Selection of Standards for Bio-Plex Cytokine Assays

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Introduction

Bio-Plex cytokine assays are bead-based quantitative multiplex immunoassays. The concentrations of analytes in these assays are quantitated using a calibration or standard curve. A regression analysis is performed to derive an equation that is used to predict the concentrations of unknown samples. The specific concentrations of the standards used to construct the standard curve have a direct effect on the quality of the results. Therefore, selection of appropriate standards is a critical component of immunoassay performance. In this tech note, the effect of the selection of standards on the accuracy, sensitivity, and dynamic range of the results is discussed. The effect on assay performance of reading assays on a Bio-Plex system set at two different photomultiplier tube (PMT) voltages as well as criteria for selecting a custom set of standards are also addressed.

Regression Analysis for Bio-Plex Assays

Because the typical response of an immunoassay is nonlinear, the nonlinear or logistic regression routine is the preferred method for sandwich immunoassay data analysis (Baud 1993). The four-parameter (4PL) and five-parameter (5PL) logistic equations contain parameters (the midpoint of the curve, the slope where the curve is linear, and the upper and lower points) that are used to predict the response of the assay (relative median fluorescence intensity, or MFI; Figure 1). Use of logistic regression yields the widest range of predictable concentrations for Bio-Plex immunoassays (Nix and Wild 2001).

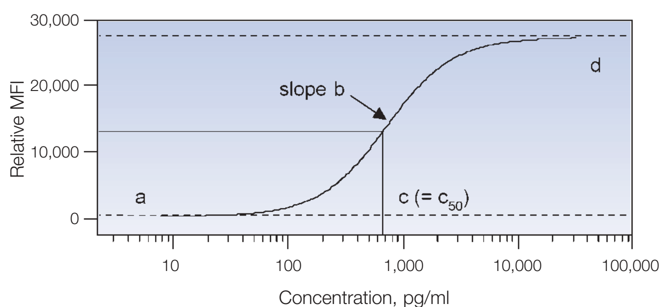

Fig. 1. A sample standard curve showing curve-fit parameters for a 4PL logistic equation. Parameters include a, estimated response at zero concentration, b, slope of the tangent at the midpoint, c, midrange concentration, and d, estimated response at infinite concentration. The 5PL equation contains an additional variable, g, to correct for asymmetry in the curve shape.

Standard Curve Ranges for Bio-Plex Assays

Two sets of standards are recommended for Bio-Plex cytokine assays, a high-sensitivity range (0.2–3,200 pg/ml) and a broad range (1.95–32,000 pg/ml). The specific concentrations for the two sets of standards are shown in Table 1. The high-sensitivity standards are recommended for use when the concentrations of the unknowns fall within the physiological range of cytokines in serum. The high-sensitivity standards are suitable for quantitation of low levels of cytokines and provide the greatest sensitivity. The broad range standards yield a wide dynamic range and are used when quantitation of a wide range of cytokine concentrations is desired or when the range of expected concentrations of cytokines is unknown. The broad range standards enable the detection of the widest range of concentrations and yield a wide dynamic range. A comparison of the high-sensitivity and broad range standard curves plotted as concentration versus fluorescence intensity is shown in Figure 2.

Table 1. Concentrations in pg/ml of broad range and high-sensitivity standards used in Bio-Plex assays.

Broad Range	High-Sensitivity
1.95	0.20
7.8	0.78
31.25	3.13
125	12.5
500	50
2,000	200
8,000	800
32,000	3,200

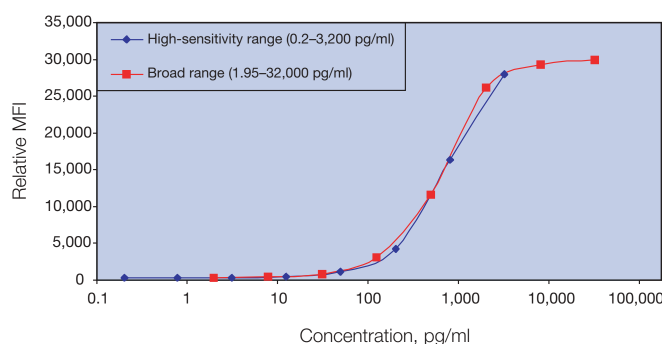

Fig. 2. Comparison of range of concentrations included in high-sensitivity and broad range standard curves using a 5PL logistic regression.

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Effect of Range on Standards Recovery

The overall dynamic range, accuracy, and sensitivity of results obtained using high-sensitivity vs. broad range standards are quite different. These differences may be illustrated using a method known as backcalculation of standards, or standards recovery (Davies 2001). In this method, a regression model is applied to a set of data and the resulting equation is used to calculate the concentrations of the standards using the response values (Nix and Wild 2001). The analysis is performed by calculating the concentration of each standard and then comparing it to the actual concentration using the following formula: (observed concentration) / (expected concentration) x 100. If the regression analysis is accurate, the calculated concentrations will be very close to the actual concentrations of the standards. This method yields information about the dynamic range, sensitivity, and accuracy of calculated concentrations of an assay (Baud 1993). The recovery value for each standard is an indication of the accuracy of the calculated concentration. A value within the range of 70–130% is considered accurate recovery. The dynamic range of the assay, or the range where accurate concentrations may be quantitated, is therefore indicated by the range of standards yielding a recovery of 70–130%.

A comparison of the standards recovery using broad range and high-sensitivity standards for three different cytokines is shown in Tables 2 and 3. The broad range standard concentrations where recovery is 70–130% is 1.95 to 8,000 or 32,000 pg/ml, depending on the cytokine assayed. The high-sensitivity standards yield a narrower dynamic range of 0.2 or 0.78 to 3,200 pg/ml. In all three assays, the broad range standards enable the quantitation of a much wider range of concentrations.

A general indication of assay sensitivity is given by the lowest concentration in a set of standards that yields accurate recovery (70–130%). Since the lowest concentration of the high-sensitivity standards is lower than the broad range standards (i.e., 0.2 pg/ml vs. 1.95 pg/ml, respectively), it would be expected that the high-sensitivity standards would yield better recovery at a lower concentration, and that the high-sensitivity standards should be chosen to maximize sensitivity in an assay. Tables 2 and 3 illustrate some typical recovery values for broad range and high-sensitivity standards.

Table 2. Recovery of broad range standards in Bio-Plex assays. Recovery refers to backcalculated standards recovery. Recovery values between 70 and 130% are shaded. OOR refers to a calculated concentration that is out of the range of the regression model.

Broad Range Standards			
Concentration (pg/ml)	IL-13	IL-10	TNF-α
1.95	100	107	101
7.81	101	90	99
31.25	99	106	98
125	102	103	105
500	98	96	97
2,000	105	107	102
8,000	88	77	79
32,000	87	OOR	OOR

Table 3. Recovery of high-sensitivity standards in Bio-Plex assays. Recovery refers to backcalculated standards recovery. Recovery values between 70 and 130% are shaded.

High-Sensitivity Standards			
Concentration (pg/ml)	IL-13	IL-10	TNF-α
0.2	107	140	95
0.78	95	87	97
3.13	101	100	107
12.5	102	100	95
50	97	103	100
200	101	98	104
800	99	101	98
3,200	101	100	100

Effect of PMT Voltage on Assay Performance

Bio-Plex assays are analyzed on a Bio-Plex system that contains a PMT. The PMT detects assay signals in the reporter or RP1 channel. The PMT voltage on the Bio-Plex system is automatically adjusted during CAL2 calibration using the RP1 target value from a bead with stable fluorescence. This method yields a reproducible signal output from day to day and between instruments. Two RP1 target values are recommended for use with Bio-Plex assays, a low target value for the broad range standards and a high target value for the high-sensitivity standards. Following calibration, the low RP1 target value yields a low PMT voltage and the high RP1 target value yields a higher PMT voltage. Table 4 compares the resulting PMT voltage on a Bio-Plex system following calibration with the low and high target values.

Table 4. Comparison of PMT voltage following CAL2 calibration with the low and high RP1 target values on a Bio-Plex system.

Target Value Used	Resulting PMT Voltage
3,713	545.3
16,894	669.8

The PMT voltage is increased in assays in which higher sensitivity is desired. However, when using the broad range standards, the signals of the high concentration standards on certain cytokines may become saturated and result in

a reduced dynamic range. Figures 3 and 4 show a broad range standard IL-8 assay analyzed with the high and low PMT settings. The standard points are labeled with the corresponding recovery. In Figure 3, the recovery at the two highest points is beyond the range of the regression, due to a saturated signal at the high end of the standard curve, and thus OOR (out of range) is displayed. When the same assay is analyzed at the low PMT setting, there is no saturation and the standards recovery is accurate across the entire range of standards (Figure 4).

The high-sensitivity standards yield a much lower fluorescence intensity signal since the concentration range is much lower. When using the low range standards, reading the assay with the high PMT voltage may improve the sensitivity. Table 5 shows a comparison of the recovery of a low range GM-CSF assay run using the low and high PMT voltage settings. The low PMT setting yielded acceptable recovery down to only 3.13 pg/ml. When the same assay was run at the high PMT setting, accurate recovery down to 0.2 pg/ml was achieved.

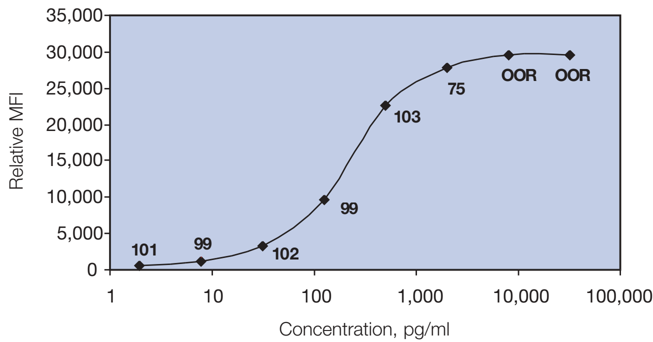

Fig. 3. Human IL-8 broad range standard assay run at the high PMT setting and analyzed using a 5PL regression routine. Each standard is labeled with the corresponding recovery. The two highest concentrations were saturated and beyond the range of the 5PL regression equation.

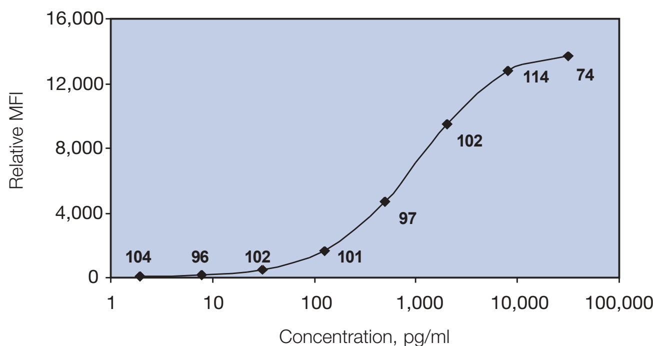

Fig. 4. Human IL-8 broad range standards assay run at the low PMT setting and analyzed using a 5PL regression routine. Each standard is labeled with the corresponding recovery. The low PMT setting yielded accurate recovery (70–130%) across the entire range of standards.

Table 5. Comparison of the recovery of GM-CSF high-sensitivity standards assayed at low and high PMT settings and analyzed using a 5PL regression. The higher PMT setting yielded accurate recovery across the entire range of standards. The recoveries of lower concentrations (0.78 and 0.2 pg/ml) were not accurate using the low PMT setting.

Concentration (pg/ml)	Recovery at Low PMT	Recovery at High PMT
3,200	100	100
800	100	100
200	100	100
50	99	100
12.5	108	102
3.13	95	102
0.78	OOR	81
0.2	1,489	85

Guidelines for Running Cytokine Assays

The overall assay performance must be taken into consideration when determining how to set up both the cytokine assay standards and the Bio-Plex array reader. In Table 6, the guidelines for optimizing the Bio-Plex system (including assays and the array reader) are shown. The standards as well as the PMT voltage chosen for reading the assay may significantly affect results.

Table 6. Guidelines for running cytokine assays on the Bio-Plex system.

Predicted Fluorescence Intensities	Standard Curve to Use	Assay Results	RP1 Target for CAL2 Calibration	Resulting PMT Voltage
Low	High-sensitivity range	Maximum sensitivity	High RP1 target	Higher
Varied	Broad range	Wide dynamic range	Low RP1 target	Lower

Selecting a Custom Set of Standards

The two sets of standards and RP1 target values discussed in this tech note have been validated using the Bio-Plex system and have been shown to yield optimal results. Efficient assay performance may also be achieved using a custom set of standards as long as a few simple rules are followed.

1. Select a minimum of 8 standard curve points.

The logistic regression model requires a minimum of 5 standards for the 4PL and 6 standards for the 5PL to fulfill the analysis requirements (Motulsky 1996). A total of 8 points are recommended to ensure an accurate fit, with a maximum of 12 points (Baud 1993). This practice allows the flexibility to remove standard points with erroneous results due to errors in assay preparation.

2. Choose standards evenly distributed across the entire range of concentrations.

As noted earlier, the response of a Bio-Plex assay is nonlinear; there is a flattening of the response near zero and at the high end of the curve with a linear portion in the middle as shown in Figure 2. Due to the nature of this response, the chosen standards concentrations should be evenly distributed across the entire range (Nix and Wild 2001). The regression routine will then yield the most accurate representation of the assay response.

3. Choose a 5PL regression routine.

When conditions are optimal, the upper and lower portions of the curve are symmetrical, and the 4PL logistic routine is appropriate. In reality, many conditions, such as a high-dose hook effect and inadequate washing, can cause asymmetry in the curve (Nix and Wild 2001). The 5PL regression contains an additional parameter to accommodate asymmetry in the curve, and thus is the more robust type of logistic regression for Bio-Plex assays.

4. Prepare standards using serial dilutions.

When selecting the range of a custom set of standards, determine the lowest and highest desired concentrations, then determine what dilution series should be used to yield a total of 8 points within the range. For example, if the low and high desired values are 5 and 10,000 pg/ml respectively, a three-fold dilution series would yield 8 points (10,000, 3,333, 1,111, 370, 123.4, 41.1, 13.7, and 4.6). A dilution series is simple to prepare and less prone to errors compared to preparation of individual concentrations.

5. Determine which PMT setting is optimal.

Read the assay on the Bio-Plex array reader after calibrating with the low RP1 target value, then the high value, to determine which setting yields the best results. An assay plate may be read a second time by aspirating the contents of the wells after the first reading and resuspending in 125 µl of buffer.

Summary

Bio-Plex assays may be run with a variety of standard curve concentrations. Two sets of concentrations are recommended, a high-sensitivity range for maximum sensitivity or for samples such as serum that fall within the biological range of cytokines, and a broad range for samples where a wide range of cytokine concentrations are expected. The voltage of the reporter PMT may also be varied to maximize assay performance. The high-sensitivity standards should be run following calibration with the high RP1 target value and the broad range standards perform best following calibration with the low RP1 target value. Overall, the high-sensitivity standards yield better sensitivity, while maximum dynamic range is achieved using the broad range standards. Excellent assay performance may also be achieved using a unique set of standards if a few simple rules are followed: 1) select a minimum of 8 standard curve concentrations, 2) select a set of concentrations that are evenly distributed across the entire selected range, 3) select a 5PL regression in Bio-Plex Manager™ software for the most robust assay analysis, 4) prepare the standard curve points using a serial dilution method, and 5) determine which PMT setting is optimal.

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