Analysis of multiplex immunoassay data subject to left and right censoring

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1 Introduction

The four- and five-parameter logistic models are examples of nonlinear dose-response curves commonly used for calibration and dose-estimation in multiplex immunoassays (e.g. Bio-Rad's Bioplex© system). Let y be the response (e.g. flourescence) at dose x. Then the form of the five-parameter logistic (5PL) model is

$$y = f(x, \boldsymbol{\beta}) \quad = \quad d + \frac{a-d}{\left(1 + \left(\frac{x}{c}\right)^b\right)^g}, \tag{1}$$

where $\boldsymbol{\beta} = (a, b, c, d, g)'$. The four-parameter logistic model (4PL) is equivalent to the 5PL with $g = 1$.

The dose-response curve is fit based on replicates of known standards. In my experience, the number of replicates is typically two and the number of standards is eight to ten. The standard concentrations are typically manufacturer-recommended serial dilutions of the analyte of interest. Figure 1 shows the fitted standard curve based on two replicates for each of eight standard concentrations of IL7. The shape of the curve is sigmoidal when the response is graphed as a function of log-dose. The standard curve is used to estimate analyte concentrations for unknowns (e.g. patient urine or serum samples) based on the flourescent signal from the unknown. The procedure to estimate concentration from flourescence amounts to extending a horizontal line from the observed flourescence and dropping a vertical line at the point of intersection with the standard curve.

For immunoassays, the dose-response curve is monotonically increasing. Interpretations of the 4- and 5PL model parameters are described in Table 1. See Gottschalk and Dunn[1] for additional details.

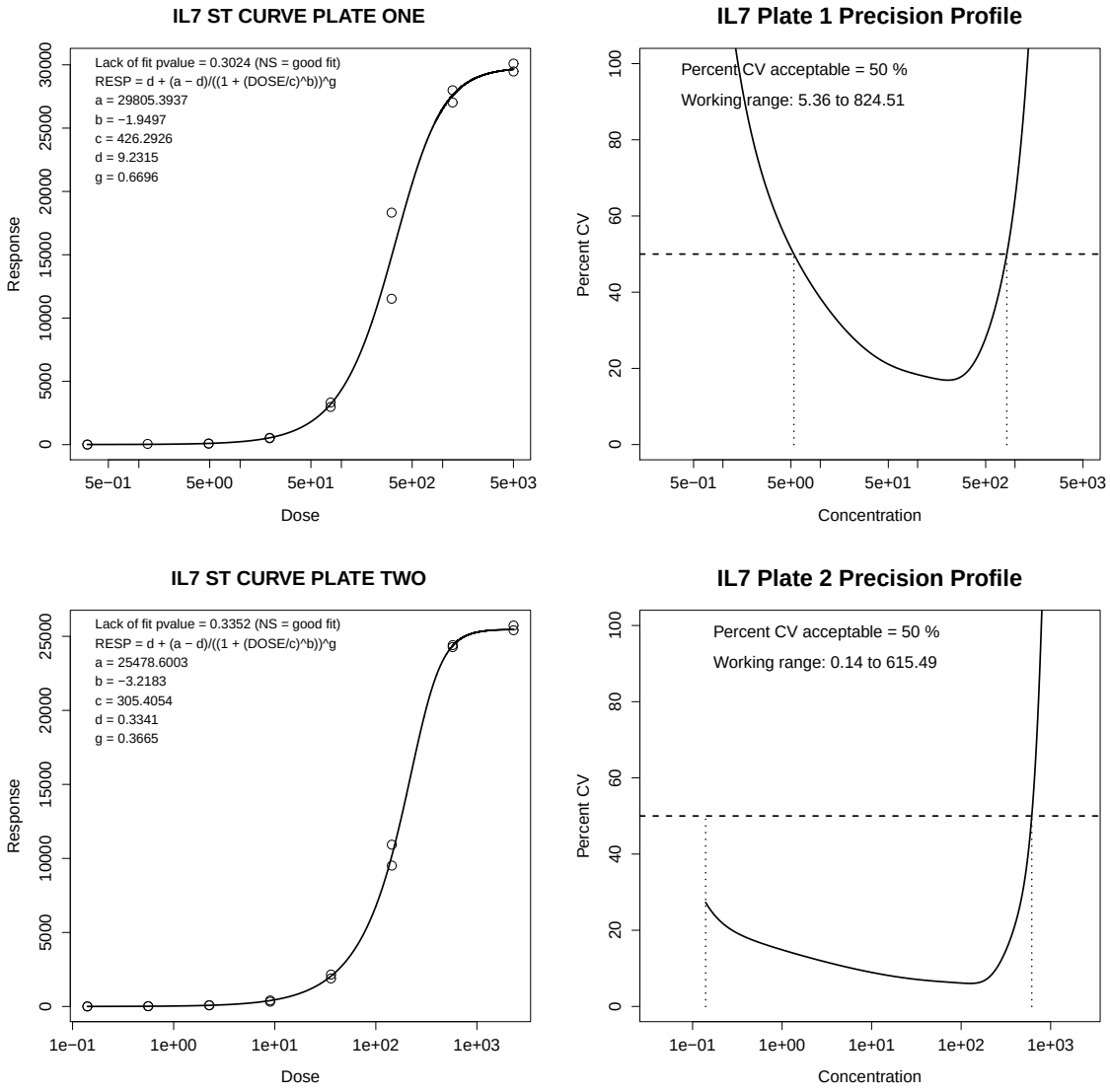

Figure 1: Five-parameter logistic dose-response curves and corresponding precision profiles for two different 96-well plates measuring IL7 .

2 The general dose-response model

The general dose-response model is

$$y_{ij} \quad = \quad f(x_i, \boldsymbol{\beta}) + \sigma g(\mu_i, \boldsymbol{\theta})\varepsilon_{ij}. \tag{2}$$

For our purposes, we assume

- y_{ij} is the jth replicate response at the ith dose, $j = 1, \ldots, m_i$ and $i = 1, \ldots, N$ where m_i is the number of replicates for the ith dose and N is the number of doses.
- x_i is the ith dose
- $\boldsymbol{\beta} = (a, b, c, d, g)'$
- $f(x_i, \boldsymbol{\beta}) = \mu_i$ and is the mean response at dose x_i. Here $f(x_i, \boldsymbol{\beta})$ is the function described in Equation 1.
- σ is a an unknown constant
- $\boldsymbol{\theta}$ is a vector of parameters for the variance function described by g
- $g(\mu_i, \boldsymbol{\theta})$ is a variance function that depends on both the mean response at dose i and parameter vector $\boldsymbol{\theta}$
- $\varepsilon \sim \text{Normal}(0, 1)$

Model 2 accommodates non-constant variance with

$$\text{Var}(y_{ij}) \quad = \quad \sigma^2 g(\mu_i, \boldsymbol{\theta})^2. \tag{3}$$

As noted by Gottschalk and Dunn[1], the variance at the high-response end of the curve is often three- to four-times larger than at the low end, due to: 1) signal detectors producing noise with a standard deviation proportional to the magnitude of the response; and 2) the kinetics associated with antibody binding are non-linear. For immunoassays, the 'power

of the mean' variance structure has been shown useful for 4- and 5PL dose-response models[1,2], and is modeled as

$$g(\mu_i, \theta) = \mu_i^{\theta}. \tag{4}$$

Here, θ is constrained to be positive.

3 Fitting the standard curve

Typically, the standard curve is fit to 'background corrected' response measures. This is achieved by measuring (usually from replicate samples) the flourescent signal from matrix-only samples (i.e. analyte concentration equals 0), and subtracting the average of these readings from all response measures. The standard curve is fit to the background-corrected data using the method of generalized least squares with variance function estimation described in detail by Carroll and Ruppert[3], Davidian and Haaland[4], and O'Connell, Belanger and Haaland[2]. Generalized least squares refers to standard weighted least squares with estimated (rather than known) weights. Here, appropriate weights are given by

$$\begin{aligned} w_i &= 1/g^2(\mu_i, \theta) \\ &= 1/\mu_i^{2\theta}. \end{aligned} \tag{5}$$

For completeness, we describe model fitting for the general model with mean function given by Equation 1 and variance function given by Equation 4.

1. Davidian and Haaland[4] suggest $\hat{\boldsymbol{\beta}}^{(0)}$ be obtained by minimizing $\sum_{i=1}^{N}\sum_{j=1}^{m_i}(y_{ij} - \mu_i)^2$. Note $\hat{\boldsymbol{\beta}}^{(0)}$ is the least-squares estimate of $\boldsymbol{\beta}$. Alternatively, good starting values may already be available for $\boldsymbol{\beta}$ from the fit obtained from the commercial software. In practice, I have found generating $\hat{\boldsymbol{\beta}}^{(0)}$ from least-squares estimation to be problematic for 5PL. An ad hoc approach I've successfully employed uses the approach outlined below:

(a) $a_0 = \max(\text{response}) \times 1.01$

(b) $d_0 = \min(\text{response}) \times 0.99$

(c) $g_0 = 1$

(d) Let $\tilde{y} = \frac{a-d}{y-d} - 1$. Then from Equation 1, $\log \tilde{y} = b \log x - b \log c$. Let $\hat{\alpha_0}$ and $\hat{\alpha_1}$ be the fitted intercept and slope from the simple linear regression of $\log \tilde{y}$ on $\log x$. Then $b_0 = \hat{\alpha_1}$ and $c_0 = e^{-\hat{\alpha_0}/b_0}$.

2. Obtain $\hat{\theta}^{(0)}$ as the slope parameter from a linear regression of $\log s_i$ on $\log \bar{y}_i$, where s_i is the estimated standard deviation of the responses at dose i and $\bar{y}_i$ is the average response at dose i. This is based on the fact that $\log\{\text{Var}(y_{ij})\}^{1/2} = \log \sigma + \theta \log \mu_i$.

3. Obtain $\hat{\sigma}^{2(0)} = e^{2\alpha_0}$ where γ_0 is the estimated intercept from the linear regression in Step 2.

4. Let $k = 1$.

5. Obtain $\hat{\boldsymbol{\beta}}^{(k)}$ by minimizing $\sum_{i=1}^{N} \sum_{j=1}^{m_i} \hat{w}_i^{(k-1)} (y_{ij} - \mu_i)^2$, where $\hat{w}_i^{(k-1)} = 1/g^2(\hat{\mu}_i^{(k-1)}, \hat{\theta}^{(k-1)})$.

6. From $\hat{\boldsymbol{\beta}}^{(k)}$, construct estimates of $r_{ij}^{(k)} = y_{ij} - \hat{\mu}_i^{(k)}$.

7. Obtain $\hat{\theta}^{(k)}$ by minimizing

$$\sum_{i=1}^{N} \sum_{j=1}^{m_i} \left\{ \frac{r_{ij}^{(k)} \hat{g}^{(k)}}{g(\hat{\mu}_i^{(k)}, \theta)} \right\}^2,$$

where

$$\hat{\dot{g}}^{(k)} = \exp\left\{ \frac{\theta}{n} \sum_{i=1}^{N} m_i \log \hat{\mu}_i^{(k)} \right\}.$$

8. Obtain

$$\hat{\sigma}^{2(k)} = \frac{1}{n-p} \sum_{i=1}^{N} \sum_{j=1}^{m_i} \frac{(r_{ij}^{(k)})^2}{g^2(\hat{\mu}_i^{(k)}, \hat{\theta}^{(k)})},$$

where p is the number of parameters in the mean model ($p = 4$ or 5 for 4- and 5PL, respectively).

Iterate steps 5 through 8 to convergence. Call the estimates $\hat{\boldsymbol{\beta}}_{\text{GLS}}$, $\hat{\theta}_{\text{GLS}}$ and $\hat{\sigma}^2_{\text{GLS}}$. The variance-covariance matrix of $\hat{\boldsymbol{\beta}}_{\text{GLS}}$ is

$$\Sigma_{\text{GLS}} \quad = \quad \sigma^2(\mathbf{X}'\mathbf{G}^{-1}\mathbf{X})^{-1} \tag{6}$$

where $\mathbf{X}$ is an $n \times p$ matrix ($n = \sum_i m_i$) such that the m_i rows corresponding to the ith dose contain the row vector

$$\left(\frac{\partial}{\partial\beta_1}f(x_i,\boldsymbol{\beta}),\ldots,\frac{\partial}{\partial\beta_p}f(x_i,\boldsymbol{\beta})\right)',$$

and $\mathbf{G}$ is an $n\times n$ diagonal matrix with the diagonal elements of the m_i rows corresponding to the ith dose equal to $g^2(\mu_i,\theta)$. Equation 6 is estimate by replacing σ^2 with $\hat{\sigma}^2_{\text{GLS}}$, evaluating $\mathbf{X}$ at $\hat{\boldsymbol{\beta}}_{\text{GLS}}$, and evaluating $\mathbf{G}$ at $\hat{\boldsymbol{\beta}}_{\text{GLS}}$ and $\hat{\theta}_{\text{GLS}}$.

For the 5PL model, the partial derivatives defining $\mathbf{X}$ are as follows:

- $\partial f(x_i,\boldsymbol{\beta})/\partial a = \left(1+(\frac{x_i}{c})^b\right)^{-g}$
- $\partial f(x_i,\boldsymbol{\beta})/\partial b = (a-d)(-g)\left(1+(\frac{x_i}{c})^b\right)^{-g-1}\left(\frac{x_i}{c}\right)^b\ln\left(\frac{x_i}{c}\right)$
- $\partial f(x_i,\boldsymbol{\beta})/\partial c = (a-d)(-g)\left(1+(\frac{x_i}{c})^b\right)^{-g-1}\left(\frac{-bx_i^b}{c^{b+1}}\right)$
- $\partial f(x_i,\boldsymbol{\beta})/\partial d = 1-\left(1+(\frac{x_i}{c})^b\right)^{-g}$
- $\partial f(x_i,\boldsymbol{\beta})/\partial g = -(a-d)\left(1+(\frac{x_i}{c})^b\right)^{-g}\ln\left(1+(\frac{x_i}{c})^b\right)$

4 Constructing the precision profile

Background-corrected flourescence readings from subject samples are converted to concentration estimates based on the inverse of Equation 1,

$$x = f^{-1}(y) \quad = \quad c\left[\left(\frac{a-d}{y-d}\right)^{1/g}-1\right]^{1/b}. \tag{7}$$

Specifically, the estimated concentration for the ith subject's jth response is $\hat{x}_{ij} = f^{-1}(y_{ij},\hat{\boldsymbol{\beta}})$. If concentration estimates are based on the backfit of the *average* of replicate

flourescence readings (which is typically the case for commercially available software), then a subject-level concentration estimate is $\hat{x}_i = f^{-1}(\bar{y}_i, \hat{\boldsymbol{\beta}})$, where $\bar{y}_i = \sum_{j=1}^{n_i} y_{ij}$ and n_i is the number of replicates for the ith subject. Table 4 shows a sample list of (fictitious) concentrations from a multiplex immunoassay. There are three types of 'data': the values that look 'real' (IDs 2, 3 and 4), the 'OOR <' values (IDs 1, 6 and 7), and the starred values (ID 5). 'OOR' stands for *out of range* and indicates flourescence readings smaller than the lower asymptote. Flourescence readings exceeding the upper asymptote are denoted as 'OOR >' in the output. In our experience, the majority of out-of-range values occur at lower concentrations. We therefore restrict our discussion to this case, although our results generalize to the analysis of values exceeding the upper asymptote as well. Since it is impossible to obtain a concentration estimate for these response values, the software simply flags them accordingly. The starred values indicate observations that extrapolate beyond the standard range, a practice that is ill-advised.

Correct analysis of subject concentration measures requires determinination of minimum and maximum acceptable concentrations (MinAC and MaxAC, respectively). These thresholds represent concentrations such that values less than (greater than) MinAC (MaxAC) are considered too variable to be reported reliably. Minimum and maximum acceptable concentrations are typically defined as those values for which the coefficient of variation (CV) of the estimated concentration equals a pre-specified cutoff. Figure 1 shows the *precision profiles* associated with the fitted 5-PL dose-response models for IL7 for two different 96-well plates. The precision profile is a plot of the percent CV in estimated concentration versus concentration, and is typically 'U-shaped' for sigmoidal dose-response models. The shape reflects the fact that small changes in flourescent signals in the flat regions of the curve result in highly variable predicted concentrations, while responses in the linear range of the curve predict concentrations with considerably less variability. Traditional thresholds for MinAC and MaxAC are 10% CV (limit of quantitation) and 30% CV (limit of detection) (include references here from the interna-

Table 1: Description of parameters for 4- and 5PL dose-response models.

Parameter	4PL	5PL
a	Asymptote at high dose	Asymptote at high dose
d	Asymptote at low dose	Asymptote at low dose
b	Slope parameter	1: Controls rate of approach to a asymptote
		2: bg controls rate of approach to d asymptote
c	ED50	Jointly with b and g estimates ED_ν,
		where $\text{ED}_\nu = c((100/\nu)^{1/g} - 1)^{1/b}$
g		1: Controls asymmetry
		2: bg controls rate of approach to d asymptote

Table 2: Sample multiplex concentration estimates.

Sample	Concentration (pg/ml)
1	OOR <
2	2.65
3	4.6
4	1.09
5	*0.70
6	OOR <
7	OOR <

tional standards literature). However, it has been our observation that thresholding the data at 30% CV or lower can result in little data available for analysis. Gottschalk and Dunn[5] use a 50% CV threshold in their examples, but make no formal recommendation for establishing MinAC and MaxAC.

Construction of the precision profile requires estimation of $\mathrm{Var}(\hat{x})$. Specifically

$$\mathrm{Var}(\hat{x}) \doteq \left(\frac{\partial f^{-1}}{\partial y}\right)^2 \mathrm{Var}(y) + \left(\frac{\partial f^{-1}}{\partial \boldsymbol{\beta}}\right)' \mathrm{Var}(\hat{\boldsymbol{\beta}}) \left(\frac{\partial f^{-1}}{\partial \boldsymbol{\beta}}\right), \tag{8}$$

where

- $\mathrm{Var}(y)$ is given by Equation 3.
- $\mathrm{Var}(\hat{\boldsymbol{\beta}})$ is given by Equation 6.
- $\frac{\partial f^{-1}}{\partial y} = \frac{c}{b}\left[\left(\frac{a-d}{y-d}\right)^{1/g} - 1\right]^{\frac{1}{b}-1} \times \frac{1}{g}\left(\frac{a-d}{y-d}\right)^{\frac{1}{g}-1} \times \frac{d-a}{(y-d)^2}$
- $\frac{\partial f^{-1}}{\partial \boldsymbol{\beta}} = \left(\frac{\partial f^{-1}}{\partial a}, \frac{\partial f^{-1}}{\partial b}, \frac{\partial f^{-1}}{\partial c}, \frac{\partial f^{-1}}{\partial d}, \frac{\partial f^{-1}}{\partial g}\right)'$ with
  - $\frac{\partial f^{-1}}{\partial a} = \frac{c}{b}\left[\left(\frac{a-d}{y-d}\right)^{1/g} - 1\right]^{\frac{1}{b}-1} \times \frac{1}{g}\left(\frac{a-d}{y-d}\right)^{\frac{1}{g}-1} \times \left(\frac{1}{y-d}\right)$
  - $\frac{\partial f^{-1}}{\partial b} = -c\left[\left(\frac{a-d}{y-d}\right)^{1/g} - 1\right]^{\frac{1}{b}} \times \log\left[\left(\frac{a-d}{y-d}\right)^{\frac{1}{g}} - 1\right] \times b^{-2}$
  - $\frac{\partial f^{-1}}{\partial c} = \left[\left(\frac{a-d}{y-d}\right)^{1/g} - 1\right]^{\frac{1}{b}}$
  - $\frac{\partial f^{-1}}{\partial d} = \frac{c}{b}\left[\left(\frac{a-d}{y-d}\right)^{1/g} - 1\right]^{\frac{1}{b}-1} \times \frac{1}{g}\left(\frac{a-d}{y-d}\right)^{\frac{1}{g}-1} \times \frac{a-y}{(y-d)^2}$
  - $\frac{\partial f^{-1}}{\partial g} = \frac{c}{b}\left[\left(\frac{a-d}{y-d}\right)^{1/g} - 1\right]^{\frac{1}{b}-1} \times \left[-\left(\frac{a-d}{y-d}\right)^{\frac{1}{g}}\right] \times \log\left(\frac{a-d}{y-d}\right) \times g^{-2}.$

Estimation of Equation 8 is achieved by substituting $\hat{\sigma}^2 g^2(x, \hat{\boldsymbol{\theta}})$ for $\mathrm{Var}(y)$ and evaluating Equation 8 for a grid of $\hat{x}$ and y values covering the domain of the standards. The estimated percent coefficient of variation is $\sqrt{\widehat{\mathrm{Var}}(\hat{x})}/\hat{x} \times 100$ and is plotted against $\hat{x}$ for the entire domain of standard concentrations as the precision profile. Precision profiles for two different standard curves are shown in Figure 1. A threshold of 50% CV is drawn indicating MinAC and MaxAC values.

5 Simulation study

The purpose of the current study is to investigate operating characteristics of various choices of MinAC and MaxAC with the goal of establishing guidelines for investigators implementing this technology. We conducted the following simulation study.

1. Assume the *uncorrected* dose-response model is

$$z_{ij} \quad = \quad f(x_i, \boldsymbol{\beta}) + k + \sigma g(\mu_i, \boldsymbol{\theta})\varepsilon_{ij}, \tag{9}$$

   where z_{ij} is the uncorrected fluorescence measure corresponding to the jth replicate of the ith concentration. Here, k is the *background* parameter reflecting a vertical shift in fluorescence readings based on blanks. Fix $(\boldsymbol{\beta}, k, \sigma, \boldsymbol{\theta})' = (a, b, c, d, g, k, \sigma, \theta)$. These are treated as the *true* dose-response parameters. In (9), $f(x_i, \boldsymbol{\beta}) = \mu_i$ is given by Equation 1, and $g(\mu_i, \boldsymbol{\theta}) = \mu_i^{\theta}$ and is given by Equation 4.

2. Fix nine standards - eight with known concentrations and one blank (i.e. 0 concentration). Call these standards $s_0, s_1, \ldots, s_8$, with s_0 indicating the blank.

3. Generate two blank fluorescent readings based on the following assumed model:

$$z_{0j} = k + \sigma\varepsilon_{ij},$$

   where $j = 1, 2$. Compute the average of these readings, $\bar{z}_0 = (z_{01} + z_{02})/2$. This average is our estimate of k; that is to say, $\hat{k} = \bar{z}_0$.

4. For each of the standards $s_1, \ldots, s_8$, use Equation 9 with the true parameter values to obtain duplicate uncorrected flourescent readings, resulting in the sixteen data pairs

$$((s_1, z_{11}), (s_1, z_{12}), (s_2, z_{21}), (s_2, z_{22}), \ldots, (s_8, z_{81}), (s_8, z_{82}))\,.$$

5. Use the background estimate obtained from Step 3 to 'correct' the fluorescent readings obtained in Step 4. Specifically, let $y_{ij} = z_{ij} - \hat{k}$. The corrected readings, $\{y_{ij}\}$, will be used to fit the standard curve and to conduct inference.

6. Using the data pairs from Step 5, fit the 5PL standard curve given by Equation 2 using the methods described in Section 3. Generate the estimated dose-response parameters $(\hat{\boldsymbol{\beta}}, \hat{\sigma}, \hat{\theta})' = (\hat{a}, \hat{b}, \hat{c}, \hat{d}, \hat{g}, \hat{\sigma}, \hat{\theta})$.

7. Construct the precision profile for the standard curve constructed in Step 6 using methods described in Section 4. Identify MinAC and MaxAC corresponding to 10, 20, 30, 40, and 50% CV. Use Equation 1 to find corresponding values of MinAFl and MaxAFl (AFL = acceptable fluorescence). That is to say, MinAFl $= f(\text{MinAC}, \hat{\boldsymbol{\beta}})$ and MaxAFl $= f(\text{MaxAC}, \hat{\boldsymbol{\beta}})$.

8. Let c_i be the *true* concentration for the ith subject $(i = 1, \ldots, n)$ where the c_i arise from the model

$$\log(c_i) = \kappa + \alpha g_i + \nu_i,$$

so that

$$c_i = \exp\{\kappa + \alpha g_i + \nu_i\}.$$

Here, κ is the overall mean, g_i is a group indicator for the ith subject, α is the group-effect, and $\nu_i \sim \text{Normal}(0, \sigma_\nu^2)$. If $\alpha = 0$, the marker is not differentially expressed between the two groups. Generate n c_i values.

9. Use Equation 9 with the true parameter values to obtained duplicate uncorrected subject fluorescent readings for each true concentration. The resulting pairs are

$$\left((c_1, z_{11}), (c_1, z_{12}), \ldots, (c_n, z_{n1}), (c_n, z_{n2})\right),$$

where n is the number of subjects.

10. From the $\{z_{ij}\}$ obtained in Step 9, construct corrected subject fluorescent readings $\{y_{ij}\}$, where $y_{ij} = z_{ij} - \hat{k}$.

11. Using the corrected duplicate subject fluorescent readings, construct a censoring indicator τ_{ij} such that $\tau_{ij} = 1$ if $y_{ij} >$ MinAFl and $\tau_{ij} = 0$ if $y_{ij} \leq$ MinAFl, where $i = 1, \ldots, n$ and $j = 1, 2$.

12. Using Equation 7 and $\hat{\boldsymbol{\beta}}$ obtained in Step 6, generate *observed* duplicate concentrations, x_{ij}, as follows:

$$x_{ij} = \begin{cases} f^{-1}(y_{ij}, \hat{\boldsymbol{\beta}}) & \text{if } \tau_{ij} = 1 \\ \text{MinAC} & \text{if } \tau_{ij} = 0 \end{cases}$$

The *observed* data for analysis is represented by the following ordered pairs:

$$((x_{11}, \tau_{11}), (x_{12}, \tau_{12}), \ldots, (x_{n1}, \tau_{n1}), (x_{n2}, \tau_{n2}))\,.$$

13. Using the observed data from Step 12, we assume

$$\log(x_{ij}) = \eta + \gamma g_i + \zeta_i + \delta_{ij}$$

where

- η is the overall mean
- γ is the group effect
- g_i is a group indicator
- ζ_i is a subject-specific random effect such that $\zeta_i \sim \text{Normal}(0, \sigma^2_\zeta)$
- $\delta_{ij} \sim \text{Normal}(0, \sigma^2_\delta)$
- ζ_i and δ_{ij} are independent

Then the likelihood is given by

$$L(\boldsymbol{\Psi}|\mathbf{X}) \;=\; \prod_{i=1}^{n} \left[\int_{-\infty}^{\infty} \left\{ \prod_{j=1}^{2} h(\log(x_{ij})|\zeta_i)^{\tau_{ij}} H(\log(x_{ij})|\zeta_i)^{1-\tau_{ij}} \; r(\zeta_i) \right\} \mathrm{d}\zeta_i \right], \quad (10)$$

where

- $\boldsymbol{\Psi} = \left(\eta, \gamma, \sigma^2_\zeta, \sigma^2_\delta\right)'$
- $\mathbf{X} = (x_{11}, x_{12}, \ldots, x_{n1}, x_{n2})'$
- $h(\log(x_{ij})|\zeta_i)$ is a normal probability density function (conditional on the random effect) with mean $\eta + \gamma g_i + \zeta_i$ and variance σ^2_δ

- $H(\log(x_{ij})|\zeta_i)$ is the normal cumulative density function (conditional on the random effect) corresponding to $h(\log(x_{ij})|\zeta_i)$
- $r(\zeta)$ is the distribution of the random effect and is a normal probability density function with mean 0 and variance σ_ζ^2

The MLE of Equation 10 is used to conduct group-level inference.

14. Repeating Steps 4 through 13 for the same values of $(a, b, c, d, g, k, \sigma, \theta)'$ (the true standard curve parameters) and the same values of $(\kappa, \alpha, \sigma_\nu^2)'$ (the true subject log concentration parameters) is equivalent to a complete analysis of a single plate. If multiple plates are used in an experiment, then Step 13 should be conducted from the aggregation of subject data from multiple plates.

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