

Modeling error in experimental assays using the bootstrap principle: Understanding discrepancies between assays using different dispensing technologies

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All experimental assay data contains error, but the magnitude, type, and primary origin of this error is often not obvious. Here, we describe a simple set of assay modeling techniques based on the bootstrap principle that allow sources of error and bias to be simulated and propagated into assay results. We demonstrate how deceptively simple operations—such as the creation of a dilution series with a robotic liquid handler—can significantly amplify imprecision and even contribute substantially to bias. To illustrate these techniques, we review an example of how the choice of dispensing technology can impact assay measurements, and show how large contributions to discrepancies between assays can be easily understood and potentially corrected for. These simple modeling techniques—illustrated with an accompanying IPython notebook—can allow modelers to understand the expected error and bias in experimental datasets, and even help experimentalists design assays to more effectively reach accuracy and imprecision goals.

Keywords: error modeling; assay modeling; Bootstrap principle; dispensing technologies; liquid handling; direct dispensing; acoustic droplet ejection

I. INTRODUCTION

Measuring the activity and potency of compounds—whether in biophysical or cell-based assays—is an important tool in the understanding of biological processes. However, understanding assay data for the purpose of optimizing small molecules for use as chemical probes or potential therapeutics is complicated by the fact that all assay data are contaminated with error from numerous sources.

Often, the dominant contributions to assay error are simply not known. This is unsurprising, given the number and variety of potential contributing factors. Even for what might be considered a straightforward assay involving fluorescent measurements of a ligand binding to a protein target, this might include (but is by no means limited to): compound impurities and degradation [1–4], imprecise compound dispensing [5, 6], unmonitored water absorption by DMSO stocks [4], the effect of DMSO on protein stability [7], intrinsic compound fluorescence [8, 9], compound insolubility [10] or aggregation [9, 11–14], variability in protein concentration or quality, pipetting errors, and inherent noise in any fluorescence measurement—not to mention stray lab coat fibers as fluorescent contaminants [15]. Under ideal circumstances, control experiments would be performed to measure the magnitude of these effects, and data quality tests would either reject flawed data or ensure that all contributions to error have been carefully accounted for in producing an assessment of error and confidence for each assayed value. Multiple independent replicates of the experiment would ideally be performed to verify the true uncertainty and replicability of the assay data¹.

Unfortunately, by the time the data reach the hands of a computational chemist (or other data consumer), the opportunity to perform these careful control experiments has usually long passed. In the worst case, the communicated assay data may not contain any estimate of error at all. Even when error has been estimated, it is often not based on a holistic picture of the assay, but may instead reflect historical estimates of error or statistics for a limited panel of control measurements. As a last resort, one can turn to large-scale analyses that examine the general reliability of datasets across many assay types [17, 18], but this is to be avoided unless absolutely necessary.

When multiple independent measurements are not available, but knowledge of how a particular assay was conducted is available, this knowledge can inform the construction of an assay-specific model incorporating some of the dominant contributions to error in a manner that can still be highly informative. Using the *bootstrap principle*—where we construct a simple computational replica of the real experiment and simulate virtual realizations of the experiment to understand the nature of the error in the experimental data—we often do a good job of accounting for dominant sources of error. Using only the assay protocol and basic specifications of the imprecision and inaccuracy of various operations such as weighing and volume transfers, we show how to construct and simulate a simple assay model that incorporates these important (often dominant) sources of error. This approach, while simple, provides a powerful tool to understand how assay error depends on both the assay protocol and the imprecision and inaccuracy of basic operations, as well as the true value of the quantity being

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¹ Care must be taken to distinguish between fully independent replicates

and partial replicates that only repeat part of the experiment (for example, repeated measurements performed using the same stock solutions), since partial measurements can often underestimate true error by orders of magnitude [16].

69 measured (such as compound affinity). This strategy is not
70 limited to computational chemists and consumers of assay
71 data—it can also be used to help optimize assay formats be-
72 fore an experiment is performed, help troubleshoot prob-
73 lematic assays after the fact, or ensure that all major sources
74 of error are accounted for by checking that variation among
75 control measurements match expectations.

76 We illustrate these concepts by considering a recent ex-
77 ample from the literature: a report by Ekins et al. [19] on how
78 the choice of dispensing technology impacts the apparent
79 biological activity of the same set of compounds under oth-
80 erwise identical conditions. The datasets employed in the
81 analyses [20, 21] were originally generated by AstraZeneca
82 using either a standard liquid handler with fixed (washable)
83 tips or an acoustic droplet dispensing device to prepare
84 compounds at a variety of concentrations in the assay, re-
85 sulting in highly discrepant assay results (Figure 1). The as-
86 say probed the effectiveness of a set of pyrimidine com-
87 pounds as anti-cancer therapeutics, targeting the EphB4 re-
88 ceptor, thought to be a promising target for several cancer
89 types [22, 23]. While the frustration for computational mod-
90 elers was particularly great, since quantitative structure ac-
91 tivity relationship (QSAR) models derived from these oth-
92 erwise identical assays produce surprisingly divergent pre-
93 dictions, numerous practitioners from all corners of drug
94 discovery expressed their frustration in ensuing blog posts
95 and commentaries [24–26]. Hosts of potential explanations
96 were speculated, including sticky compounds absorbed by
97 tips [27] and compound aggregation [13, 14].

98 For simplicity, we ask whether the simplest contribu-
99 tions to assay error—imprecision and bias in material trans-
100 fer operations and imprecision in measurement—might ac-
101 count for some component of the discrepancy between as-
102 say techniques. We make use of basic information—the as-
103 say protocol as described (with some additional inferences
104 based on fundamental concepts such as compound solubil-
105 ity limits) and manufacturer specifications for imprecision
106 and bias—to construct a model of each dispensing process
107 in order to determine the overall inaccuracy and impreci-
108 sion of the assay due to dispensing errors, and identify the
109 steps that contribute the largest components to error. To
110 better illustrate these techniques, we also provide an an-
111 notated IPython notebook² that includes all of the compu-
112 tations described here in detail. Interested readers are en-
113 couraged to download these notebooks and explore them
114 to see how different assay configurations affect assay error,
115 and customize the notebooks for their own scenarios.

116 II. EXPERIMENTAL ERROR

117 Experimental error can be broken into two components:
118 The *imprecision* (quantified by standard deviation or vari-

119 ance), which characterizes the random component of the er-
120 ror that causes different replicates of the same assay to give
121 slightly different results, and the *inaccuracy* (quantified by
122 bias), which is the deviation of the average over many repli-
123 cates from the true value of the quantity being measured.

124 There are a wide variety of sources that contribute to ex-
125 perimental error. Variation in the quantity of liquid deliv-
126 ered by a pipette, errors in the reported mass of a dry com-
127 pound, or noise in the measured detection readout of a well
128 will all contribute to the error of an assay measurement. If
129 the average (mean) of these is the true or desired quantity,
130 then these variations all contribute to imprecision. If not—
131 such as when a calibration error leads to a systematic devi-
132 ation in the volume delivered by a pipette, the mass mea-
133 sured by a balance, or the average signal measured by a
134 plate reader—the transfers or measurements will also con-
135 tribute to inaccuracy or bias. We elaborate on these con-
136 cepts and how to quantify them below.

137 MODELING EXPERIMENTAL ERROR

138 1. The hard way: Propagation of error

139 There are many approaches to the modeling of error and
140 its propagation into derived data. Often, undergraduate
141 laboratory courses provide an introduction to the tracking
142 of measurement imprecision, demonstrating how to prop-
143 agate imprecision in individual measurements into derived
144 quantities using Taylor series expansions—commonly re-
145 ferred to simply as *propagation of error* [28]. For example,
146 for a function $f(x, y)$ of two measured quantities x and y with
147 associated standard errors σ_x and σ_y (which represent our
148 estimate of the standard deviation of repeated measure-
149 ments of x and y), the first-order Taylor series error propa-
150 gation rule is,

$$\delta^2 f = \left[\frac{\partial f}{\partial x} \right]_x^2 \sigma_x^2 + \left[\frac{\partial f}{\partial y} \right]_y^2 \sigma_y^2 + \left[\frac{\partial f}{\partial x} \right]_x \left[\frac{\partial f}{\partial y} \right]_y \sigma_{xy}^2 \quad (1)$$

151 where the correlated error $\sigma_{xy}^2 = 0$ if the measurements of
152 x and y are independent. The expression for $\delta^2 f$, the es-
153 timated variance in the computed function f over experi-
154 mental replicates, in principle contains higher-order terms
155 as well, but first-order Taylor series error propagation pre-
156 sumes these higher-order terms are negligible and all error
157 can be modeled well as a Gaussian (normal) distribution.

158 For addition or subtraction of two independent quanti-
159 ties, this rule gives a simple, well-known expression for the
160 additivity of errors in quadrature,

$$f = x \pm y \\ \delta^2 f = \sigma_x^2 + \sigma_y^2 \quad (2)$$

161 For more complex functions of the data, however, even the
162 simple form of Eq. 1 for just two variables can be a struggle
163 for most scientists to apply, since it involves more complex
164 derivatives that may not easily simplify.

² The companion IPython notebook is available online at: <http://github.com/choderalab/dispensing-errors-manuscript>

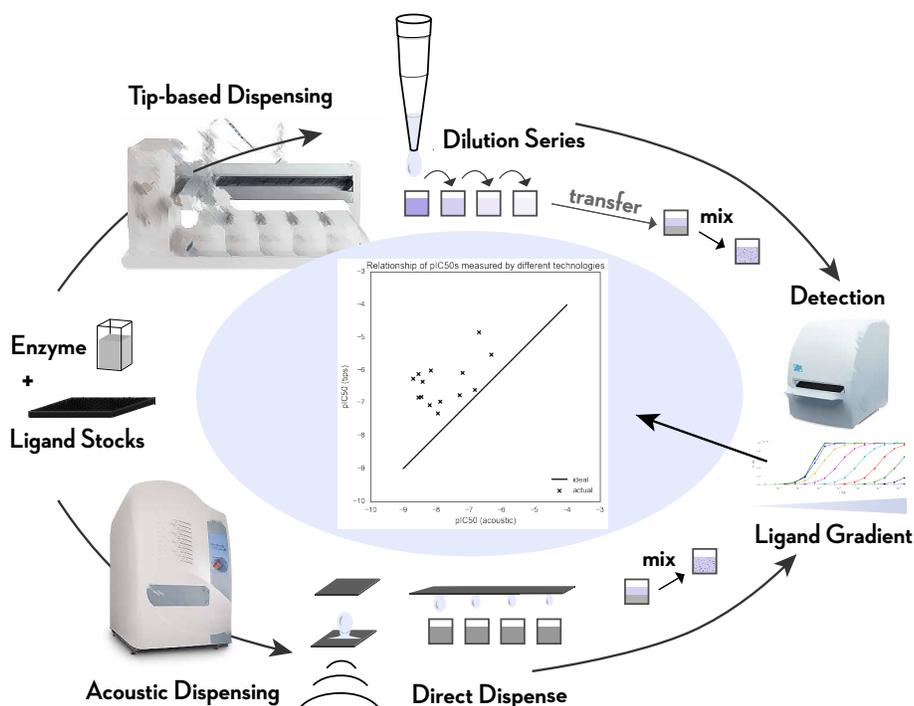


FIG. 1. Illustration of the stages of the two assay protocols considered here, utilizing either tip-based or acoustic droplet dispensing.

Two different assay protocols—utilizing different dispensing technologies—were used to perform the same assay [19–21]. In the case of tip-based dispensing, a Tecan Genesis liquid handler was used to create a serial dilution of test compounds using fixed washable tips, and a small quantity of each dilution was pipetted into the enzyme assay mixture prior to detection. In the case of acoustic dispensing (sometimes called acoustic droplet ejection), instead of creating a serial dilution, a Labcyte Echo was used to directly dispense nanoliter quantities of compound stock into the enzyme assay mixture prior to detection. The detection phase measured product accumulation after a fixed amount of time (here, detection of accumulated phosphorylated substrate peptide using AlphaScreen), and the resulting data were fit to obtain pIC_{50} estimates. Ekins et al. [19] noted that the resulting pIC_{50} data between tip-based dispensing and acoustic dispensing were highly discrepant, as shown in the central figure where the two sets of assay data are plotted against each other.

2. The easy way: The bootstrap principle

Instead, we adopt a simpler approach based on the *bootstrap principle* [29]. *Bootstrapping* allows the sampling distribution to be approximated by simulating from a good estimate (or simulacrum) of the real process. While many computational chemists may be familiar with *resampling bootstrapping* for a large dataset, where resampling values from the dataset with replacement provides a way to simulate a replica of the real process, it is also possible to simulate the process in other ways, such as from a parametric or other model of the process. Here, we model sources of random error using simple statistical distributions, and *simulate* multiple replicates of the experiment, examining the distribution of experimental outcomes in order to quantify error. Unlike propagation of error based on Taylor series approximations (Eq. 1), which can become nightmarishly complex for even simple models, quantifying the error by bootstrap simulation is straightforward even for complex assays. While there are theoretical considerations, practical application of the bootstrap doesn't even require that the function f be differentiable or easily written in closed form—as long as we can compute the function f on a dataset, we can bootstrap it.

For example, for the case of quantities x and y and associated errors σ_x and σ_y , we would conduct many realizations $n = 1, \dots, N$ of an experiment in which we draw *bootstrap replicates* x_n and y_n from normal (Gaussian) distributions

$$\begin{aligned} x_n &\sim \mathcal{N}(x, \sigma_x^2) \\ y_n &\sim \mathcal{N}(y, \sigma_y^2) \\ f_n &\equiv f(x_n, y_n) \end{aligned} \quad (3)$$

where the notation $x \sim \mathcal{N}(\mu, \sigma^2)$ denotes that we draw the variable x from a normal (Gaussian) distribution with mean μ and variance σ^2 ,

$$x \sim \mathcal{N}(\mu, \sigma^2) \Leftrightarrow p(x) = \frac{1}{\sqrt{2\pi}\sigma} \exp\left[-\frac{(x-\mu)^2}{2\sigma^2}\right] \quad (4)$$

We then analyze the statistics of the $\{f_n\}$ samples as if we had actually run the experiment many times. For example, we can quantify the statistical uncertainty δf using the standard deviation over the bootstrap simulation realizations, $\text{std}(f_n)$. Alternatively, presuming we have simulated enough bootstrap replicates, we can estimate 68% or 95% confidence intervals, which may sometimes be very lopsided if the function f is highly nonlinear.

202 Since most instruments we deal with in a laboratory—
203 such as pipettes or liquid handlers or balances—have read-
204 ily available manufacturer-provided specifications for im-
205 precision and accuracy, we will generally make use of the
206 normal (Gaussian) distribution³ in modeling the error Δx ,

$$\Delta x \sim \mathcal{N}(\mu, \sigma^2), \quad (5)$$

207 where $\Delta x \equiv x - x_*$ is the deviation from the true or de-
208 sired value x_* , the mean μ represents the inaccuracy, and
209 the standard deviation σ represents the imprecision.

210 We will generally quantify the error from our bootstrap
211 simulation replicates in terms of two primary statistics:

212 **Relative bias (RB).** As a measure of inaccuracy, we will com-
213 pute the relative expected deviation from the true value f ,

$$\text{RB} \equiv \frac{E[f_n - f]}{f}. \quad (6)$$

214 This is often expressed as a percentage (RB%) by multiplying
215 RB by 100. Note that, for cases where $f = 0$, this can be a
216 problematic measure, in which case the absolute bias (just
217 the numerator) is a better choice.

218 **Coefficient of variation (CV).** As a measure of imprecision,
219 we will compute the relative standard deviation,

$$\text{CV} \equiv \frac{\text{std}(f_n)}{E[f_n]}, \quad (7)$$

220 which can again be estimated from the mean over many
221 bootstrap replicates, and is often also represented as a per-
222 cent (CV%) by multiplying CV by 100.

223 Simple liquid handling: Mixing solutions

224 *The bootstrap principle*

225 Consider the simplest kind of liquid transfer operation,
226 in which we use some sort of pipetting instrument (hand-
227 held or automated) to combine two solutions. For simplic-
228 ity, we presume we combine a volume v_{stock} of compound
229 stock solution of known true concentration c_0 with a quan-
230 tity of buffer of volume v_{buffer} .

231 Initially, we presume that these operations are free of
232 bias, but have associated imprecisions σ_{stock} and σ_{buffer} . To
233 simulate this process using the bootstrap principle, we sim-
234 ulate a number of realizations $n = 1, \dots, N$, where we again
235 assume a normal distribution for the sources of error, ne-
236 glecting bias and accounting only for imprecision,

$$\begin{aligned} v_{\text{stock}}^{(n)} &\sim \mathcal{N}(v_{\text{stock}}, \sigma_{\text{stock}}^2) \\ v_{\text{buffer}}^{(n)} &\sim \mathcal{N}(v_{\text{buffer}}, \sigma_{\text{buffer}}^2) \\ v_{\text{tot}}^{(n)} &= v_{\text{stock}}^{(n)} + v_{\text{buffer}}^{(n)} \\ c^{(n)} &= c_0 / v_{\text{tot}}^{(n)}. \end{aligned} \quad (8)$$

³ Volumes, masses, and concentrations must all be positive, so it is more appropriate in principle to use a *lognormal* distribution to model these processes to prevent negative values. In practice, however, if the relative imprecision is relatively small and negative numbers do not cause large problems for the functions, a normal distribution is sufficient.

237 We can then compute statistics over the bootstrap repli-
238 cates of the resulting solution concentrations, $\{c^{(n)}\}$, $n =$
239 $1, \dots, N$ to estimate the bias and variance in the concentra-
240 tion in the prepared solution.

241 *Relative imprecision*

242 Manufacturer specifications⁴ often provide the impreci-
243 sion in relative terms as a coefficient of variation (CV), from
244 which we can compute the imprecision σ in terms of transfer
245 volume v via $\sigma = \text{CV} \cdot v$,

$$\begin{aligned} \sigma_{\text{stock}} &= v_{\text{stock}} \cdot \text{CV} \\ \sigma_{\text{buffer}} &= v_{\text{buffer}} \cdot \text{CV} \\ v_{\text{stock}}^{(n)} &\sim \mathcal{N}(v_{\text{stock}}, \sigma_{\text{stock}}^2) \\ v_{\text{buffer}}^{(n)} &\sim \mathcal{N}(v_{\text{buffer}}, \sigma_{\text{buffer}}^2) \\ v_{\text{tot}}^{(n)} &= v_{\text{stock}}^{(n)} + v_{\text{buffer}}^{(n)} \\ c^{(n)} &= c_0 / v_{\text{tot}}^{(n)}. \end{aligned} \quad (9)$$

246 We remind the reader that a CV specified as a % (CV% or
247 %CV) should be divided by 100 to obtain the CV we use here.

248 *Relative inaccuracy*

249 Similarly, the expected inaccuracy might also be stated
250 in terms of a relative percentage of the volume being trans-
251 ferred. The inaccuracy behaves differently from the impreci-
252 sion in that the inaccuracy will *bias* the transferred volumes
253 in a consistent way throughout the whole experiment. To
254 model bias, we draw a single random bias for the instrument
255 from a normal distribution, and assume all subsequent op-
256 erations with this instrument are biased in the same rela-
257 tive way. We presume the relative bias (RB)—expressed as
258 a fraction, rather than a percent—is given as RB, and draw a
259 specific instrumental bias $b^{(n)}$ for each bootstrap replicate of
260 the experiment, simulating the effect of many replications
261 of the experiment where the instrument is randomly recal-
262 ibrated,

$$\begin{aligned} b^{(n)} &\sim \mathcal{N}(0, \text{RB}^2) \\ \sigma_{\text{stock}} &= v_{\text{stock}} \cdot \text{CV} \\ \sigma_{\text{buffer}} &= v_{\text{buffer}} \cdot \text{CV} \\ v_{\text{stock}}^{(n)} &\sim \mathcal{N}(v_{\text{stock}}(1 + b^{(n)}), \sigma_{\text{stock}}^2) \\ v_{\text{buffer}}^{(n)} &\sim \mathcal{N}(v_{\text{buffer}}(1 + b^{(n)}), \sigma_{\text{buffer}}^2) \\ v_{\text{tot}}^{(n)} &= v_{\text{stock}}^{(n)} + v_{\text{buffer}}^{(n)} \\ c^{(n)} &= c_0 / v_{\text{tot}}^{(n)}. \end{aligned} \quad (10)$$

⁴ While manufacturer-provided specifications for imprecision and inaccuracy are often presented as the maximum-allowable values, we find these are a reasonable starting point for this kind of modeling.

Obviously, if the instrument is never recalibrated, the bias will be the *same* over many realizations of the experiment, but we presume that a calibration process is repeated frequently enough that its effect can be incorporated as a random effect over many replicates of the assay over a long timespan.

Uncertainty in initial concentration

We could further extend this model to include uncertainty σ_c in the stock concentration c_0 (where the concentration may be stated $c_0 \pm \sigma_c$), and begin to see how powerful and modular the bootstrap scheme is. In this new model, each simulation realization n includes one additional initial step,

$$\begin{aligned} c_0^{(n)} &\sim \mathcal{N}(c_0, \sigma_c^2) \\ b^{(n)} &\sim \mathcal{N}(0, \text{RB}^2) \\ \sigma_{\text{stock}} &= v_{\text{stock}} \cdot \text{CV} \\ \sigma_{\text{buffer}} &= v_{\text{buffer}} \cdot \text{CV} \\ v_{\text{stock}}^{(n)} &\sim \mathcal{N}(v_{\text{stock}}(1 + b^{(n)}), \sigma_{\text{stock}}^2) \\ v_{\text{buffer}}^{(n)} &\sim \mathcal{N}(v_{\text{buffer}}(1 + b^{(n)}), \sigma_{\text{buffer}}^2) \\ v_{\text{tot}}^{(n)} &= v_{\text{stock}}^{(n)} + v_{\text{buffer}}^{(n)} \\ c^{(n)} &= c_0^{(n)} / v_{\text{tot}}^{(n)}. \end{aligned} \quad (11)$$

All we had to do was add one additional step to our bootstrap simulation scheme (Eq. 10) in which the stock concentration $c_0^{(n)}$ is independently drawn from a normal distribution with each bootstrap realization n . The model can be expanded indefinitely with additional independent measurements or random variables in the same simple way.

Below, we exploit the modularity of bootstrap simulations to design a simple scheme to model a real assay—the measurement of $p\text{IC}_{50}$ s for compounds targeting the EphB4 receptor [19–21]—without being overwhelmed by complexity. This assay is particularly interesting because data exists for the same assay performed using two *different* dispensing protocols that led to highly discrepant assay $p\text{IC}_{50}$ data, allowing us to examine how different sources of error arising from different dispensing technologies can impact an otherwise identical assay. We consider only errors that arise from the transfer and mixing of volumes of solutions with different concentrations of compound, using the same basic strategy seen here to model the mixing of two solutions applied to the complex liquid handling operations in the assay. To more clearly illustrate the impact of imprecision and inaccuracy of dispensing technologies, we neglect considerations of the completeness of mixing, which can itself be a large source of error in certain assays⁵.

Modeling an enzymatic reaction and detection of product accumulation

The EphB4 assay we consider here [19–21], illustrated schematically in Figure 1, measures the rate of substrate phosphorylation in the presence of different inhibitor concentrations. After mixing the enzyme with substrate and inhibitor, the reaction is allowed to progress for one hour before being quenched by the addition of a quench buffer containing EDTA. The assay readout (in this case, AlphaScreen) measures the accumulation of phosphorylated substrate peptide. Fitting a binding model to the assay readout over the range of assayed inhibitor concentrations yields an observed $p\text{IC}_{50}$.

A simple model of inhibitor binding and product accumulation for this competition assay can be created using standard models for competitive inhibition of a substrate S with an inhibitor I . Here, we assume that in excess of substrate, the total accumulation of product in a fixed assay time will be proportional to the relative enzyme turnover velocity times time, $V_0 t$, and use an equation derived assuming Michaelis-Menten kinetics,

$$V_0 t = \frac{V_{\text{max}}[S]t}{K_m(1 + [I]/K_i) + [S]}, \quad (12)$$

where the Michaelis constant K_m and substrate concentration $[S]$ for the EphB4 system are pulled directly from the assay methodology description [20, 21]. To simplify our modeling, we divide by the constants $V_{\text{max}} t$, and work with the simpler ratio,

$$\frac{V_0}{V_{\text{max}}} = \frac{[S]}{K_m(1 + [I]/K_i) + [S]}, \quad (13)$$

In interrogating our model, we will vary the true inhibitor affinity K_i to determine how the assay imprecision and inaccuracy depend on true inhibitor affinity.

In reality, detection of accumulated product will also introduce uncertainty. First, there is a minimal detectable signal below which the signal cannot be accurately quantified—below this threshold, a random background signal or “noise floor” is observed. Second, any measurement will be contaminated with noise, though changes to the measurement protocol—such as collecting more illumination data at the expense of longer measurement times—can affect this noise. While simple calibration experiments can often furnish all of the necessary parameters for a useful detection error model—such as measuring the background signal and signal relative to a standard for which manufacturer specifications are available—we omit these effects to focus on the potential for the discrepancy between liquid handling technologies to explain the difference in assay results.

Advanced liquid handling: Making a dilution series

Because the affinities and activities of compounds can vary across a dynamic range that spans several orders of

⁵ A surprising amount of effort is required to ensure thorough mixing of two solutions, especially in the preparation of dilution series [30–32]. We have chosen not to explicitly include this effect in our model, but it could similarly be added within this framework given some elementary data quantifying the bias induced by incomplete mixing.

magnitude, it is common for assays to use a dilution series to measure the activity and potency of ligands. To create a dilution series, an initial compound stock is diluted into buffer in the first well of the series, and the contents mixed; for each subsequent well, a volume from the previous well is transferred into a well containing only buffer, and mixed (Figure 2). Commonly, each subsequent dilution step uses fixed ratios, such as 1:2 or 1:10 of solute solution to total volume⁶.

It is easy to see how the creation of a dilution series by pipetting can amplify errors: because each dilution step involves multiple pipetting operations, and the previous dilution in the series is used to prepare the next dilution, errors will generally grow with each step. As a result, the liquid handling instrumentation can have a substantial impact on the results obtained. Here, we compare an aqueous dilution series made with a liquid handler that makes use of fixed, washable tips (a Tecan Genesis) with an assay prepared directly via direct-dispensing using an acoustic dispensing instrument (a Labcyte Echo).

Tip-based liquid handling

To create a serial dilution series (Figure 2), we first transfer an aliquot of compound in DMSO stock solution to the first well, mixing it with buffer, to prepare the desired initial concentration c_0 at volume $v_{\text{intermediate}}$ for the dilution series. Next, we sequentially dilute a volume v_{transfer} of this solution with a volume v_{buffer} of buffer, repeating this process to create a total of $n_{\text{dilutions}}$ solutions. To model the impact of imprecision and inaccuracy on the serial dilution process, we again use manufacturer-provided specifications for the Tecan Genesis: the relative imprecision is stated to be 3% and the inaccuracy as 3-5% for the volumes in question [33]. The resulting concentration c_m of each dilution m is determined by both the previous concentration c_{m-1} and by the pipetted volumes v_{transfer} and v_{buffer} , each of which is randomly drawn from a normal distribution. Putting this together in the same manner as for the simple mixing of solutions, we have

$$\begin{aligned}
 b^{(n)} &\sim \mathcal{N}(0, \text{RB}^2) \\
 \sigma_{\text{transfer}} &= v_{\text{transfer}} \cdot \text{CV} \\
 \sigma_{\text{buffer}} &= v_{\text{buffer}} \cdot \text{CV} \\
 v_{\text{transfer},m}^{(n)} &\sim \mathcal{N}(v_{\text{transfer}}(1 + b^{(n)}), \sigma_{\text{transfer}}^2) \\
 v_{\text{buffer},m}^{(n)} &\sim \mathcal{N}(v_{\text{buffer}}(1 + b^{(n)}), \sigma_{\text{buffer}}^2) \\
 v_{\text{intermediate},m}^{(n)} &= v_{\text{transfer},m}^{(n)} + v_{\text{buffer},m}^{(n)} \\
 v_{\text{final},(m-1)}^{(n)} &\sim v_{\text{intermediate},(m-1)} - v_{\text{transfer},m}^{(n)} \\
 c_m^{(n)} &= c_{m-1}^{(n)} / v_{\text{intermediate},m}^{(n)}, \quad (14)
 \end{aligned}$$

⁶ Note that a 1:2 dilution refers to combining one part solute solution with one part diluent.

where the last five steps are computed for dilution $m = 1, \dots, (n_{\text{dilutions}} - 1)$. In the companion notebook, we make comparisons easier by also removing a final volume v_{transfer} from the last well so all wells have the same intended final volume.

In the EphB4 protocol [20, 21], the initial dilution step from 10 mM DMSO stocks is not specified, so we choose initial concentration $c_0 = 600 \mu\text{M}$ in order to match the maximum assay concentration used in the direct dispense version of the assay (described in the next section). We presume an initial working volume of $v_{\text{intermediate}} = 100 \mu\text{L}$, and model this dispensing process using Eq. 10. We presume this solution is then serially 1:2 diluted with 5% DMSO for a total of $n_{\text{dilutions}} = 8$ dilutions⁷ with $v_{\text{transfer}} = v_{\text{buffer}} = 50 \mu\text{L}$, which after dilution into the assay plate will produce a range of assay concentrations from 800 nM to $100 \mu\text{M}$ ⁸. We estimate the appropriate coefficient of variation (CV) and relative bias (RB) for the Tecan Genesis liquid handling instrument used in this assay using a linear interpolation over the range of volumes in a manufacturer-provided table [33]. Sampling over many bootstrap replicates, we are then able to estimate the CV and RB in the resulting solution concentrations for the dilution series. Figure 5 (left panel) shows the estimated CV and RB for the resulting concentrations in the dilution series. While the CV for the volume is relatively constant since we are always combining only two transferred aliquots of liquid, the CV for both the concentration and the total quantity of compound per well grow monotonically with each subsequent dilution. On the other hand, because the bias is assumed to be zero on average, the average dilution series bias over many bootstrap replicates with randomly calibrated instruments will be free of bias. This situation may be different, of course, if the *same* miscalibrated instrument is used repeatedly without frequent recalibration.

Once the dilution series has been prepared, the assay is performed in a 384-well plate, with each well containing 2 μL of the diluted compound in buffer combined with 10 μL of assay mix (which contains EphB4, substrate peptide, and cofactors) for a total assay volume of 12 μL . This liquid transfer step is easily modeled using the steps for modeling the mixing of two solutions in Eq. 10.

Direct dispensing technologies

Using a direct dispensing technology such as acoustic droplet ejection (ADE), we can eliminate the need to prepare an intermediate dilution series, instead adding small quantities of the compound DMSO stock solution directly into the

⁷ The published protocol [20, 21] does not specify how many dilutions were used, so for illustrative purposes, we selected $n_{\text{dilutions}} = 8$.

⁸ We note that real assays may encounter solubility issues with such high compound concentrations, and that the nonideal nature of water:DMSO solutions means that serial dilution of DMSO stocks will not always guarantee all dilutions will readily keep compound soluble. Here, we also presume the DMSO and EDTA control wells are not used in fitting to obtain ρIC_{50} values.

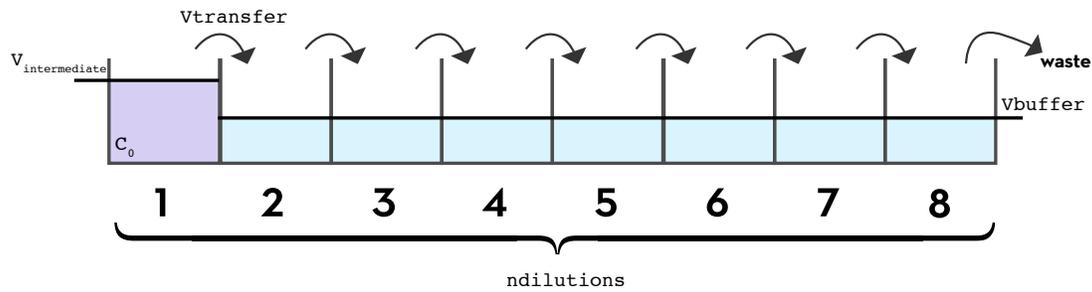


FIG. 2. Preparation of a serial dilution series with a fixed-tip liquid handler. To create a dilution series with a fixed-tip liquid handler, a protocol similar to the preparation of a dilution series by hand pipetting is followed. Starting with an initial concentration c_0 and initial volume V_{initial} in the first well, a volume V_{transfer} is transferred from each well into the next well, in which a volume of buffer, V_{buffer} , has already been pipetted. In the case of a 1:2 dilution series, V_{transfer} and V_{buffer} are equal, so the intended concentration in the second well will be $c_0/2$. This transfer is repeated for all subsequent wells to create a total of $n_{\text{dilutions}}$ dilutions. For convenience, we assume that a volume V_{transfer} is removed from the last well so that all wells have the same final volume of $V_{\text{transfer}} = V_{\text{buffer}}$, and that the error in the initial concentration (c_0) is negligible.

431 assay plate. For the LabCyte Echo used in the EphB4 assay,
 432 the smallest volume dispensed is 2.5 nL droplets; other in-
 433 struments such as the HP D300/D300e can dispense quan-
 434 tities as small as 11 pL using inkjet technology. To construct
 435 a model for a direct dispensing process, we transfer a vol-
 436 ume V_{dispense} of ligand stock in DMSO at concentration c_0
 437 into each well already containing assay mix at volume V_{mix}
 438 (presumed to be pipetted by the Tecan Genesis), and back-
 439 fill a volume V_{backfill} with DMSO to ensure each well has the
 440 same intended volume and DMSO concentration (Figure 3).
 441 We again incorporate the effects of imprecision and bias us-
 442 ing manufacturer-provided values; for the Labcyte Echo, the
 443 relative imprecision (CV) is stated as 8% and the relative in-
 444 accuracy (RB) as 10% for the volumes in question [34],

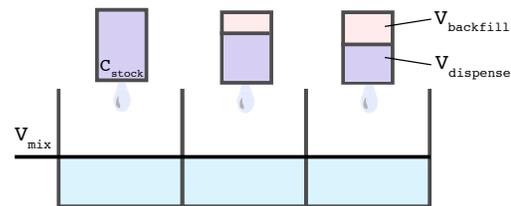


FIG. 3. Preparation of a dilution series with direct-dispense technology. With a direct-dispense liquid handler—such as the LabCyte Echo, which uses acoustic droplet ejection—instead of first preparing a set of compound solutions at different concentrations via serial dilution, the intended quantity of compound can be dispensed into the assay plates directly without the need for creating an intermediate serial dilution. We model this process by considering the process of dispensing into each well independently. A volume V_{dispense} of compound stock in DMSO at concentration c_0 is dispensed directly into an assay plate containing a volume V_{mix} of assay mix. To maintain a constant DMSO concentration throughout the assay—in this case of the EphB4 assay, 120 nL—a volume V_{backfill} of pure DMSO is also dispensed via acoustic ejection.

$$\begin{aligned}
 b_{\text{Echo}}^{(n)} &\sim \mathcal{N}(0, \text{RB}_{\text{Echo}}^2) \\
 b_{\text{Genesis}}^{(n)} &\sim \mathcal{N}(0, \text{RB}_{\text{Genesis}}^2) \\
 \sigma_{\text{dispense}} &= V_{\text{dispense}} \cdot \text{CV}_{\text{Echo}} \\
 \sigma_{\text{backfill}} &= V_{\text{backfill}} \cdot \text{CV}_{\text{Echo}} \\
 \sigma_{\text{mix}} &= V_{\text{mix}} \cdot \text{CV}_{\text{Genesis}} \\
 V_{\text{dispense}}^{(n)} &\sim \mathcal{N}(V_{\text{dispense}}(1 + b_{\text{Echo}}^{(n)}), \sigma_{\text{dispense}}^2) \\
 V_{\text{backfill}}^{(n)} &\sim \mathcal{N}(V_{\text{backfill}}(1 + b_{\text{Echo}}^{(n)}), \sigma_{\text{backfill}}^2) \\
 V_{\text{mix}}^{(n)} &\sim \mathcal{N}(V_{\text{mix}}(1 + b_{\text{Genesis}}^{(n)}), \sigma_{\text{mix}}^2) \\
 V_{\text{assay}}^{(n)} &= V_{\text{dispense}}^{(n)} + V_{\text{backfill}}^{(n)} + V_{\text{mix}}^{(n)} \\
 C_m^{(n)} &= C_{\text{stock}} / V_{\text{assay}}^{(n)} \cdot
 \end{aligned}
 \tag{15}$$

445 Since the maximum specified backfilled volume was 120 nL,
 446 we presume that V_{dispense} consisted of 8 dilutions ranging
 447 from 2.5 nL (the minimum volume the Echo can dispense)
 448 to 120 nL in a roughly logarithmic series. Note that this
 449 produces a much narrower dynamic range than the dilu-
 450 tion series experiment, with the minimum assay intended
 451 concentration being 2.5 μM assuming a 10 mM DMSO stock
 452 solution concentration C_{stock} . We can then produce an es-

453 timate for the errors in volumes and concentrations (Fig-
 454 ure 5, middle panels) by generating many synthetic repli-
 455 cates of the experiment. Because direct dispensing tech-
 456 nologies can dispense directly into the assay plate, rather
 457 than creating an intermediate dilution series that is then
 458 transferred into the assay wells, direct dispensing experi-
 459 ments can utilize fewer steps (and hence fewer potential
 460 inaccuracy- and imprecision-amplifying steps) than the tip-
 461 based assays that are dependent on the creation of an inter-
 462 mediate dilution series.

463

Fixed tips and the dilution effect

464 Simply including the computed contributions from in-
 465 accuracy and imprecision in our model of the Ekins et
 466 al. dataset [19], it is easy to see that the imprecision is
 467 not nearly large enough to explain the discrepancies be-
 468 tween measurements made with the two dispensing tech-
 469 nologies (Figure 7). Multichannel liquid-handlers such as
 470 the Tecan Genesis that utilize liquid-displacement pipet-
 471 ting with fixed tips actually have a nonzero bias in liquid
 472 transfer operations due to a dilution effect. This effect was
 473 previously characterized in work from Bristol Myers Squibb
 474 (BMS) [35, 36], where it was found that residual system
 475 liquid—the liquid used to create the pressure differences re-
 476 quired for pipetting—can cling to the interior of the tips after
 477 washing and mix with sample when it is being aspirated (Fig-
 478 ure 4). While the instrument can be calibrated to dispense
 479 volume without bias, the concentration of the dispensed so-
 480 lution can be measurably diluted.

481 To quantify this effect, the BMS team used both an Ar-
 482 tel dye-based Multichannel Verification System (MVS) and
 483 gravimetric methods, concluding that this dilution effect
 484 contributes a -6.30% inaccuracy for a target volume of 20
 485 μL [35]. We can expand our bootstrap model of dilution with
 486 fixed tips (Eq. 15) to include this effect with a simple modifi-
 487 cation to the concentration of dilution solution m ,

$$c_m^{(n)} = (1 + d) c_{m-1}^{(n)} / v_{\text{intermediate},m}^{(n)} \quad (16)$$

488 where the factor $d = -0.0630$ accounts for the -6.30% dilu-
 489 tion effect. The resulting CV and RB in volumes, concentra-
 490 tions, and quantities (Figure 5, middle) indicate a significant
 491 accumulation of bias. This is especially striking when con-
 492 sidered alongside the corresponding values for disposable
 493 tips (Figure 5, left)—which lack the dilution effect—and the
 494 acoustic-dispensing model (Figure 5, right), both of which
 495 are essentially free of bias when the average over many ran-
 496 dom instrument recalibrations is considered.

497 This dilution effect also must be incorporated into the
 498 transfer of the diluted compound solutions ($2 \mu\text{L}$) into the
 499 enzyme assay mix ($10 \mu\text{L}$) to prepare the final $12 \mu\text{L}$ assay
 500 volume, further adding to the overall bias of the assay re-
 501 sults from the fixed-tips instrument.

Fitting the assay readout to obtain $p\text{IC}_{50}$ data

503 While the IC_{50} reported in the EphB4 assay [19–21] in prin-
 504 ciple represents the stated concentration of compound re-
 505 quired to inhibit enzyme activity by half, this value is esti-
 506 mated in practice by numerically fitting a model of inhibi-
 507 tion to the measured assay readout across the whole range
 508 of concentrations measured using a method such as least-
 509 squared (the topic of another article in this series [37]).

510 To mimic the approach used in fitting the assay data,
 511 we use a nonlinear least-squares approach (based on the
 512 simple `curve_fit` function from `scipy.optimize`) to fit
 513 V_0/V_{max} computed from the competitive inhibition model
 514 (Eq. 13, shown in Fig. 6, top panels) using the true assay

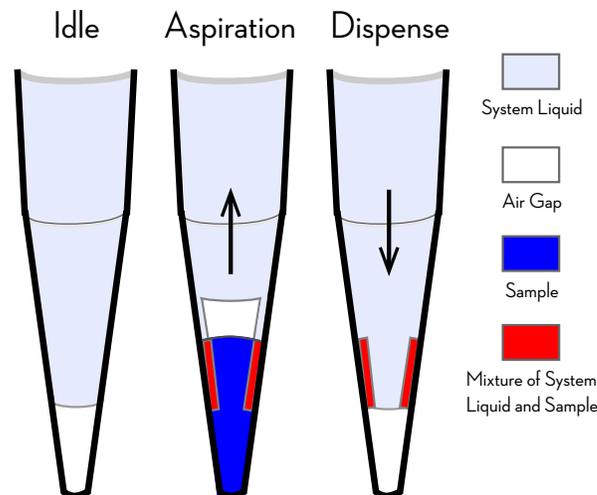


FIG. 4. Fixed tips dilute aspirated samples with system liquid. Automated liquid handlers with fixed tips utilizing liquid-displacement pipetting technology (such as the Tecan Genesis used in the EphB4 assay described here) use a washing cycle in which system liquid (generally water or buffer) purges samples from the tips in between liquid transfer steps. Aspirated sample (blue) can be diluted by the system liquid (light purple) when some residual system liquid remains wetting the inside walls of the tip after purging. This residual system liquid is mixed with the sample as it is aspirated, creating a mixture of system liquid and sample (red) that dilutes the sample that is dispensed. While the use of an air gap (white) reduces the magnitude of this dilution effect, dilution is a known issue in fixed tip liquid-based automated liquid handling technologies, requiring more complex liquid-handling strategies to eliminate it [36]. Diagram adapted from Ref. [36].

515 well concentrations to obtain a K_i and then compute the IC_{50}
 516 from this fit value. We can then use a simple relation be-
 517 tween IC_{50} and K_i to compute the reported assay readout,

$$\text{IC}_{50} = K_i \left(1 + \frac{[S]}{K_m} \right). \quad (17)$$

518 The reported results are not IC_{50} values but $p\text{IC}_{50}$ values,

$$p\text{IC}_{50} = \log_{10} \text{IC}_{50}. \quad (18)$$

519 Note that no complicated manipulation of these equations
 520 is required. As can be seen in the companion IPython note-
 521 book, we can simply use the `curve_fit` function to obtain
 522 a K_i for each bootstrap replicate, and then store the $p\text{IC}_{50}$
 523 obtained from the use of Eqs. 17 and 18 above (Fig. 6, mid-
 524 dle panels). Repeating this process for a variety of true com-
 525 pound affinities allows the imprecision (CV) and bias (RB) to
 526 be quantified as a function of true compound affinity (Fig. 6,
 527 bottom panels).

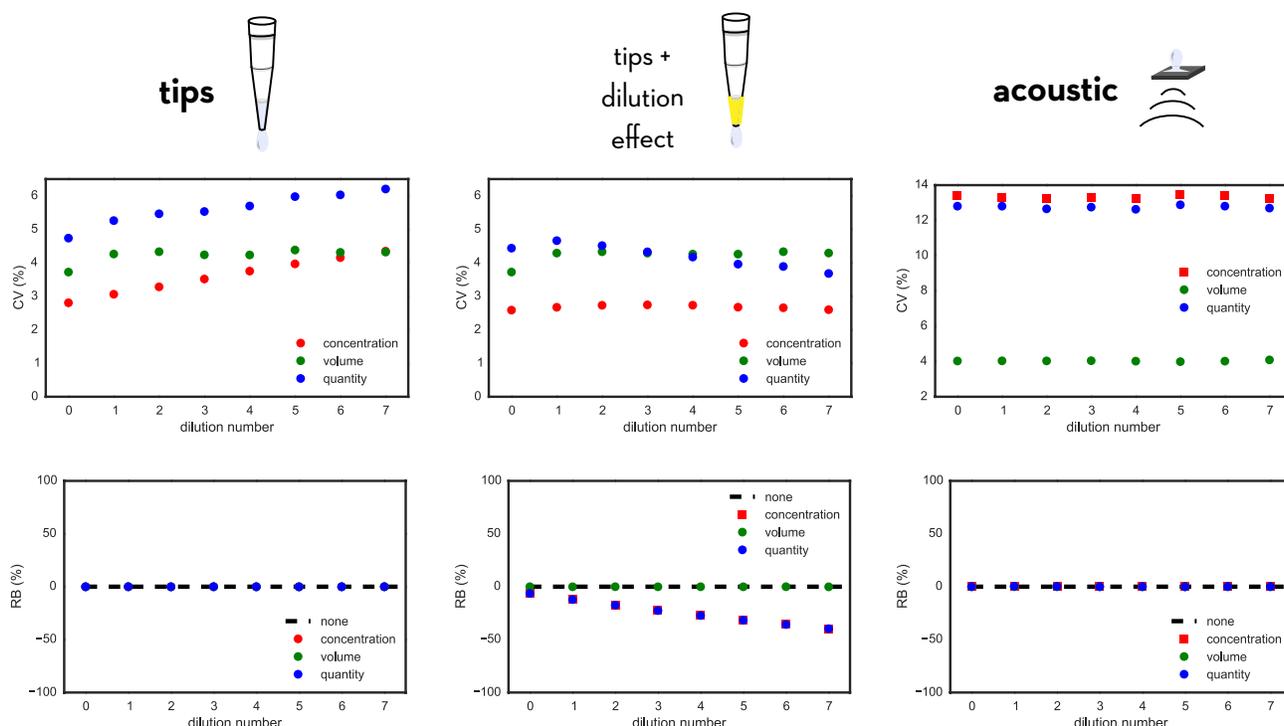


FIG. 5. Modeled accumulation of random and systematic error in creating dilution series with fixed tips and acoustic dispensing. The model predicts how errors in compound concentration, well volume, and compound quantity accumulate for a dilution series prepared using fixed tips neglecting dilution effects (*left*) or including dilution effects (*middle*) compared with an acoustic direct-dispensing process (*right*). Imprecision and inaccuracy parameters appropriate for a Tecan Genesis (fixed tips dispensing) or Labcyte Echo (acoustic dispensing) were used, and assume that the initial compound stocks had negligible concentration error; see text for more details. The top panels show the average relative random error via the coefficient of variation (CV) of concentration, volume, or quantity, while the bottom panels depict the relative bias (RB); both quantities are expressed as a percentage. For tip-based dispensing, relative random concentration error (CV) accumulates with dilution number, while for acoustic dispensing, this is constant over all dilutions. When the dilution effect is included for fixed tips, there is significant bias accumulation over the dilution series. Note that the CV and RB shown for acoustic dispensing are for the final assay solutions, since no intermediate dilution series is created.

III. DISCUSSION

Use of fixed washable tips can cause significant accumulation of bias due to dilution effects

The most striking feature of Fig. 5 is the significant accumulation of bias in the preparation of a dilution series using fixed washable tips (Fig. 5, bottom middle panel). Even for an 8-point dilution series, the relative bias (RB) is almost -50% in the final well of the dilution series. As a result, the measured pIC_{50} values also contain significant bias toward weaker affinities (Fig. 6, bottom middle panel) by about 0.25 \log_{10} units for a large range of compound affinities. At weaker compound affinities, this effect is diminished by virtue of the fact that the first few wells of the dilution series have a much smaller RB (Fig. 5, bottom middle panel).

This cumulative dilution effect becomes more drastic if the dilution series is extended beyond 8 points. If instead a dilution series is created across 16 or 32 wells and assayed, the RB in the final well of the dilution series can reach nearly

-100% (see accompanying IPython notebook for 32-well dilution series). As a result, the bias in the measured pIC_{50} as a function of true pK_i also grows significantly for these larger dilution series (Fig. 8).

Imprecision is greater for direct dispensing with the Echo

As evident from the top panels of Fig. 5, the CV for concentrations in the assay volume for direct acoustic dispensing (right) is significantly higher than the CV of the dilution series prepared with tips (left and middle). This effect manifests itself in the CV of measured pIC_{50} values as a higher imprecision (Fig. 6, bottom panels), where the CV for acoustic dispensing is nearly twice that of tip-based dispensing. Despite the increased CV, there are still numerous advantages to the use of direct dispensing technology: Here, we have ignored a number of difficulties in the creation of a dilution series beyond this dilution effect, including the difficulty of attaining good mixing [30–32], the time required to prepare the serial dilution series (during which evaporation may be

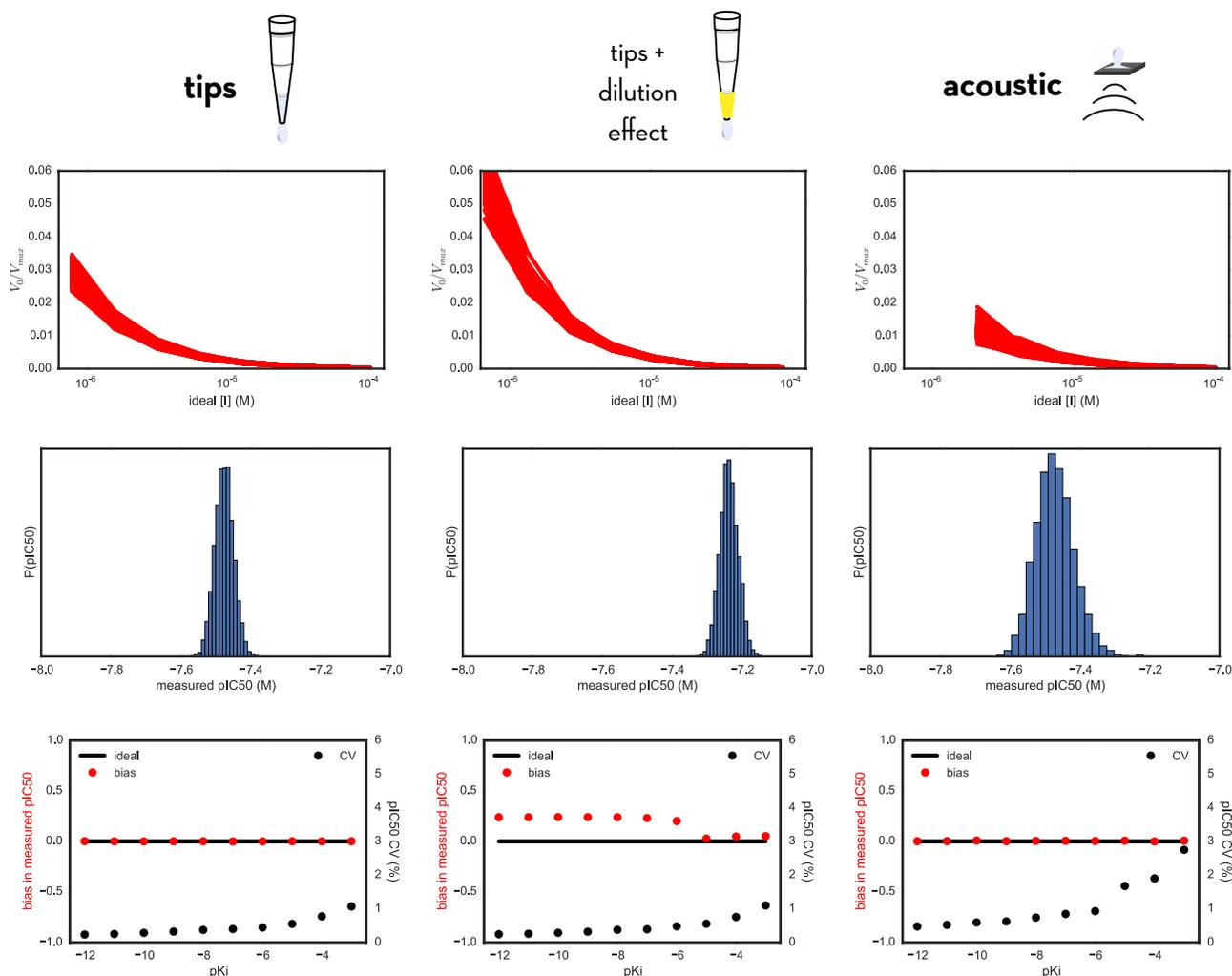


FIG. 6. Comparing modeled errors in measured pIC_{50} values using tip-based or acoustic direct dispensing. *Top row:* Bootstrap simulation of the entire assay yields a distribution of V_0/V_{max} (proportional to measured product accumulation) vs ideal inhibitor concentration $[I]$ curves for many synthetic bootstrap replicates of the assay. Here, the inhibitor is modeled to have a true K_i of 1 nM ($pK_i = -9$). *Middle row:* For the same inhibitor, we obtain a distribution of measured pIC_{50} values from fitting the Using our model we can look at the variance in activity measurements as a function of inhibitor concentration $[I]$ (top), which then directly translates into a distribution of measured pIC_{50} values. *Bottom row:* Scanning across a range of true compound affinities, we can repeat the bootstrap sampling procedure and analyze the distribution of measured pIC_{50} values to obtain estimates of the relative bias (red) and CV (black) for the resulting measured pIC_{50} s. For all methods, the CV increases for weaker affinities; for tip-based dispensing using fixed tips and incorporating the dilution effect, a significant bias is notable.

564 problematic), and a host of other issues.

565 Imprecision is insufficient to explain the discrepancy between 566 assay technologies

567 Fig. 7 depicts the reported assay results [19–21] aug-
568 mented with error bars and corrected for bias using models
569 appropriate for disposable tips (blue circles) or fixed wash-
570 able tips (green circles) that include the dilution effect de-
571 scribed in Fig. 4. Perfect concordance of measured pIC_{50} s

572 between assay technologies would mean all points fall on
573 the black diagonal line. We can see that simply adding the
574 imprecision in a model with fixed tips (Fig. 4, blue circles,
575 horizontal and vertical bars denote 95% confidence inter-
576 vals) is insufficient to explain the departure of the dataset
577 from this diagonal concordance line.

578 When the tip dilution effect for washable tips is incorpo-
579 rated (Fig. 4, green circles), there is a substantial shift toward
580 higher concordance. If, instead of an 8-point dilution series,
581 a 16- or 32-point dilution series was used, this shift toward

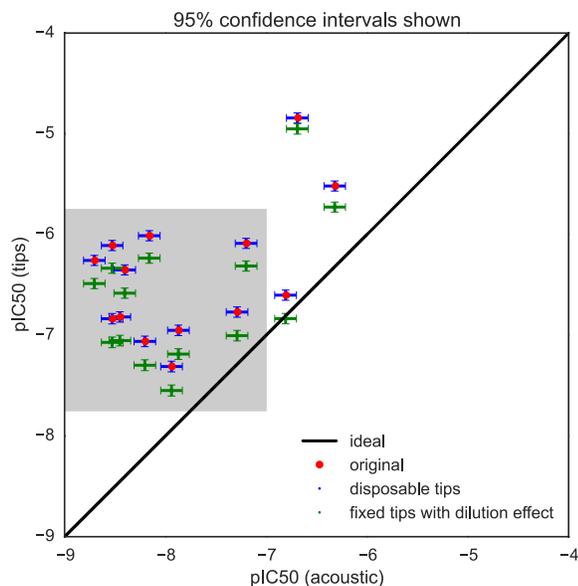


FIG. 7. Adding bias shifts pIC_{50} values closer to equivalence.

The original experimental pIC_{50} values obtained using from fixed tips (red) are plotted against pIC_{50} values from acoustic dispensing, with errors bars representing the uncertainty (shown as 95% confidence intervals) estimated by bootstrapping from our models. Since the bias is relatively sensitive to pIC_{50} value, here it is determined by including both the experimental value and the estimated bias. Incorporating the dilution effect from tip-based dispensing (green) shifts the experimental pIC_{50} values closer to concordance between tip-based and acoustic-based measurements. While this does not entirely explain all discrepancies between the two sets of data, it shifts the root mean square error between the tip-based and acoustic-based dispensing methods from 1.56 to 1.37 pIC_{50} units. The model also demonstrates that (1) the bias induced by the fixed tips explains much of the pIC_{50} shift between the two datasets, and (2) there is still a large degree of variation among the measurements not accounted for by simple imprecision in liquid transfers. This demonstrates the power of building simple error models to improve our understanding of experimental data sets. Grey box indicates portion of graph shown in Fig. 8.

concordance is even larger (Fig. 8). While this effect may explain a substantial component of the divergence between assay technologies, there is no doubt a significant discrepancy remains.

Other contributions to the discrepancy are likely relevant

Serial dilutions are commonly used in the process of determining biologically and clinically relevant values such as inhibition concentrations (IC_{50}) and dissociation constants (K_d). While high-throughput automation methods can improve the reproducibility of these measurements over manual pipetting, even robotic liquid handlers are victim to the accumulation of both random and systematic error. Since the AstraZeneca dataset [20, 21] and the related analysis by

Ekins et al. [19], several studies have posited that acoustic dispensing results in fewer false positives and negatives than tip-based dispensing and that this phenomenon is not isolated to EphB4 receptor inhibitors [38–41].

The power of bootstrapping

We have demonstrated how a simple model based on the bootstrap principle, in which nothing more than the manufacturer-provided imprecision and inaccuracy values and a description of the experimental protocol were used to simulate virtual replicates of the experiment for a variety of simulated compound affinities allowed us to estimate the imprecision and inaccuracy of measured pIC_{50} s. It also identified the difficulty in creating an accurate dilution series using washable fixed tips, with the corresponding dilution effect being a significant contribution to discrepancies in measurements between fixed pipette tips and direct dispensing technologies. In addition to providing some estimate for the random error in measured affinities, the computed bias can even be used to correct for the bias introduced by this process after the fact, though it is always safer to take steps to minimize this bias before the assay is performed.

The EphB4 assay considered here is just one example of a large class of assays involving dilution or direct dispensing of query compounds followed by detection of some readout. The corresponding bootstrap model can be used as a template for other types of experiments relevant to computational modelers.

This approach can be a useful general tool for both experimental and computational chemists to understand common sources of error within assays that use dilution series and how to model and correct for them. Instead of simply relying on intuition or historically successful protocol designs, experimentalists could use bootstrap simulation models during assay planning stages to verify that the proposed assay protocol is capable of appropriately discriminating among the properties of the molecules in question given the expected range of IC_{50} or K_i to be probed, once known errors are accounted for. Since the model is quantitative, adjusting the parameters in the assay protocol could allow the experimentalist to optimize the protocol to make sure the data is appropriate to the question at hand. For example, in our own laboratory, it has informed the decision to use only direct dispensing technologies—in particular the HP D300 [42]—for fluorescent ligand-binding assays that require preparation of a range of compound concentrations.

This modeling approach can also be extremely useful in determining appropriate tests and controls to use to be sure errors and biases are properly taken into account in general. If one is not certain about the primary sources of error in an experiment, one is arguably not certain about the results of the experiment in general. Understanding these errors, and being certain they are accounted for via clear benchmarks in experimental assays could help ensure the reproducibility of assays in the future, which is currently a topic of great

650 interest. Especially with such a wide ranging set of assays
651 that use dilution series, most notably toward the develop-
652 ment of proteins and small molecules to study and treat dis-
653 ease, this is a very important category of experiments to un-
654 derstand how to make more clearly reproducible and inter-
655 pretable.

656 While here we have illustrated the importance of model-
657 ing to the specific case of liquid handling with fixed tips in
658 the context of measuring IC_{50} values for EphB4 inhibitors,
659 there are still large discrepancies that have not been ex-
660 plained, and perhaps variations on this model could explain
661 everything, but perhaps the full explanation comes from
662 parts of the assay yet to be incorporated into this model.
663 As experiments become more automated and analysis be-
664 comes more quantitative, understanding these errors will
665 be increasingly important both for the consumers (com-
666 putational modelers) and producers (experimentalists) of
667 these data.

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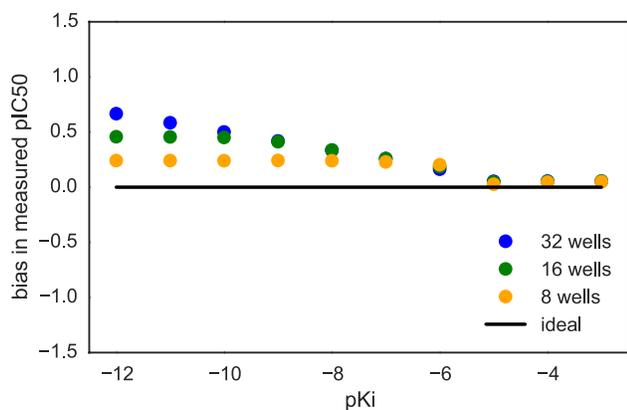
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691 V. CONFLICTS OF INTEREST

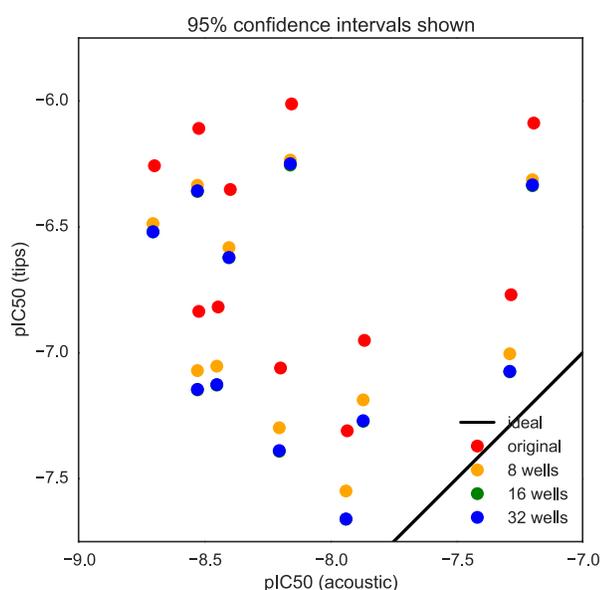
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(a) Bias as a function of wells in dilution series.



(b) Zoom of pIC_{50} data with bias as a function of wells plotted.

FIG. 8. Bias in measured pIC_{50} depends on number of wells in dilution series when using fixed washable tips. (a) If the dilution series is extended beyond 8 wells (yellow) to instead span 16 (green) or 32 (blue) wells, the bias effect in the measured pIC_{50} increases as the cumulative effect of the dilution effect illustrated in Fig. 4 shifts the apparent affinity of the compound. Because the dilution bias is greater for lower compound concentrations, this effect is more drastic for compounds with high affinity. (b) Applying these biases to the pIC_{50} from the sample dataset shows the bias increases with both the 16 (green) and 32 (blue) well dilution series, shifting the points even further toward the line of ideal equivalence of the two types of liquid handling. Note these points overlap exactly.