Principles of Curve Fitting for Multiplex Sandwich Immunoassays

Diana Davis, PhD, Aiguo Zhang, PhD, Chloe Etienne, Ivan Huang,
and Michele Malit, Bio-Rad Laboratories, Inc., Hercules, CA 94547 USA

Introduction

Bio-Plex cytokine assays are bead-based multiplex sandwich immunoassays. The concentrations of analytes in a Bio-Plex cytokine immunoassay are quantitated using a calibration or standard curve. More specifically, a series of known concentrations of an analyte is used to construct a plot of signal intensity vs. concentration. The plot is mathematically modeled to derive an equation that may be used to predict the concentrations of unknown samples. The type of mathematical or curve-fitting model as well as the fit of the model have a direct effect on the accuracy of the results. Therefore, curve fitting is a critical component of immunoassay performance. In this technical note, curve-fitting methods as well as methods used to determine the quality of the curve fitting are discussed. Advantages and disadvantages of each method and the effects on the predicted cytokine concentrations are illustrated using representative Bio-Plex cytokine assay data.

Methods of Curve Fitting

Linear Regression

The simplest method for determining concentrations from a standard curve is to construct a plot of the concentration vs. the response using the linear portion of the response curve. This method has been used traditionally to quantitate results of ELISA and other immunoassays (Nix and Wild 2001). An example of a GM-CSF Bio-Plex cytokine assay plotted using a linear regression is shown in Figure 1. The R^2 value is used to determine the overall goodness of the linear fit. A linear regression with an R^2 value >0.99 is considered a very good fit (Nix and Wild 2001). The primary advantage of this method is that it is extremely simple. Once the linear range of an assay is determined, additional standard concentrations within the specified range may be added to improve the accuracy of the fit.

Logistic Regression

Immunoassay data may also be modeled using a nonlinear regression routine, most commonly known as a logistic regression (Baud 1993). An example of a logistic regression of a standard curve from a Bio-Plex cytokine assay, with the log of the concentration plotted on the x-axis and the response (relative median fluorescence intensity, or MFI) plotted on the y-axis, is presented in Figure 2. The logistic regression is commonly used for many assays, including sandwich immunoassays (Baud 1993). Two common logistic equations are used, four-parameter (4PL) and five-parameter (5PL) (Baud 1993). Depending on the data, one regression may yield better results than another.

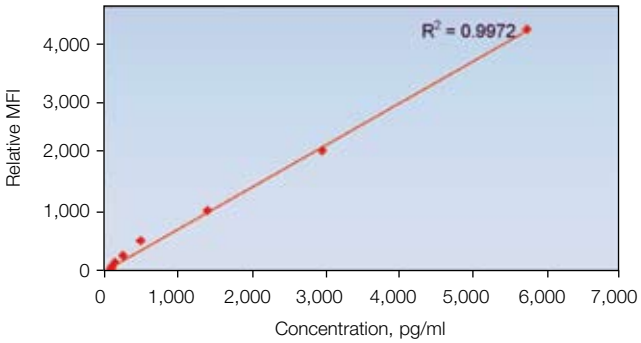

Fig. 1. GM-CSF Bio-Plex cytokine assay plotted using a linear regression.

Four-Parameter Logistic (4PL)

The 4PL equation contains four parameters or variables related to the graphical properties of the curve, as illustrated in Figure 2.

One derivation of the 4PL equation may be expressed as follows (Baud 1993):

$$y = d + \frac{a - d}{\left[1 + \left(\frac{x}{c}\right)^b\right]}$$

Where:
a = estimated response at zero concentration
b = slope factor
c = mid-range concentration (c_{50})
d = estimated response at infinite concentration

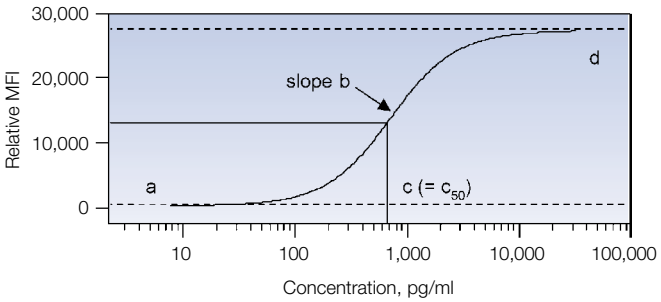

Fig. 2. Example of Bio-Plex cytokine standard curve fitted with 4PL regression.

BIO-RAD

Once a 4PL equation is created from a set of data, values for all four of the parameters will be determined. The equation may then be used to calculate unknown concentrations (x) from the assay data (y), much like the well-known linear equation y = mx + b.

Five-Parameter Logistic (5PL)

The 5PL equation is equivalent to the 4PL equation with an additional parameter added for asymmetry (Baud 1993). This additional parameter provides a better fit when the response curve is not symmetrical.

One derivation of the 5PL equation may be expressed as follows:

$$y = d + \frac{a - d}{\left[1 + \left(\frac{x}{c}\right)^{b}\right]^{g}}$$

Where:
a = estimated response at zero concentration
b = slope factor
c = mid-range concentration (c_{50})
d = estimated response at infinite concentration
g = asymmetry factor

4PL vs. 5PL

The type of logistic equation that will yield the best fit through a set of points is dependent on the response or the shape of the standard curve of an assay. Three different types of response curves may be encountered when analyzing Bio-Plex cytokine immunoassays: a sigmoidal or S-shaped curve (Figure 3A), a low-response curve (Figure 3B), or a high-response curve (Figure 3C). If the curve is S-shaped and symmetrical (i.e., similar shapes on both ends of the "S"), a 4PL or 5PL regression will yield similar results. When the curve is not symmetrical, as in Figures 3B and 3C, a better fit will be achieved using a 5PL regression.

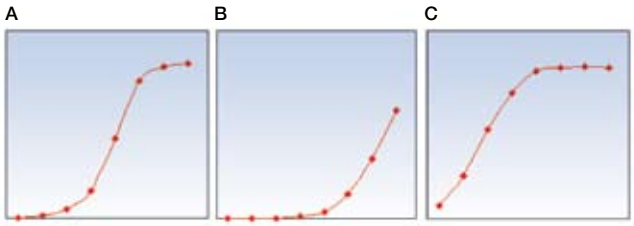

Fig. 3. Three standard curve shapes are commonly encountered when analyzing Bio-Plex cytokine assay data. A, sigmoidal or S-shaped curve; **B**, low-response curve; **C**, high-response curve.

Measuring Goodness of Fit

"Goodness of fit" is a term that describes how well a curve fits a given set of data. Goodness of fit using linear regression is commonly assessed by the R^2 value (Motulsky 1996). When using logistic regression, there are other statistical parameters for measuring goodness of fit, such as fit probability and residual variance. Two methods more practical for measuring goodness of fit are typically used, backcalculation of standards and spiked recovery (Nix and Wild 2001, Davies 2001).

Backcalculation of Standards (Standards Recovery)

A practical method for assessing the quality of a curve fit is to calculate the concentrations of the standards after the regression has been completed (Nix and Wild 2001, Baud 1993). This procedure is also known as standards recovery and is performed by calculating the concentration of each standard and then comparing it to the actual concentration using the formula: Observed concentration/ expected concentration x 100. This method yields information about the relative error in the calculation of samples. Backcalculation of standards is automatically performed with Bio-Plex Manager™ software, and the results are displayed in the report table in the (Obs/Exp) * 100 column (Figure 4). It is most desirable to have each standard fall between 70 and 130% of the actual value, although more stringent ranges may be applied if greater accuracy is desired. The limitation of using backcalculation as the sole method of evaluating goodness of fit is the existence of a bias toward the concentrations of the standards. More specifically, only the standard concentrations are used to assess the quality of the fit; the portions of the curve between each of the standard points are ignored (Nix and Wild 2001).

Spiked Recovery

Spiked recovery is used to assess the overall accuracy of an assay (Davies 2001). This method incorporates variables in assay preparation as well as the regression analysis. Samples are spiked with known concentrations of cytokine and analyzed to determine the closeness of the calculated value to the actual value. The chosen concentrations are usually between the concentrations of the standards, thus removing the bias inherent to the backcalculation of standards method. The results are assessed in the same manner as the standards recovery, using the Obs/Exp x 100 formula. A spiked recovery value between 80 and 120% is considered acceptable. Spiked recovery results may be analyzed using Bio-Plex Manager software by formatting the spiked samples as controls and specifying the concentration of each sample in the Enter Controls Info dialog box of the protocol settings. The recovery values are displayed in the report table in the (Obs/Exp) * 100 column (Figure 5). The disadvantage of this method is that it is affected by variables other than curve fitting. Errors in sample preparation or assay preparation (pipetting, adding reagents) may affect overall recovery. In addition, it is difficult to accurately spike low levels of cytokines into samples due to the relative imprecision of pipets that deliver small volumes. All of these variables should be taken into consideration when analyzing spiked recovery data.
For example, if standards recovery is accurate, but spiked recovery is poor, the error is most likely due to the spiked samples themselves. This method should be performed in addition to, and not in place of, backcalculation of standards when evaluating assay performance.

Type	Well	Description	FI	FI - Bkgd	Std Dev	%CV	Obs Conc	Exp Conc	(Obs/Exp) * 100
S1	C1,C2,C3	32000.00 pg/ml Std	16005.0	16005.0	741.77	4.63	34155.37	32000.00	107
S2	E1,E2,E3	8000.00 pg/ml Std	10016.3	10016.3	243.64	2.43	7599.59	8000.00	95
S3	H1,H2,H3	2000.00 pg/ml Std	3864.7	3864.7	256.59	6.64	2151.77	2000.00	108
S4	D4,D5,D6	500.00 pg/ml Std	850.5	850.5	19.50	2.29	456.19	500.00	91
S5	F4,F5,F6	125.00 pg/ml Std	341.7	341.7	11.85	3.47	145.96	125.00	117
S6	H4,H5,H6	31.25 pg/ml Std	188.7	188.7	15.37	8.15	31.01	31.25	99
S7	B7,B8,B9	7.80 pg/ml Std	160.3	160.3	1.53	0.95	1.18	7.80	15
S8	D7,D8,D9	1.95 pg/ml Std	161.3	161.3	9.07	5.62	2.71	1.95	139

Fig. 4. Bio-Plex Manager report table showing backcalculation of standards (standards recovery) in the (Obs/Exp) * 100 column. A value between 70 and 130% indicates a good fit.

Type	Well	Description	FI	FI - Bkgd	Std Dev	%CV	Obs Conc	Exp Conc	(Obs/Exp) * 100
S1	C1,C2,C3	32000.00 pg/ml Std	24479.7	24479.7	251.96	1.03	14298.99	32000.00	45
S2	E1,E2,E3	8000.00 pg/ml Std	24218.3	24218.3	79.20	0.33	9014.21	8000.00	113
S3	H1,H2,H3	2000.00 pg/ml Std	18417.2	18417.2	345.47	1.88	2017.31	2000.00	101
S4	D4,D5,D6	500.00 pg/ml Std	6906.0	6906.0	188.05	2.72	485.12	500.00	97
S5	F4,F5,F6	125.00 pg/ml Std	2288.0	2288.0	41.33	1.81	132.10	125.00	106
S6	H4,H5,H6	31.25 pg/ml Std	683.0	683.0	22.59	3.31	31.10	31.25	100
S7	B7,B8,B9	7.80 pg/ml Std	231.8	231.8	8.81	3.80	7.44	7.80	95
S8	D7,D8,D9	1.95 pg/ml Std	108.7	108.7	5.69	5.23	2.02	1.95	103
C1	A10,A11,A12	5000 pg Control	23817.2	23817.2	114.62	0.48	6494.56	5000.00	130
C2	B10,B11,B12	2500 pg Control	18599.7	18599.7	462.18	2.48	2063.33	2500.00	83
C3	C10,C11,C12	1250 pg Control	13209.8	13209.8	139.46	1.06	1124.53	1250.00	90
C4	D10,D11,D12	625 pg Control	8170.3	8170.3	449.25	5.50	595.50	625.00	95
C5	E10,E11,E12	312.50 pg Control	4992.7	4992.7	267.72	5.36	329.68	312.50	105
C6	F10,F11,F12	156.20 pg Control	2843.7	2843.7	14.05	0.49	170.42	156.20	109
C7	G10,G11,G12	78.00 pg Control	1680.3	1680.3	33.25	1.98	91.87	78.00	118
C8	H10,H11,H12	39 pg/ml Control	914.8	914.8	28.44	3.11	44.45	39.00	114

Fig. 5. Spiked recovery results shown in a report table in Bio-Plex Manager software. The wells formatted as controls (C1–C8) represent samples that have been spiked with known amounts of cytokines. The recovery for these samples is shown in the (Obs/Exp) * 100 column.

Linear vs. Logistic Regression

Linear and logistic regression methods have distinct advantages and disadvantages. Linear regression may be readily used when analyzing serum or plasma cytokine levels. The biological range of most cytokines in serum is within the linear range of the standard curve and thus linear regression is appropriate for analysis. Some samples may need to be diluted and reanalyzed if the result is above the range covered by the standard curve. Although linear regression requires fewer data points or standards (as few as 3) compared to logistic regression (4PL and 5PL require 6 data points), a more accurate fit is obtained by using at least 6 points for any of the regression types (Motulsky 1996). Linear regression may not be as useful when analyzing samples in a multiplex setting (e.g., Bio-Plex cytokine assays are available in an 18-plex panel). Each analyte exhibits a different response and resulting linear range, and as a result it is difficult to select a universal set of standards that covers all of the analytes in a panel. This problem may be circumvented in the data analysis step of Bio-Plex Manager by deleting specific standards for each analyte; however, selection of standards that yield an R^2 value >0.99 for each analyte is a tedious and time-consuming task. It is also important to analyze the backcalculated standards when using linear regression. Even if the R^2 value is very high (>0.99), the accuracy of the fit as determined by standards recovery may indicate otherwise. For example, Figure 6 shows a cytokine assay exhibiting an R^2 value of 1.000, indicating an excellent fit through the points; however, the associated backcalculated standards data (Table 1) indicate that the goodness of fit is not optimal throughout the entire range of the standards.

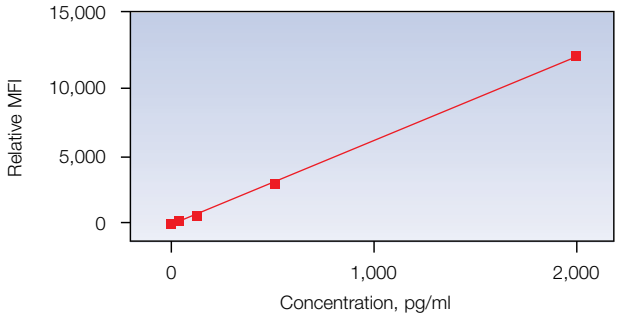

Fig. 6. Standard curve of mouse IL-2 in a Bio-Plex cytokine assay. Even though the R^2 value is 1.000, the accurate range as determined by backcalculation of standards (Table 1) is 31.25–2,000 pg/ml.

Table 1. Standards recovery. Data are from standard curve in Figure 6. The shaded cells indicate a recovery range of 70–130%.

Concentration (pg/ml)	Obs/Exp x 100
2,000	100
500	99
125	96
31.25	104
7.9	146
1.95	266

Logistic regression yields accurate quantitation across a wider range of concentrations compared to linear regression. This is the primary advantage of using a logistic regression. In Table 2, the standards recovery of a mouse cytokine assay using both linear and logistic regression methods is shown. The cells corresponding to 70–130% standards recovery for linear and logistic regression are shaded. The range using logistic regression is much broader

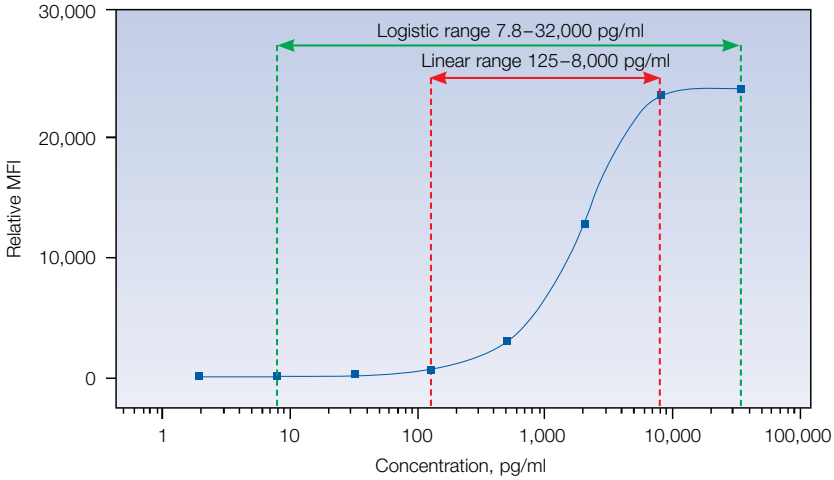

Fig. 7. Comparison of the dynamic range of linear and logistic regression routines. The dynamic ranges of linear and logistic regression routines of a mouse IL-2 assay are indicated by the red and green arrows, respectively. The arrows indicate the concentration ranges where the standards recovery is 70–130%.

Table 2. Comparison of the recovery of standards for a Bio-Plex IL-5 mouse cytokine assay using linear and logistic regressions. The shaded cells indicate a recovery range of 70–130%.

Concentration (pg/ml)	Standards Recovery (Obs/Exp x 100)	
	Linear (R^2 = 0.9996)	Logistic (5PL)
32,000	Out of range	100
8,000	100	100
2,000	96	100
500	82	99
125	106	114
31.25	216	102
7.8	745	133
1.95	2,418	Out of range

compared to the linear regression range. These data are shown graphically in Figure 7. The red bars indicate the range of concentrations showing 70–130% recovery using linear regression (R^2 = 0.9996), while the green bars indicate the range of concentrations showing 70–130% recovery using logistic regression. The dynamic range using linear regression is narrower than that achieved using logistic regression. From a practical perspective, logistic regression is much more flexible with respect to the standard concentrations used in a multiplex setting. A range of 1.95–32,000 pg/ml is appropriate for all analytes in a Bio-Plex cytokine assay panel, which facilitates analysis of the data compared to linear regression. It should be noted that although one may transform both the signal and the concentration to yield a linear plot, linear transformation of data is less accurate (Baud 1993). This inaccuracy is due to distortion of the experimental error and alteration of the relationship between x and y (Baud 1993).

Summary

Linear and logistic regressions are the two most commonly used curve-fitting models for sandwich immunoassays. Although linear regression may be useful when analyzing samples that fall within the linear portion of the response curve, logistic regression is the preferred regression type for multiplex immunoassays. The logistic regression yields the broadest range of concentrations at which unknown samples may be accurately predicted, and it allows the selection of a single set of standards that may be simultaneously applied to multiple analytes such as cytokines. The 5PL regression, with a fifth parameter to accommodate curve asymmetry, yields the best results in most cases. Assessment of the quality of curve-fitting routines is best achieved using two methods, standards recovery and spiked recovery. Both methods should be included when evaluating immunoassay performance to ensure accurate results.

References

Baud M, Data analysis, mathematical modeling, pp 656–671 in Methods of Immunological Analysis Volume 1: Fundamentals (Masseyeff RF et al., eds), VCH Publishers, Inc., New York, NY (1993)

Davies C, Concepts, pp 78–110 in The Immunoassay Handbook, 2nd ed (David Wild, ed), Nature Publishing Group, New York, NY (2001)

Motulsky H, The GraphPad guide to nonlinear regression, in GraphPad Prism Software User Manual, GraphPad Software Inc., San Diego, CA (1996)

Nix B and Wild D, Calibration curve-fitting, pp 198–210 in The Immunoassay Handbook, 2nd ed (David Wild, ed), Nature Publishing Group, New York, NY (2001)

Information in this tech note was current as of the date of writing (2004) and not necessarily the date this version (rev B, 2007) was published.

BIO-RAD

Bio-Rad Laboratories, Inc.

Life Science Group

Web site *www.bio-rad.com* ***USA*** *800 4BIORAD* ***Australia*** *61 02 9914 2800* ***Austria*** *01 877 89 01* ***Belgium*** *09 385 55 11* ***Brazil*** *55 21 3237 9400* ***Canada*** *905 712 2771* ***China*** *86 21 6426 0808* ***Czech Republic*** *420 241 430 532* ***Denmark*** *44 52 10 00* ***Finland*** *09 804 22 00* ***France*** *01 47 95 69 65* ***Germany*** *089 318 84 0* ***Greece*** *30 210 777 4396* ***Hong Kong*** *852 2789 3300* ***Hungary*** *36 1 455 8800* ***India*** *91 124 4029300* ***Israel*** *03 963 6050* ***Italy*** *39 02 216091* ***Japan*** *03 5811 6270* ***Korea*** *82 2 3473 4460* ***Mexico*** *52 555 488 7670* ***The Netherlands*** *0318 540666* ***New Zealand*** *0508 805 500* ***Norway*** *23 38 41 30* ***Poland*** *48 22 331 99 99* ***Portugal*** *351 21 472 7700* ***Russia*** *7 495 721 14 04* ***Singapore*** *65 6415 3188* ***South Africa*** *27 861 246 723* ***Spain*** *34 91 590 5200* ***Sweden*** *08 555 12700* ***Switzerland*** *061 717 95 55* ***Taiwan*** *886 2 2578 7189* ***United Kingdom*** *020 8328 2000*