


Figure 8. Application of ACI-K_d to results of ACTIS experiments with BSA–fluorescein as the binding pair: (a) influence of change in [fluorescein]₀ (nominal L_0) on binding isotherms; (b) dependence of computed ACI and PCI on [fluorescein]₀ with 68.3% confidence; (c) dependence of computed ACI and PCI on [fluorescein]₀ with 99.7% confidence. Note the log–log scale in (b) and (c).

scenario, we used the error combination " $\Delta L_0/L_0 = -2\delta L_0/L_0 < -1.36\delta L_0/L_0$ and $\Delta T_0/T_0 = 2\delta T_0/T_0 > 1.36\delta T_0/T_0$ " (i.e., true $\Delta^* > \Delta^*_{\max}$) which theoretically results in the true K_d be greater than the upper limit of calculated ACI. Here, quotation marks are used for the ">" and "<" symbols to emphasize that vectors cannot be directly compared in magnitude. By inputting the corresponding data into ACI-K_d, we observed that true $K_d = 1$ was consistently outside the computed ACI across the entire range of L_0/K_d studied (Figure 7c1, c2), and the relationships between true K_d and the calculated ACI aligned with theoretical expectations. In Figure 7c1, c2, we also observed that with the increase of L_0/K_d , the computed PCI kept shifting away from the true K_d , and the PCI width increased with the increasing L_0/K_d (similar to Figure 7b1,b2).

To sum up the above, since the relationship between true K_d and computed ACI agreed with the theoretical predictions for all three scenarios, we confirmed that ACI-K_d can produce theoretically predicted results. The results in Figure 7 also confirmed that the range of ACI increases with the increase of L_0/K_d , which was suggested by Figure 3. Additionally, Figure 4b and eq 14 imply that, when there exists systematic error in L_0 and/or T_0 , the difference between $K_{d,\det}$ and true K_d (i.e., $|K_{d,\det} - \text{true } K_d|$) increases with the increase of L_0/K_d . This dependence of $|K_{d,\det} - \text{true } K_d|$ on L_0/K_d was confirmed by the dependence of PCI (the center of PCI was $K_{d,\det}$) on L_0/K_d in Figure 7b1,b2,c1,c2. Without adding any random errors to variables in the simulation, the PCI width increases with increasing L_0/K_d in Figure 7b1,b2,c1,c2 because as L_0/K_d rises,

$K_{d,\det}$ values calculated with different data points in a binding isotherm are subject to larger systematic errors and thus distribute over a wider range, resulting in lower precision. However, this may not be the case in real experiments which always include random errors in variables. Note that, since there was no systematic error in either L_0 or T_0 in the simulated binding isotherms for the study in Figure 7a, PCI in Figure 7a was always close to true K_d with negligible width as expected. Figure 7b1,b2,c1,c2 indicate that when there exist systematic errors in L_0 and T_0 , which is typically the case, solely relying on PCI can potentially either highly underestimate or overestimate (depending on the systematic errors in independent variables) K_d values. Our results in Figure 7 show that, compared to PCI, ACI computed with ACI-K_d is much more reliable to show the possible value range of accurate K_d when there are errors in concentrations (which is always the case in experiments). Now, let us apply ACI-K_d to real experiments and provide a comprehensive interpretation of the experimental results.

Examples of ACI-K_d Application to Results of Binding Experiments

To demonstrate the application and verify the capability of ACI-K_d in K_d -determination experiments, we conducted both virtual and real experiments involving various binding pairs. The results of the virtual experiments (Note S7, including Figure S4) confirmed that ACI-K_d can accurately compute the confidence intervals of K_d when provided with the correct confidence intervals of relative systematic errors in concentrations. In the real experiments, we studied two binding pairs

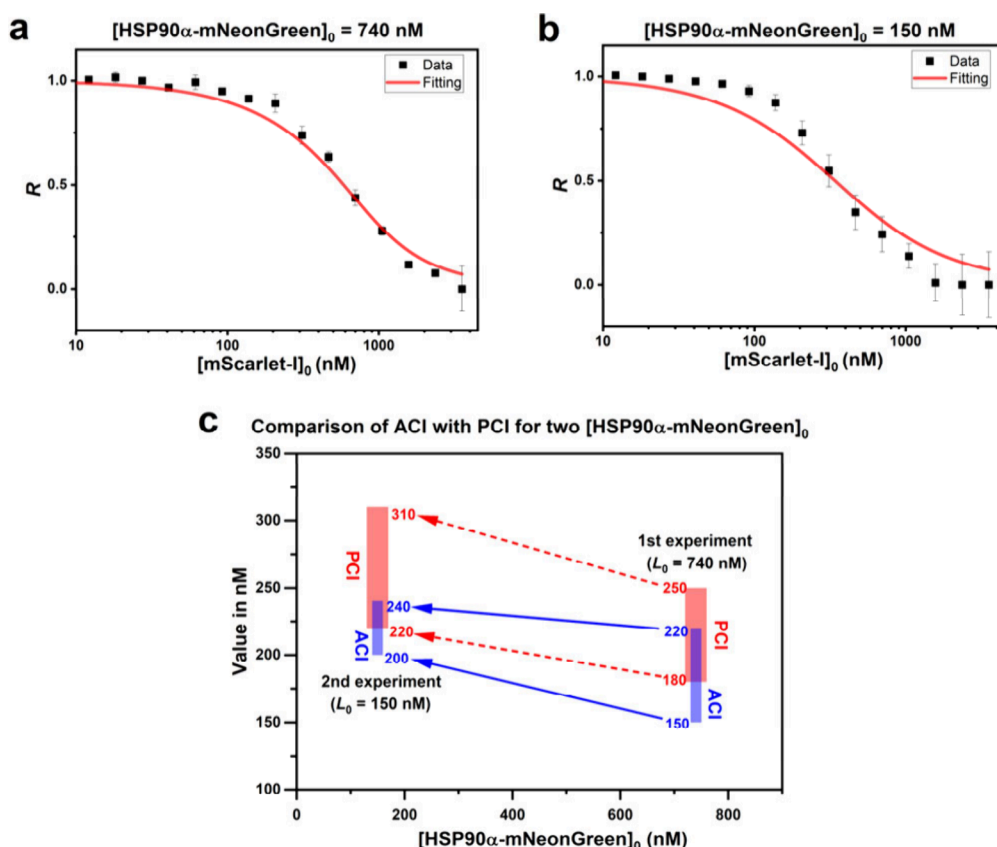


Figure 9. Application of ACI-Kd to results of FRET experiments with HSP90 α -mNeonGreen–mScarlet-I-CDC37 as the binding pair: (a) binding isotherm of the experiment with total ligand concentration $L_0 = [HSP90\alpha\text{-mNeonGreen}]_0 = 740 \text{ nM}$; (b) binding isotherm of the experiment with total ligand concentration $L_0 = [HSP90\alpha\text{-mNeonGreen}]_0 = 150 \text{ nM}$; (c) comparison of ACI with PCI for the experiments with $[HSP90\alpha\text{-mNeonGreen}]_0 = 150$ and 740 nM . All the ACI and PCI shown in (c) are with a confidence level of 68.3%.

with two different K_d -determination methods: (i) BSA–fluorescein with the ACTIS method (accurate constant via transient incomplete separation)^{50,64–66} and (ii) HSP90 α -mNeonGreen–mScarlet-I-CDC37 with the Förster resonance energy transfer (FRET) method.⁶⁷ Experimental details can be found in Notes S8–S12 (including Figures S5–S7 and Tables S2–S8).

First, we demonstrate the application of ACI-Kd to the results of ACTIS experiments. We chose BSA and fluorescein as binding partners because this pair has been well studied and has a relatively large consensus K_d value in the micromolar range,^{50,68–70} which can give us enough room for varying L_0/K_d while still satisfying the requirement that L_0 should be greater than the LOQ of the ACTIS instrument for fluorescein (in the picomolar range). In four ACTIS experiments, the total fluorescein concentrations $[fluorescein]_0$ (nominal L_0) were prepared to be 0.10, 10, 100, and $300 \mu\text{M}$. The purity of the product was 70% according to the product specification of the used fluorescein sodium salt. Thus, $[fluorescein]_0$ (nominal L_0) was corrected to be 0.070, 7.0, 70, and $210 \mu\text{M}$, respectively. By measuring the signals of 5 independently prepared fluorescein stock solutions with a spectrophotometer, $\delta L_0/L_0$ was estimated to be 0.023 (Table S2). For all four experiments, total BSA concentration $[BSA]_0$ (nominal T_0) was varied from 0 (the lowest nonzero concentration was $0.1 \mu\text{M}$ or $0.5 \mu\text{M}$) to 1000 (or 1024) μM , which was much greater than the expected K_d value to ensure the binding was saturated. The high purity of the BSA protein used in this study was confirmed by SDS-PAGE (Figure S5). By measuring the UV–

vis absorbance of BSA stock solutions with a spectrophotometer in 5 independent sample preparations, $\delta T_0/T_0$ was estimated to be 0.082 (Table S3). Then, with ACTIS experiments, the experimental binding isotherms “ R vs $[BSA]_0$ ” for different $[fluorescein]_0$ were produced (Figure 8a). By inputting $\delta L_0/L_0 = 0.023$, $\delta T_0/T_0 = 0.082$, and the binding-isotherm data corresponding to $[fluorescein]_0 = 0.070$, 7.0, 70, and $210 \mu\text{M}$ into ACI-Kd, PCI and ACI (with 68.3% confidence) of K_d were found (Figure 8b). Note that, all the boundary values of ACI and PCI presented in Figure 8 were with no more than two significant figures and the rest of this paper will follow this rule because errors in K_d were unlikely smaller than 10%.

Figure 8b indicates that, with high ligand concentration (e.g., $[fluorescein]_0 = 210 \mu\text{M}$), if we conclude the value of K_d only based on the underestimated narrow range of PCI (e.g., 9.9–15 μM), we may overestimate the level of affinity between BSA and fluorescein. The large range of ACI (e.g., 5.6–22 μM) with 68.3% confidence at $[fluorescein]_0 = 210 \mu\text{M}$ suggests that the accurate K_d can be different from PCI with high probability, and researchers should pay attention to the large uncertainty of accurate K_d suggested by wide ACI and decide if additional experiments are required to obtain a narrower ACI. Therefore, our results clearly show the necessity of assessing ACI in K_d determinations since the conclusions based on PCI can be misleading. It was noticed (from Figure 8b) that, with 68.3% confidence, the upper limit of ACI (i.e., 22 μM) determined with $[fluorescein]_0 = 210 \mu\text{M}$ was smaller than the lower limit of ACI (i.e., 27 μM) determined with $[fluorescein]_0 = 0.070$ or

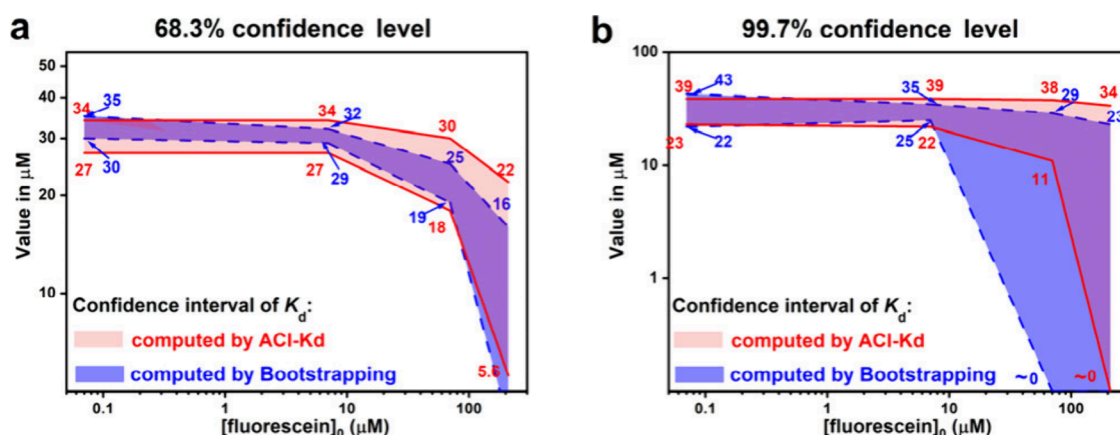


Figure 10. Comparison of the confidence intervals of K_d determined with ACI-Kd and bootstrapping approach for two confidence levels: 68.3% (a) and 99.7% (b). The binding isotherms obtained from the ACTIS experiments for BSA–fluorescein were studied. A total of 5,000 iterations were used in the bootstrapping procedure, which was confirmed to be sufficient for achieving convergent results. Note the log–log scale in both panels.

7.0 μM. The possible reasons for this phenomenon include the underestimated width of confidence intervals for relative systematic errors in L_0 and T_0 and/or disregarding the systematic errors of R . This emphasizes that the ACI of K_d computed with ACI-Kd workflow is the narrower limit of confidence interval of accurate K_d as we explained in the theory-development part. To obtain the range of accurate K_d with a higher confidence level, we increased the confidence level of ACI to 99.7%. With the increased confidence level of 99.7%, ACI of K_d for nominal [fluorescein]₀ = 0.070, 7.0, 70, and 210 μM were calculated to be 23–39 μM, 22–39 μM, 11–38 μM, and ~0.0–34 μM, respectively (Figure 8c). As expected, with the higher confidence interval, the range of computed ACI became wider and the ACI determined with different ligand concentrations were highly overlapped.

Then, to further confirm the capability of ACI-Kd in the research on molecular interactions, we applied ACI-Kd to the results of K_d -determination experiments conducted with FRET method for a protein–protein binding pair of HSP90α-mNeonGreen–mScarlet-I-CDC37. Since HSP90α–CDC37 is a potential drug target in cancer, knowing the accurate K_d of this binding pair is crucial for corresponding drug (e.g., inhibitor) development.^{71–73} Therefore, our goal was to find a reasonably narrow ACI (68.3% confidence) of K_d with the ratio between the upper-limit value and lower-limit value being <1.5 since two K_d values different by less than 1.5 folds are often considered indistinguishable. Note that, users of ACI-Kd can define their own targeted width of ACI based on the intended application of the evaluated ligand.

To find the ACI of K_d for HSP90α-mNeonGreen–mScarlet-I-CDC37, we first conducted the FRET K_d -determination experiment using a Tecan Infinite M200Pro plate reader (Männedorf, Switzerland). In this experiment, the total ligand concentration [HSP90α-mNeonGreen]₀ (i.e., nominal L_0) was chosen to be 990 nM, while the total target concentration [mScarlet-I-CDC37]₀ (i.e., nominal T_0) varied from 0 (the lowest nonzero concentration was 15 nM) to 4,500 nM. With SDS-PAGE, the purity of HSP90α-mNeonGreen protein was determined to be 74.5%, and the purity of mScarlet-I-CDC37 was determined to be 79.5% (Figure S6). Thus, the nominal L_0 was corrected to be 740 nM, and the nominal T_0 was corrected to be from 0 (the lowest nonzero concentration was 10 nM) to 3,500 nM. With a spectrophotometer, $\delta L_0/L_0$ and $\delta T_0/T_0$ for the protein stock solutions were estimated to be 0.024 and

0.054, respectively (Tables S7 and S8). By inputting the obtained binding isotherm (Figure 9a) and relative errors of L_0 and T_0 into ACI-Kd, PCI of K_d was determined to be 180–250 nM and ACI of K_d was determined to be 150–220 nM. Both PCI and ACI demonstrated here are with 68.3% confidence. The ACI found here was slightly wider than our target defined by the condition that the ratio between the upper-limit value and lower-limit value of K_d be <1.5; in our case, this ratio is 220/150 ≈ 1.5. Since L_0 = 740 nM approaches the LOQ (≈ 630 nM) of Tecan Infinite M200Pro plate reader for HSP90α-mNeonGreen in the HSP90α-mNeonGreen–mScarlet-I-CDC37 binding experiment (Figure S7a), we decided to use a FRET instrument with lower LOQ to reconduct the K_d -determination experiment with lower L_0 (i.e., [HSP90α-mNeonGreen]₀).

In the second experiment, we used a Tecan Infinite F200Pro plate reader (Männedorf, Switzerland) with LOQ ≈ 140 nM for HSP90α-mNeonGreen (Figure S7b) to conduct the study. Similar to the first experiment, the total target concentration [mScarlet-I-CDC37]₀ (i.e., nominal T_0) was varied from 0 (the lowest nonzero concentration was 10 nM) to 3,500 nM in the second experiment, while the total ligand concentration [HSP90α-mNeonGreen]₀ (i.e., nominal L_0) was lowered to 150 nM (after concentration correction based on the product purity) and kept constant. By inputting the binding isotherm obtained from the second experiment (Figure 9b), $\delta L_0/L_0$ = 0.024 and $\delta T_0/T_0$ = 0.054 into ACI-Kd, PCI and ACI (68.3% confidence) of K_d were determined to be 220–310 nM and 200–240 nM, respectively (demonstrated in Figure 9c). Since the ACI found in the second experiment achieved our targeted width (240/200 = 1.2 < 1.5), no more experiment was required, and we can finally conclude the K_d of HSP90α-mNeonGreen–mScarlet-I-CDC37 as 200–240 nM with 68.3% confidence. The comparison of ACI and PCI for the two FRET experiments (Figure 9c) reveals the following. First, the width of ACI decreased with decreasing the ligand concentration, which was expected. Second, ACI determined with the two different ligand concentrations greatly overlapped each other. Third, the width of PCI increased with decreasing ligand concentration due to the increased random errors for the data points with the lower ligand concentration. Notably, all the observations of the experimental results for ACTIS and FRET experiments agree with our theory and corresponding discussions.

Finally, we compared the confidence intervals produced by ACI-Kd with those obtained through bootstrapping, a statistical method based on Monte Carlo simulation. For this comparison, we developed a Python script utilizing publicly available packages, incorporating the fitting model for K_d determination (eq 11). The computational workflow for bootstrapping is illustrated in Note S13 (including Figure S8), which differs fundamentally from the computational algorithm for ACI-Kd shown in Figure S2. Figure 10 presents the results of the comparison for two confidence levels: 68.3% and 99.7%. In Figure 10, the confidence intervals of K_d computed by both the ACI-Kd and bootstrapping methods are comparable and exhibit similar trends: the width of the confidence intervals increases, while the values of both interval bounds tend to decrease as the ligand concentration rises. We propose that the observed quantitative differences arise from the distinct strategies each method employs to define confidence levels. However, further investigation is necessary to fully understand the nature of these differences, which is beyond the scope of this study.

CONCLUSION

Nonlinear regression is widely used for data analysis in physical sciences, engineering, and economics. This mathematical procedure can produce largely inaccurate values of determined parameters with apparently small uncertainties of regressions. The uncertainties of regression define PCI which may exclude the accurate value of the sought parameter with a very high probability. To account for this inherent problem of nonlinear regression, we introduce ACI, a range that includes the accurate value of the determined parameter with a chosen confidence level (probability). We propose ACI-Concept, an approach for finding ACI, which uses language and concepts comprehensible to molecular scientists. ACI-Concept includes a strategy for developing practical workflows for computing ACI.

PCI and ACI are not correlated as they result from different types of errors: PCI results from random errors of the dependent variable, while ACI results from systematic errors of variables. PCI has a normal (Gaussian) probability distribution (Figure 1) with the width depending on random errors of the dependent variable (assuming that the dependent variable does not have systematic errors and the regression model is correct). The random errors can be very small (e.g., relative standard deviation <10%) even for largely inaccurate values of the determined parameters. In contrast, ACI is expected to have a non-Gaussian probability distribution in most cases because the contributions of the normally distributed variables to the determined parameter are very likely to be unequal.¹⁷ ACI depends only on how much the systematic errors in all variables are magnified by a specific choice of the fixed independent variable. ACI may be wider than PCI for one range of fixed variables, and narrower for another range (e.g., Figure 9).

At the first glance, it may be attractive to integrate all errors in one parameter and produce a single confidence interval as a combination of ACI and PCI; however, this is a conceptual mistake as accuracy and precision are not “miscible”. Therefore, it is recommended to use both ACI and PCI as independent characteristics (of accuracy and precision) of nonlinear regression. More studies are needed to better understand a multitude of potential combinations of ACI and PCI and ways to interpret them.

Our use of ACI-Kd revealed that PCI may both overestimate and underestimate K_d , i.e., relying only on PCI may lead to both false-positive and false-negative results. Consequently, high-potency ligands can be dismissed, and low-potency ligands can instead be brought into further development of drugs and diagnostic probes if only PCI is used for decision-making in the hit-to-lead stage of drug discovery. Reporting both PCI and ACI of the determined K_d provides valuable insight into the precision and accuracy of the results. For a reliable K_d value, the PCI and ACI should be narrow and exhibit significant overlap.

The current ACI-Kd workflow computes the narrower limit of ACI of K_d , disregarding the systematic errors in R due to the lack of a strategy to estimate the confidence interval of ΔR . When such a strategy becomes available, ACI-Kd can be easily upgraded to compute ACI of K_d involving the systematic errors of all variables by slightly modifying the algorithm to establish the ACI graph. Without considering the systematic error in R , the lower limit of the relative systematic error in K_d values is a combination of relative systematic errors in L_0 and T_0 , as suggested by error propagation rules.³⁶ As we have explained, systematic errors in L_0 and T_0 stem from their random errors when stock solutions of ligand and target are prepared once for the entire series of binding experiments, which is typically the case. The relative random errors in concentrations can hardly be below 1% even with the highest diligence in experiments. Our assessment shows that, even after careful method optimization and concentration correction based on product purity, the errors in protein concentrations can approach approximately 10%. When these errors are propagated, they will inevitably lead to errors in K_d , which are almost always greater than 10% (even without considering the potential contribution of ΔR to the errors of K_d). Therefore, it is impractical to present K_d values with more than two significant figures. As a rule, the boundaries of ACI and PCI must be presented with only one to two significant figures.

It is important to emphasize that systematic errors in concentrations can lead to an incorrect ranking of ligands in high-throughput screening despite the common belief that systematic errors shift all K_d values (or PCI of K_d using our terminology) in the same direction and, thus, do not affect the ranking order of ligands. It is obvious that ligands with low K_d can have high L_0/K_d values and accordingly, judging only PCI can grossly overestimate K_d values, thus, mistakenly presenting such ligands as low-potency ones. Using both ACI and PCI for ligand ranking should greatly improve the quality of ranking and, thus, prevent waste of R&D efforts unavoidable when wrong leads are chosen for the development of drugs and diagnostic probes.

As finding ACI of K_d is now possible and simple, we recommend that reporting both ACI and PCI (ACI-Kd computes both) be adopted for publishing quantitative results of binding experiments. Taking on this recommendation will require reaching a consensus by the scientific community. We call for a constructive discussion on this subject within a public forum on Microsoft Teams (SC-ACI | General | Microsoft Teams)⁷⁴ created for this purpose. Editors of peer-reviewed journals who have expertise in this field are encouraged to discuss with their editorial boards adding the requirement of quantitative accuracy assessment for K_d to journal policies. The journals that frequently publish the results of binding experiments should advise their reviewers to pay attention to the quality of binding experiments and the need for accuracy

assessments of K_d values. Developing standard requirements for binding experiments and the presentation of their results is an urgent need.⁷⁵ The joint effort of the authors, reviewers, and editors can help us to overcome the “pandemic” of inaccuracy in K_d of biomolecular complexes.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/prechem.4c00085>.

Detailed theory development and deviation of equations, including detailed computational workflow and algorithm for ACI-Kd web app (Section S1: Notes S1–S6, Figures S1–S3, and Table S1); additional details for virtual and real experiments, instrumental setups, materials and methods, supplementary experimental results, and data analysis, including the computational workflow for the developed bootstrapping program (Sections S2 and S3: Notes S7–S13, Figures S4–S8, and Tables S2–S8) (PDF)

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Notes

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■ ABBREVIATIONS AND SYMBOLS

ACI, accuracy confidence interval
 PCI, precision confidence interval
 CI, confidence interval
 p , parameter; true/accurate value of parameter
 p_{dev} parameter determined with nominal values of variables
 p'_{dev} parameter determined by introducing a large artificial systematic error into the nominal values of variables
 Δp , systematic error of the determined parameter
 x_c , the fixed (constant) independent variable
 Δx_c , systematic error of the fixed (constant) independent variable
 x_v , the varied independent variable in a parameter-determination method
 Δx_v , systematic error of the varied independent variable
 S , measured signal; dependent variable
 ΔS , systematic error of measured signal (dependent variable)
 Δ and Δ' , two different vectors of systematic errors of variables
 Δ^* and $\Delta^{*'}_i$, two different vectors of relative systematic errors of variables
 δ , the “artificial” relative systematic error added to the “experimental” relative systematic error of x_c
 Δ^*_{min} , the vector of relative systematic errors of variables that leads to the smallest systematic error of the determined parameter
 Δ^*_{max} , the vector of relative systematic errors of variables that leads to the largest systematic error of the determined parameter
 T , equilibrium concentration of the target
 T_0 , total concentration of the target
 ΔT_0 , deviation of nominal T_0 from true (unknown) T_0
 δT_0 , random error in stock T_0
 L , equilibrium concentration of the ligand
 L_0 , total concentration of the ligand
 ΔL_0 , deviation of nominal L_0 from true (unknown) L_0
 δL_0 , random error in stock L_0
 $L_{0,\text{fit}}$, the value of L_0 used in nonlinear regression (fitting the binding isotherm); assumed to be equal to the nominal value of L_0
 $L'_{0,\text{fit}}$, the value of L_0 used in nonlinear regression (fitting the binding isotherm); chosen to be much smaller than the nominal value of L_0
 α , the ratio of $L'_{0,\text{fit}}$ to $L_{0,\text{fit}}$
 C , equilibrium concentration of the target–ligand complex
 R , fraction of unbound ligand in the equilibrium mixture of ligand and target
 ΔR , systematic error in R

K_d , the equilibrium dissociation constant and the accurate value of K_d
 $K_{d, \text{det}}$ value of K_d determined for $L_{0, \text{fit}}$
 $K'_{d, \text{det}}$ value of K_d determined for $L'_{0, \text{fit}}$
LOQ, limit of quantitation of the instrument used to detect the ligand
SD, standard deviation

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