USDA Center for Veterinary Biologics

Statistics Section

Work Instructions

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Parameterizations of the four-parameter logistic curve in software for estimating relative potency

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1 Introduction

In vitro enzyme immunoassays (eg, ELISAs) are often used as relative potency tests for lot release[1] of biological products [2, 9]. In such assays, a single 96-well microtiter plate may contain serial dilution sequences of one or more test lots and a reference preparation. The measured response in each well is an Optical Density (OD) value. An estimate of Relative Potency (RP) may be obtained by comparing the OD responses of the test lot(s) with those of the reference [14]. This comparison is facilitated by fitting mathematical curves to these responses as a function of dilution (or concentration[2]), provided that the curve fits are checked for suitability. A commonly used mathematical function for such curve fits is the *four-parameter logistic* (4PL) curve [5, 13]. CVBSOP 0102 [16] illustrates such a curve fitting procedure when it is appropriate to constrain the lower asymptote to zero. (A 4PL curve with such a constraint is termed a *three-parameter logistic*, or 3PL, curve.)

CVBSOP 0102, Section 4.3.5, states that the "CVB Statistics Section can provide a technical document providing guidance on how to work with the existing parameterizations of the 4-parameter logistic model, available in certain programs," for implementing the calculations illustrated in CVBSOP 0102 [16]. The following technical document serves that purpose. Readers should have some familiarity with differential calculus to gain the most from reading this document. Readers should also be familiar with basic concepts of relative potency assays, such as those described in Sections 1-2 of Reference [9]. The authors may be contacted if there are comments and questions about this document; please cc both authors. The authors are grateful to other members of the CVB Statistics Section, as well as an external reader, for critical readings of and considerable improvements to the manuscript.

2 Scope

This document focuses exclusively on the 4PL and 3PL curve fits, when used for lot release testing. Classical parallel line analysis is not considered. **Assay validation activities are outside the scope of this document.**

We assume that the assay is designed to consistently exhibit the full dose response, from saturation to extinction, per the appropriate Outline of Production, Special Outline, or Supplemental Assay Method. We further assume that OD data are appropriately corrected for background so that the lower asymptote is zero. Therefore, the 4PL curve *with lower asymptote constrained to be zero*, or 3PL curve, will be used. (There are some assays, such as some competitive ELISAs, that may require a nonzero lower asymptote, even after a background correction is made.) We do not discuss asymmetry [6], nonconstant variance

[1] Other terms for *lot release* include *batch release* and *serial release*. In this document we will use the term "lot" for simplicity.

[2] In this document, we use the term *concentration* to refer to the concentration in the diluent of an analyte with unknown potency. We do *not* mean concentration in the sense of a quantitative measure of potency itself, a topic outside the scope of this document.

[13], or dilution error [8] (these features can be important in statistical modeling, but are usually omitted from lot release testing for simplicity.) Finally we only discuss fitting data from a single plate, and not simultaneously modeling data from multiple plates. (The latter would be necessary, for example, in assay validation studies.)

3 Conventions

In this section, we establish the conventions used by CVB when working with commercial curve-fitting software considered for lot release testing.

3.1 Concentration and dilution

The concentration of an analyte falls as it becomes more dilute. In this subsection, new terminology will be introduced and explained: *concentration units* and *dilution units*. After reading the subsection, users should be able to relate these terms to terminology used at their own laboratories. We begin with an example.

Example. Consider diluting a preparation using a two-fold dilution sequence, starting at undilute. Material from such a dilution sequence is placed in consecutive wells on a microtiter plate, possibly with replication. The concentration of the material at each dilution corresponds to the following values, d_i, multiplied by the concentration of the initial material:[3]

$$d_i = 1,\ \frac{1}{2},\ \frac{1}{4},\ \frac{1}{8},\ \frac{1}{16}, \ldots, \tag{1}$$

or equivalently,

$$d_i = 2^0,\ 2^{-1},\ 2^{-2},\ 2^{-3},\ 2^{-4}, \ldots, \tag{2}$$

which conventional software usually displays in decimal form as

$$d_i = 1,\ 0.5,\ 0.25,\ 0.125,\ 0.0625, \ldots. \tag{3}$$

These values can be considered *relative concentrations* of the preparations, at each dilution, with respect to the starting material's concentration. If we were to take the $\log_2$ transformation of these d_i values, we would obtain

$$\log_2 d_i = 0,\ -1,\ -2,\ -3,\ -4, \ldots \tag{4}$$

Because the dilution sequence is two-fold, the *dilution factor* is $k = 2$.

In the following mathematical development, we have an independent variable x that can be expressed in one of two ways. When x is expressed as the relative concentration of the material, d_i, as in Eqs. 1-3, we say that x has *concentration*

[3] Microbiologists often describe this sequence using the notation 1:1, 1:2, 1:4, 1:8, 1:16, ... This notation is, of course, mathematically incorrect, but when truncated it then corresponds to the reciprocal sequence of Eq. 5.

units. Many software programs also provide the option of entering the dilution sequence in the form of the reciprocals of the relative concentrations:

$$d_i^{-1} = 1,\ 2,\ 4,\ 8,\ 16, \ldots \tag{5}$$

We will call x expressed in this way as having *dilution units*, d_i^{-1}.

Our notation. More generally, let $z_i = z_0 d_i = z_0 k^{u_i}$, where

- $z_0 > 0$ is the concentration of the starting material,
- z_i is the concentration of the ith dilution of the preparation ($0 < z_i \leq z_0$),
- $k > 1$ is the dilution factor,
- $d_i = k^{u_i}$ is the relative concentration of the ith dilution of the preparation ($0 < d_i \leq 1$), and
- $u_i = \log_k(d_i)$ are non-positive integers.

We define *concentration units* as the d_i's, and *dilution units* as their reciprocals, d_i^{-1}. In the sequel, the independent variable x_i may be expressed in either concentration units, $x_i = d_i$, or dilution units, $x_i = d_i^{-1}$. We will present results for both conventions in this document. (Internally, CVB uses dilution units for analysis of data for lot release.)

In the above example, $k = 2$, the d_i values are given in Eqs. 1-3, which represent concentration units; the u_i values are given by Eq. 4; and dilution units are represented by Eq. 5.

Note that Refs. [9, 14] use the term "dilution" for what we have called "relative concentration" and "concentration units," or d_i. Hence their use of "$-\log$ Dilution" as the horizontal axis of their graphs corresponds to using what we call "dilution units," or $\log d_i^{-1} = -\log d_i$. We have chosen our terminology based on common laboratory practices and the word usage of typical commercial software programs.

Though both dilution units and concentration units are described in this document, for completeness, usage of dilution units is preferred for electronic data submissions to CVB, since rounding errors are less common when using such units.

3.2 Assay response type

For purposes of discussion, we refer to assays where the OD response decreases with dilution (therefore increases with concentration) as *immunometric* [18]. (Many indirect, sandwich ELISAs are of this type.) Assays where the OD response increases with dilution (therefore decreases with concentration) are called *competitive*. These terms are not absolute, but we will use them to simplify the discussion.

For either immunometric or competitive ELISAs, the response curve for a potent lot will appear to the right of the reference when plotting ODs against

a logarithm of x in dilution units, and a subpotent lot will appear to the left of the reference. This will reverse when plotting ODs against a logarithm of x in concentration units.

3.3 4PL curve

Formula. There are a number of ways to parameterize the 4PL model [12]. One parameterization, used in several commercial software systems [1, 10] as well as the *U.S. Pharmacopeia* (USP) [17], is shown in Eq. 6, where y is the observed OD, x may be in either concentration *or* dilution units ($x = d_i$ or $x = d_i^{-1}$), and A, B, C, and D are the curve parameters.

$$y = D + \frac{A - D}{1 + (\frac{x}{C})^B}. \tag{6}$$

The values of A, B, C, and D are estimated using the data, and are not known *a priori*. The parameters A and D correspond to the *asymptotes*; B is called the *scale factor*; and C is the *location parameter*, the position (in units of x) of the inflection point, at which the curve has a response halfway between the upper and lower asymptotes.[4]

In the parameterization of Eq. 6, x is expected in either dilution units or concentration units, *not* in logarithms thereof. This may be a point of confusion for several reasons. We usually graph the data using a logarithm of x as the horizontal axis (see Subsection 3.4). Moreover, other parameterizations of the 4PL curve, not discussed here, may require $\log x$ to be used in their formulas [12]. This is the case, for example, when using the parameterizations used in the `SSfpl` and `SSlogis` functions in the `nlme` package for the S language [11] and for the Logistic 4P and 3P models in JMP [3]. (Indeed, in classical parallel line analysis, $\log x$ is the independent variable used for linear regression.) Nonetheless, *do not submit a logarithm of x to Eq. 6.* The relationship of Eq. 6 to other parameterizations is discussed further in Sec. 6.

Parameter interpretation. We interpret A and D as asymptotes, but to this point it has not been stated which one is the upper and which is the lower asymptote, corresponding to saturation (high ODs) and extinction (low ODs), respectively. The assignment of roles to A and D depends on whether the assay is immunometric or competitive, and whether x is expressed in concentration or dilution units. All four combinations of possibilities are discussed in Section 4. However, once the assay format and units of x are specified, the roles of A and D are still not yet fixed. In fact, their roles may be interchanged by flipping the sign of B [5]. In other words, a given data set may be fit with *two different* 4PL curves, one in which B is positive and one in which B is negative; the fitted values for A and D are reversed in these two curve fits, and only C has the

[4] The USP calls B the *steepness* and C the *effective concentration 50%* (or EC_{50}, sometimes also referred to as the ED_{50} or IC_{50}). The latter assumes symmetry, since the inflection point corresponds with the EC_{50} only with symmetrical response curves. The B parameter is also called *Hill's slope* due to an analogy with Hill's equation [7], although B is *not* a slope parameter per se, as can be determined by dimensional analysis.

same value in both. To proceed, we will have to arbitrarily adopt one of these possible solutions as the conventional one.

In this document we adopt the convention that the sign of B matches the sign of the slope of any tangent line to the curve, when ODs are plotted against a logarithm of x. Under this assumption, A becomes the lower asymptote (at extinction) and D becomes the upper asymptote (at saturation). This is an arbitrary choice, but one consistent with internal CVB practice.[5]

Three-parameter logistic. Finally, as stated above we will assume that the OD values, y, are suitably corrected so that the lower asymptote may be assumed to be zero. Then when fitting a 4PL curve the lower asymptote may be constrained to be zero, leaving three free parameters. The resulting mathematical curve is known as a *three-parameter logistic* (3PL) curve. Therefore:

A 3PL curve is simply a 4PL with lower asymptote constrained to be zero.

Summary. Table 1 summarizes the above findings. The "Potency increasing" column indicates that, for instance, curves for successively increasing potency lots would occur at successive locations toward the right, when plotting ODs against the logarithm of dilution units. In all four cases, A is interpreted as the lower asymptote, B is interpreted as the scale factor, C is interpreted as the inflection point, and D is interpreted as the upper asymptote.

Response type	Units of x	Constraint	Sign of B	Potency increases
immunometric	dilution	$A = 0$	$-$	to the right
competitive	dilution	$A = 0$	$+$	to the right
immunometric	concentration	$A = 0$	$+$	to the left
competitive	concentration	$A = 0$	$-$	to the left

Table 1: Summary of conventions for 4PL constraints and sign of B for enforcing a 3PL fit.

3.4 Graphing

The 4PL curve described above has a sigmoid shape when y is plotted against a logarithm of x. Thus, by convention we will always use a logarithm of x as the horizontal axis in our graphs. Note that

$$-\log_k d_i = \log_k d_i^{-1}, \tag{7}$$

[5]Some software programs do not follow this convention. Guidance for usage in such cases may be found in Section 5.

or that selecting concentration units or dilution units for the horizontal axis results in the opposite ordering of points on that axis.

In the examples of Sec. 4, we will use $\log_k(d_i^{-1}/d_1^{-1})$ for dilution units, and $\log_k(d_i/d_1)$ for concentration units, as the horizontal axis. Dividing by the starting dilution or concentration will result in integer-valued tick positions on the horizontal axis for the data points, since there may be an initial dilution that is not k-fold.

3.5 Suitability Criteria

Once values for the parameters B, C, and D are estimated from individual fits for each reference and test lot in the assay, it is possible to make comparisons to evaluate for suitability, which here means sufficiently adequate parallelism. Usually this involves a comparison of the upper asymptote and scale factor estimates for the reference and test lot. The Outline of Production or Special Outline should specify the criteria for suitability; these should be justified by data when proposed to CVB. A recommended set of criteria that may be used as a default are:

$$B_{lo} \leq \frac{B_{\text{lot}}}{B_{\text{reference}}} \leq B_{hi}, \tag{8}$$

$$D_{lo} \leq \frac{D_{\text{lot}}}{D_{\text{reference}}} \leq D_{hi}, \tag{9}$$

where B_{lo}, B_{hi}, D_{lo}, and D_{hi} are set by the firm for a particular assay. For instance, these choices could be based on data from validation study plates that are known to satisfy the parallelism criteria of Veterinary Services Memorandum 800.112, Appendix III, Sec. 2.2.5 [15]. If desired, default values could be adopted and shown to be (at least loosely) consistent with the data for a particular assay.

Both Eqs. 8 and 9 must be satisfied before relative potency estimates may be calculated per Section 3.6. *If the comparisons for a lot release test do not pass parallelism, it will not be appropriate to calculate an RP estimate for the data on the plate.*

3.6 Procedure for estimating relative potency

As outlined in CVBSOP 0102 [16], OD data from each test lot and reference must first be fit to individual 3PL curves and evaluated for suitability (Subsection 3.5). If suitability criteria are satisfied, a parallel 3PL model[6] should be fit for each test lot and reference *together*. This latter model is used to estimate the relative potency from the fitted C parameters.

Multiple test lot curves may be compared to a single reference curve on the same plate; if this is done, separate parallel models for each test lot paired with the reference should be fit.

[6] That is, constraining the upper asymptote and scale factor to be the same for both curves.

Relative potency estimates are simply the horizontal distance between the parallel curves being compared, as shown in Figure 1. The relative potency concept is meaningful if the asymptotes and scale factors for both curves are the same; then the curves may differ only by a horizontal shift.

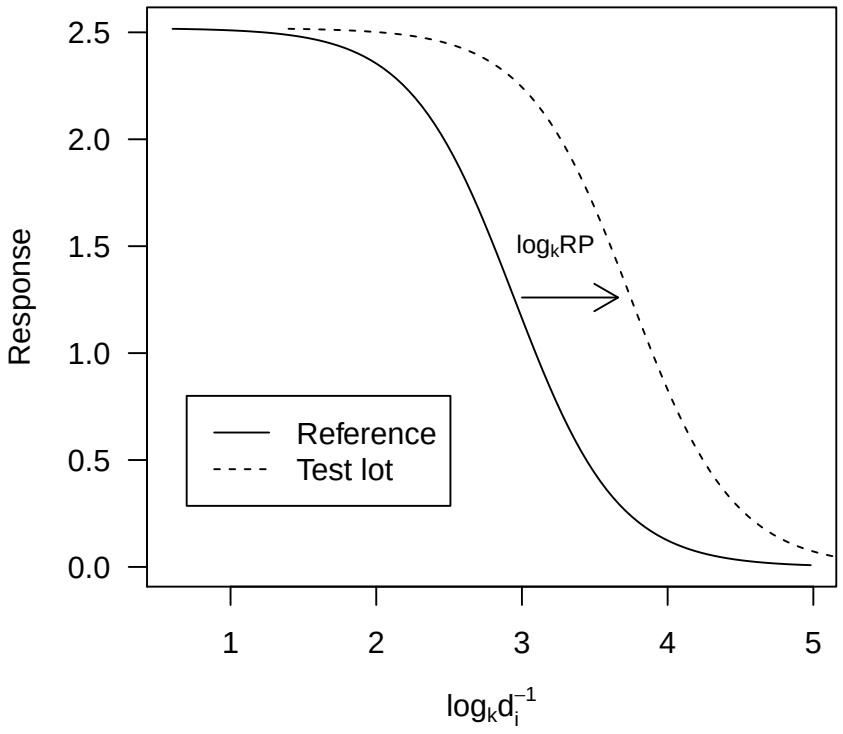

Figure 1: The RP estimate is a measure of the horizontal distance between two curves that have the same upper and lower asymptotes (A and D parameters) and have the same scale factor (B parameter).

As suggested by Fig. 1, when x is in dilution units, the relative potency, RP, is given by

$$\log_k RP = \log_k C_{\text{test lot}} - \log_k C_{\text{reference}} = \log_k \left(\frac{C_{\text{test lot}}}{C_{\text{reference}}} \right), \qquad (10)$$

where k is the dilution factor. Thus

$$RP = \frac{C_{\text{test lot}}}{C_{\text{reference}}}. \tag{11}$$

When x is in concentration units, the right hand side must be replaced by its reciprocal.

4 Examples

This section will provide examples of parameter constraints for each of the four cases that are the unique combination of choice of x units and assay response type.

Case 1: Immunometric ELISA, dilution units

In the case of an immunometric ELISA, where OD decreases with increasing dilution (i.e., decreasing concentration), when using dilution units for x, the parameter constraint is $A = 0$, corresponding to the lower asymptote. In this case, B will attain a negative value and D will be the upper asymptote.

For the example in Figure 2, we see that this choice of parameter constraints leads to a curve fit where the OD values are high at low dilutions (i.e., high concentration), approach zero as dilutions increase to infinity (i.e., low concentration), and any tangent line to the curve has a negative slope (when using a logarithm of x as the horizontal axis).

OD	x
2.52	4
2.51	10
2.49	25
2.42	62
2.25	156
1.84	391
1.18	977
0.56	2441
0.22	6104
0.08	15259
0.03	38147
0.01	95367

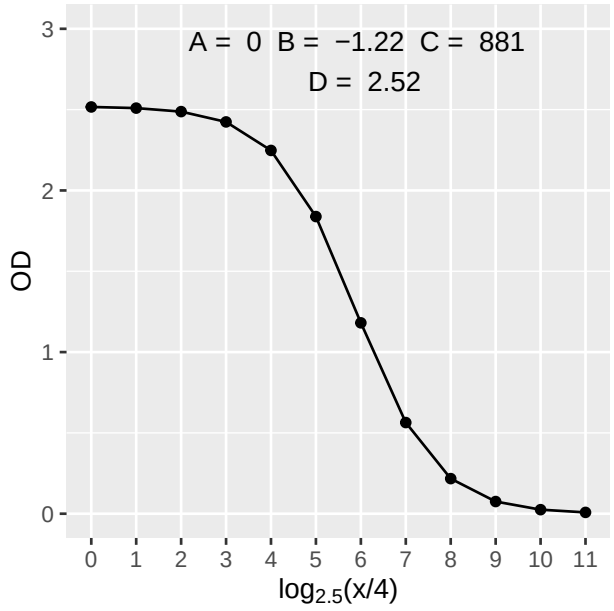

Figure 2: Case 1. (Left) Data used for curve fit, with x rounded to the nearest integer for display. (Right) 3PL fit for case of immunometric ELISA with dilution units, $x = d_i^{-1}$.

Case 2: Competitive ELISA, dilution units

In the case of a competitive ELISA, where OD increases with increasing dilution, when using dilution units for x, the parameter constraint is $A = 0$, corresponding to the lower asymptote. In this case, B will attain a positive value and D will be the upper asymptote. (Please bear in mind that for competitive ELISAs, constraining the lower asymptote to zero may not always be appropriate.)

For the example in Figure 3, we see that this choice of parameter constraints leads to a curve fit where the OD values approach zero at low dilutions (i.e. high concentration), approach an asymptotic maximum as dilutions increase to infinity (i.e. low concentration), and any tangent line to the curve has a positive slope (when using a logarithm of x as the horizontal axis).

OD	x
0.00	4
0.01	10
0.03	25
0.10	62
0.27	156
0.68	391
1.34	977
1.96	2441
2.30	6104
2.44	15259
2.49	38147
2.51	95367

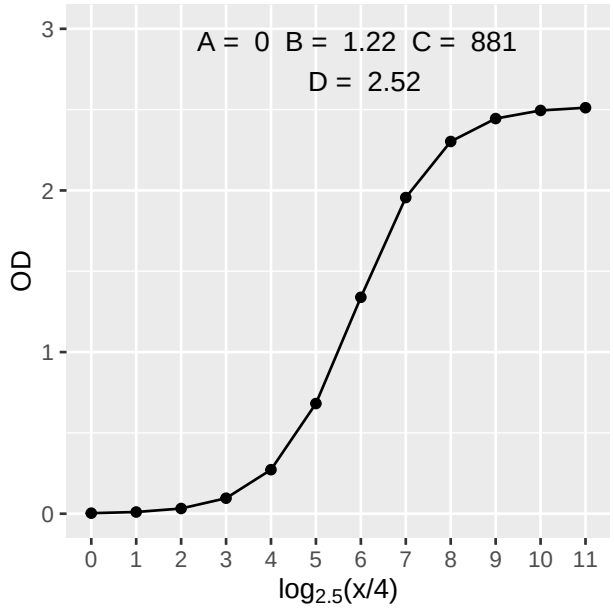

Figure 3: Case 2. (Left) Data used for curve fit, with x rounded to the nearest integer for display. (Right) 3PL fit for case of competitive ELISA with dilution units, $x = d_i^{-1}$.

Case 3: Immunometric ELISA, concentration units

In the case of an immunometric ELISA, where OD increases with increasing concentration (i.e., decreasing dilution), when using concentration units for x, the parameter constraint is $A = 0$, corresponding to the lower asymptote. In this case, B attains a positive value and D is the upper asymptote.

For the example in Figure 4, we see that this choice of parameter constraints leads to a curve fit where the OD values approach zero at low concentration (i.e. high dilution), approach an asymptotic maximum as log concentration increases to zero (i.e. low dilution), and any tangent line to the curve has a positive slope (when using a logarithm of x as the horizontal axis).

OD	x
2.52	2.50E-01
2.51	1.00E-01
2.49	4.00E-02
2.42	1.60E-02
2.25	6.40E-03
1.84	2.56E-03
1.18	1.02E-03
0.56	4.10E-04
0.22	1.64E-04
0.08	6.55E-05
0.03	2.62E-05
0.01	1.05E-05

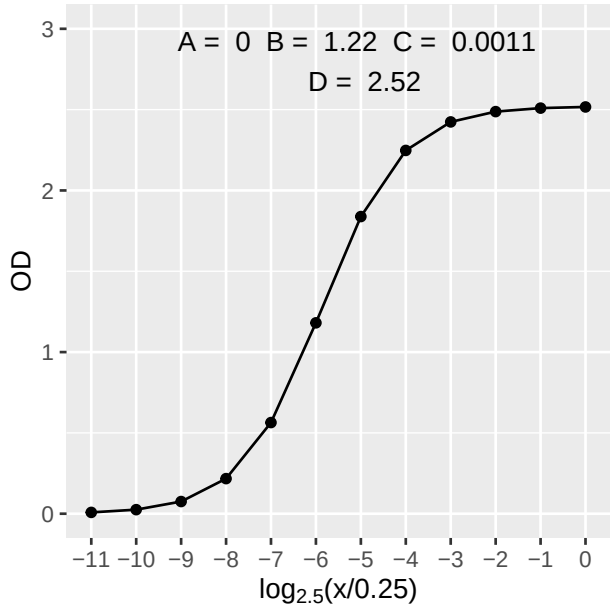

Figure 4: Case 3. (Left) Data used for curve fit, rounded to 2 decimal places for display. (Right) 3PL fit for case of immunometric ELISA with concentration units, $x = d_i$.

Case 4: Competitive ELISA, concentration units

In the case of an competitive ELISA, where OD decreases with increasing concentration (i.e., decreasing dilution), when using concentration units for x, the parameter constraint is $A = 0$, corresponding to the lower asymptote. In this case, B will attain a negative value and D will be the upper asymptote. (Please bear in mind that for competitive ELISAs, constraining the lower asymptote to zero may not always be appropriate.)

For the example in Figure 5, we see that this choice of parameter constraints leads to a curve fit where the OD values approach the asymptotic maximum at low concentration (i.e., high dilution), approach zero as log concentration increases to zero (i.e., low dilution), and any tangent line to the curve has a negative slope (when using a logarithm of x as the horizontal axis).

5 Software Implementation

While the methods described above are independent of software choice, it important to note that *not all available plate reader software allows users to make the parameter selections described above.* It is up to each firm to evaluate that their software choice is fit for purpose. The discussion below reflects CVB's

OD	x
0.00	2.50E-01
0.01	1.00E-01
0.03	4.00E-02
0.10	1.60E-02
0.27	6.40E-03
0.68	2.56E-03
1.34	1.02E-03
1.96	4.10E-04
2.30	1.64E-04
2.44	6.55E-05
2.49	2.62E-05
2.51	1.05E-05

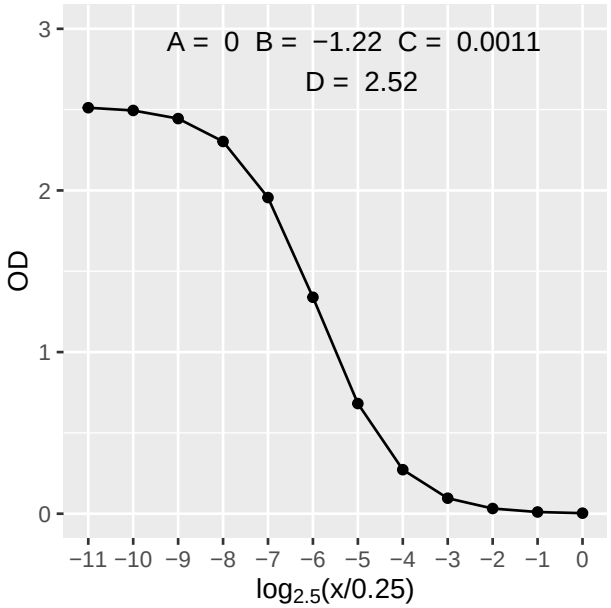

Figure 5: Case 4. (Left) Data used for curve fit, rounded to 2 decimal places for display. (Right) 3PL fit for case of competitive ELISA with concentration units, $x = d_i$.

experiences when using commercial software.

This discussion is not exhaustive. Firms are encouraged to investigate whether software best suited for organizational needs are consistent with calculations described above, or their equivalent. (For example, in some of the other commercial software products we have examined, we have not been able to determine how to constrain the lower asymptote for 4PL curves.)

5.1 SoftMax Pro 6.3 [10]

Molecular Devices' Softmax Pro (SMP) requires only constraining the lower asymptote to zero, and will automatically fit the correct sign for the scale factor, B. Setting parameter A to 0 using the curve fit dialog settings is sufficient to impose the constraints described above. Determining parameters for the purpose of assessing whether suitability criteria have been passed must be done using *independent* curve fits; these options are also found on the curve fit dialog settings (figure 6, left).[7] Calculation of the RP estimate may be evaluated from the graph of comparison curves fit using a 4-Parameter *global* curve fit[8]

[7]The SMP terminology of "Independent Fits" corresponds to what we have called individual or separate fits in this and other documentation.

[8]In SMP parlance, a "Global Fit (PLA)" corresponds to what we have called a parallel model, which constrains the upper asymptote and scale factor to be the same for both

with $A = 0$ (figure 6, right). When using dilution units for x, the RP estimate may be obtained using the function $\frac{1}{\texttt{RelPotPLA}}$ applied to the global fit's comparison graph. (This reciprocation is required because SMP's default behavior expects x expressed in concentration. If using concentration units for x, then the reciprocation should not be done.)

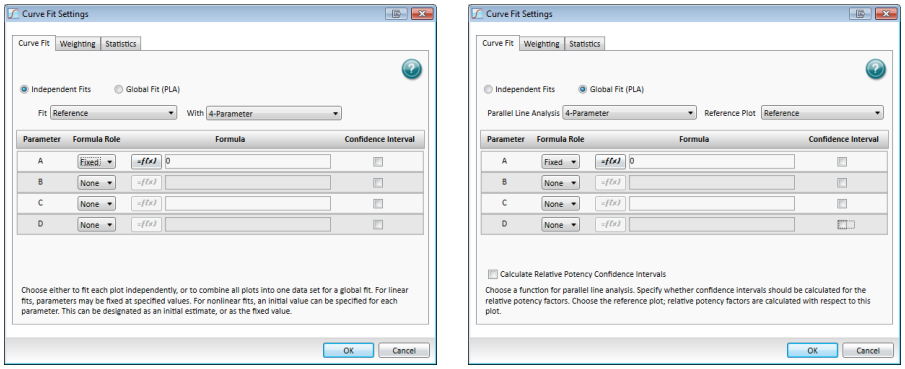

Figure 6: (Left) SMP curve fit dialog options for determining parameters to be used for evaluating suitability. (Right) SMP curve fit dialog options for evaluating RP estimate.

Please note that when we developed the SoftMax Pro protocol "Estimate Relative Potency for Serial Release PLA" posted at `www.softmaxpro.org`, the terminology introduced in subsection 3.1 had not yet been established. Thus in the protocol file the term "dilution" is used for what this document calls "dilution units."

5.2 Gen5 2.01 [1]

Biotek's Gen5 arbitrarily imposes a constraint of $B > 0$ for all curve fits. This results in a different convention than the one discussed above. Here, A corresponds to the left asymptote (at low values of $\log x$), and D corresponds to the right asymptote (at high values of $\log x$), *regardless* of whether dilution or concentration units are used.[9] The sign of B no longer indicates the sign of the slope of the pseudo-linear region; it is simply always positive.

Because Gen5 uses a different convention than the one discussed in Section 3, use Table 5.2 to identify which constraints apply to the testing scenario. The constraint listed corresponds to setting the lower asymptote to zero. For suitability criteria, Eq. 8 may continue to be used, but Eq. 9 is used only in the

preparations.

[9] Note that "left asymptote" means the one approached by the curve on the left (as $\log x$ approaches $-\infty$), regardless of whether it is the upper or lower asymptote, and similarly for the "right asymptote" (as $\log x$ approaches $+\infty$). This means that the parameters A and D do not always have the same immunochemical interpretation.

cases where the table states $A = 0$; otherwise, an analogous constraint must be imposed on the ratio of A estimates.

Response type	Units of x	Constraint in Gen5	Sign of B	Potency increases
immunometric	dilution	$D = 0$	+	to the right
competitive	dilution	$A = 0$	+	to the right
immunometric	concentration	$A = 0$	+	to the left
competitive	concentration	$D = 0$	+	to the left

Table 2: Summary of conventions for 4PL constraints and sign of B for enforcing a 3PL fit in Biotek Gen5 software.

Figure 7 shows the settings for selecting and constraining the 4PL curve fit using Gen5. To calculate the estimated RP, select for "Parallelism Analysis" in the curve analysis (Fig. 8). When this option is selected, both the individual curve fits and the parallel (global) curve fits are generated automatically. When using dilution units for x, the estimated RP is the *reciprocal* of the calculated output. This reciprocation should not be done when using concentration units for x.

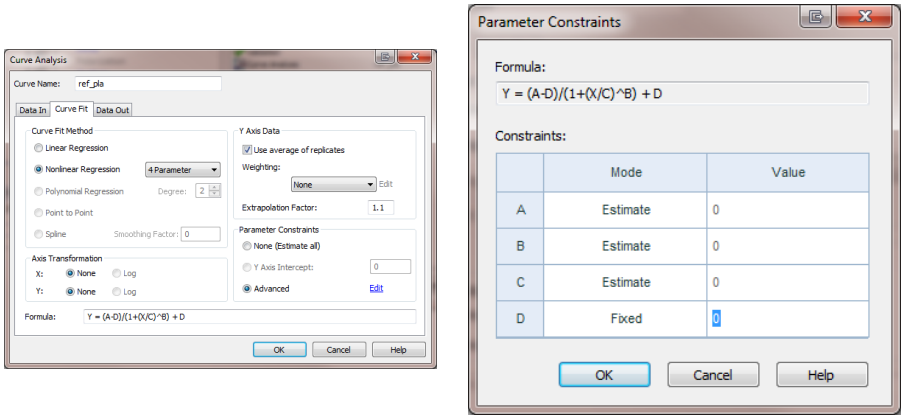

Figure 7: (Left) Gen5 dialog for curve analysis. Under "Curve Fit Method," select "Nonlinear Regression" and specify "4 parameter." Then under "Parameter Constraints," choose "Advanced" and click on "Edit" to bring up the "Parameter Constraints" dialog shown on the right. (Right) Constrain the parameter per testing condition listed in Table 5.2 in the parameter constraints dialog box.

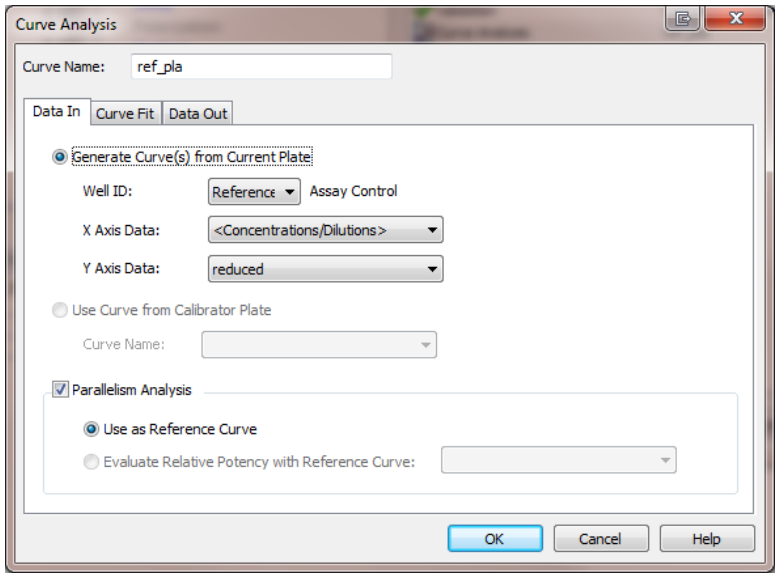

Figure 8: In Gen5, select for calculation of RP estimate by activating the Parallelism Analysis option on the curve analysis dialog box. The reported RP estimate is the *reciprocal* of this value, when x is in dilution units.

Please note that at the time the Gen5 experiment files posted at `http://www.biotek.com/resources/articles/estimating-relative-potency-sample-file.html` were developed, the terminology introduced in subsection 3.1 had not yet been established. Thus in the Gen5 experiment files and technical document [1] the term "dilution" is used for what this document calls "dilution units," and "concentration" is used for what this document calls "concentration units."

6 An alternate parameterization of the 4PL curve

In subsection 3.3 we discussed one parameterization of the 4PL curve, Eq. 6, and mentioned other parameterizations that may require inputting a logarithm of x instead of just x. Here we discuss one such parameterization, given by Pinheiro & Bates [11]:

$$y = \phi_1 + \frac{\phi_2 - \phi_1}{1 + \exp\{(\phi_3 - \log_k x)/\phi_4\}}, \tag{12}$$

where ϕ_i represent the four parameters. (For simplicity we are omitting the division by starting dilution in the expression $\log_k x$; we address this point at the end of the section.) The *European Pharmacopoeia* [4] discusses both parameterizations, Eqs. 6 and 12. Our goal in this section is to relate the parameters of Eq. 12 to those of Eq. 6.

First we immediately identify $\phi_1 = D$ and $\phi_2 = A$. It remains to equate the expressions in the denominators, leaving us with

$$\left(\frac{x}{C}\right)^B = \exp\left(\frac{\phi_3 - \log_k x}{\phi_4}\right). \tag{13}$$

The left-hand side of Eq. 13 may be rewritten as follows.

$$\begin{aligned} \left(\frac{x}{C}\right)^B &= \left(e^{\log x}\right)^B \left(\frac{1}{C}\right)^B & (14)\\ &= \left(e^{\log_k x}\right)^{B/\log_k e} \left(\frac{1}{C^{\log_k e}}\right)^{B/\log_k e}. & (15) \end{aligned}$$

The right-hand side of Eq. 13 may be rewritten as follows.

$$\begin{aligned} \exp\left(\frac{\phi_3 - \log_k x}{\phi_4}\right) &= \left(e^{\phi_3} e^{-\log_k x}\right)^{\frac{1}{\phi_4}} & (16)\\ &= \left(e^{-\phi_3}\right)^{-\frac{1}{\phi_4}} \left(e^{\log_k x}\right)^{-\frac{1}{\phi_4}}. & (17) \end{aligned}$$

A solution to setting these expressions equal is given by

$$\begin{aligned} \phi_4 &= -\frac{\log_k e}{B} = -\frac{1}{B \log k} & (18)\\ \phi_3 &= (\log_k e)(\log C) = \log_k C. & (19) \end{aligned}$$

The parameters B and ϕ_4 correspond to the scale factor, and differ only by a constant factor. Thus, for checks of parallelism per Sec. 3.5, the ratio of B parameters is mathematically equivalent to the (reciprocal of the) ratio of ϕ_4 parameters, as long as B_{lo} and B_{hi} are chosen to be reciprocals of each other.

Now, recall that for simplicity we omitted the division by the starting dilution when considering $\log_k x$. What would be the affect of dividing by the starting dilution? This would only shift the location parameter, leaving other parameters unchanged. Consider Eq.12 but with x replaced with $w = x/x_1$. The expression $\phi_3 - \log_k(w) = \phi + 3 + \log_k(x_1) - \log_k(x)$, and we may simply defined $\phi_3* = \phi_3 + \log_k(x_1)$. No other parts of expression 12 are affected.

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