Application Note

Improved analysis of multiplexed biomarker quantitation data with MILLIPLEX® Analyst 5.1 software

Introduction

Analyzing data from multiplexed biomarker assays can be difficult when working with diverse sample and analyte types. This diversity can lead to a wide range of possible analyte levels and assay signal intensity with respect to those analyte levels, both of which are not always easy to predict or determine accurately. Merck Millipore, in partnership with VigeneTech®, has updated and improved the multiplex data analysis software tool, MILLIPLEX® Analyst, resulting in the release of MILLIPLEX® Analyst 5.1 software. Our improved software is designed to generate the most reliable and meaningful quantitative output with a focus on data derived from the low and high ends of standard curves. Data in these regions can be important and are commonly deemed incalculable by existing multiplex data analysis packages.

To obtain the most appropriate curves for the widest array of multiplex experiments, several critical improvements were made to the curve fitting models in MILLIPLEX® Analyst 5.1 software. These improvements included intelligently setting initial parameters and optimizing mathematical methods to minimize fitting errors. In developing the new curve fitting algorithms simulations were run on a very large set of actual experimental standard curves to determine the curve fit models that would give the lowest CVs at the low and high end of the curves and that would work well even with standard curves of low quality.

To produce the concentration output, MILLIPLEX® Analyst makes estimates and performs iterations until convergence to the best solution. To test the ability of several existing multiplex data analysis software packages, four diverse Merck Millipore MILLIPLEX® MAP kits, representing different research focus areas, were used to analyze biological samples using the Luminex 200™ system. MILLIPLEX® Analyst 5.1 data were compared to data obtained using other commercially available analysis tools: Bio-Plex® Manager 6.1 and StatLIA® 3.2 software packages. Overall, MILLIPLEX® Analyst 5.1 software was consistently the most sensitive multiplex data analysis tool.

Materials and Methods

Following manufacturer-recommended protocols, four different MILLIPLEX® MAP multiplex kits were used to analyze serial dilutions of included purified standards and biological samples (serum, urine or tissue culture) on a Luminex 200™ system. The MILLIPLEX® MAP kits tested were:

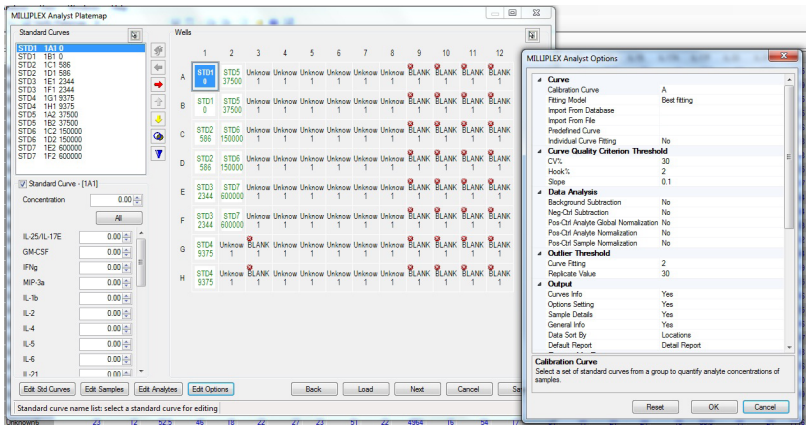
- **MILLIPLEX® MAP Human Cardiovascular Disease (CVD) Panel 1** (Cat. No. HCVD1MAG-67K)
- **MILLIPLEX® MAP Mouse TH17 Magnetic Bead Panel - Premixed 25 plex** (Cat. No. MT17MAG47K-PX25)
- **MILLIPLEX® MAP Rat Kidney Toxicity Panel 2** (Cat. No. RKTX2MAG-37K)
- **MILLIPLEX® MAP Rat Cytokine/Chemokine Magnetic Bead Panel – Premixed 27 Plex** (Cat. No. RECYMAG65K27PMX)

Kits selected for testing were based on having known low or high levels of certain analytes in biological samples. These analytes could be problematic for generation of analyte concentration data.

Samples were prepared using the dilutions recommended in the kit protocol. All calculated concentrations listed here were not adjusted for the dilution if one was required. This allowed easier interpretation, because the unadjusted sample concentrations fall on the curve fitted to the data points corresponding to standards of known concentration.

The resulting .csv data files generated from the xPonent® 3.1 software were exported and subsequently further analyzed with MILLIPLEX® Analyst 5.1, StatLIA® 3.2, and Bio-Plex® Manager 6.1 software packages. Figure 1 shows an example of the user interface for MILLIPLEX® Analyst 5.1 software. The data reduction settings used for each software are described below.

Figure 1.
Analysis options and plate layout for MILLIPLEX® MAP Mouse TH17 Magnetic Bead Panel in MILLIPLEX® Analyst 5.1 software.



MILLIPLEX® Analyst 5.1 Software

Each .csv file was analyzed twice using different curve fitting models. The first run used the "Best Fitting" option with no background subtraction. This model runs multiple types of curve-fitting equations (weighted and unweighted) and selects the one that generates the best recoveries. The second run employed 5 parameter (log scale) curve fit with no background subtraction; this is an unweighted model.

Background Subtraction: Background subtraction is typically used when there is a linear curve. However, most multiplex assays fall in a non-linear exponential curve. Hence, an arithmetic background subtraction is not a true representation of where the data point should lie. This is especially true for low affinity curves where there is a low signal-to-noise ratio for the first standard point.

Also, in some cases the background signal may actually be higher than the first standard point, which would cause the software analysis to delete the point or give it a negative value, whereas the issue may actually be with the fluorescent intensity of the background wells. Removing these values would reduce the number of standard points and in some cases cause the algorithm to not perform the calculations correctly. This can result in poor curve fittings, which are not due to poor data output.

For these reasons, in most cases, we recommend to use the "Background" wells as a "0" Standard Point and to not subtract the background mean fluorescence intensity (MFI). We also recommend determining empirically the effect of background subtraction. The effect of background subtraction was determined empirically for all analyses in this study, and in all cases, background subtraction was not applied as an option in MILLIPLEX® Analyst 5.1 software.

Bio-Plex® Manager 6.1 Software

The initial data analysis used the default settings:

- 5P Log (log(x)-linear(y) scale)
- Weighting=power law variance
- Coefficient A=auto-calculated
- Coefficient B=1.8

Upon completion of the first run, the data were reanalyzed using the Standard Curve Optimizer, which identifies and removes outliers demonstrating poor recovery.

StatLIA® 3.2 Software

Data were analyzed using a 5 parameter logistic curve with the following weighting definitions:

- Var Expression=Adj
- Variance Line=Power
- Standard & Unknown Weighting Coefficient A=0.01184
- Standard & Unknown Weighting Coefficient B=1.8
- Degrees of Freedom=999

The reportable range limits were set to 0.0000001 and 1000000.

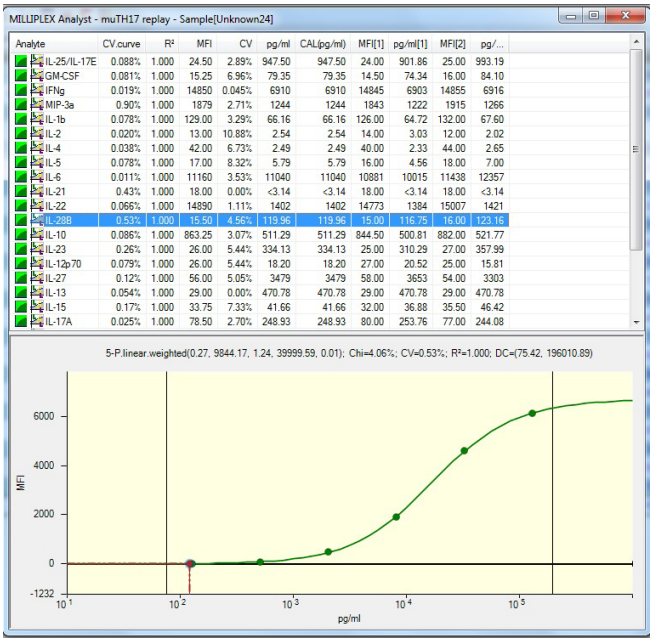


Figure 2.
Data for sample Unknown 24 for IL-28B. Although the data are at the low end of the curve, MILLIPLEX® Analyst 5.1 software "data point" is able to precisely calculate the concentration of IL-28B in this sample.

Results

Quantification of IL-28B using the Mouse TH17 Magnetic Bead Panel (Figure 2). MILLIPLEX® Analyst 5.1 software showed better sensitivity at the bottom part of the curve than did Bio-Plex® software. MILLIPLEX® Analyst 5.1 software generated similar results to those generated using StatLIA® software (Table 1). The Bio-Plex® software Optimized Data method was used because this generated the optimal curve according to the software calculation's recommendations. In MILLIPLEX® Analyst 5.1 software, the Best Fitting option selected the 5P Linear model. For most cases, the 5P Linear model leads to a better fit at lower concentrations (and thus improved sensitivity) than 5P Log due to the use of the linear scale instead of log scale. However, there are cases in which weighting used along with 5P Log could lead to a better fit at lower concentrations. The Best Fitting option in MILLIPLEX® Analyst 5.1 software tests multiple models to find the curve that best fits the data. Tested models included weighted and unweighted versions of the following equations: 4 parameter (log scale), 5 parameter (linear scale), and 5 parameter (log scale). Five-parameter curve fitting model (linear scale) and Five-parameter curve fitting model (log scale). The best curve fit is then chosen based on minDC, R^2, recovery, X^2 testing and other parameters.

Analyte: IL-28B, Kit: Mouse TH17
Units: pg/mL

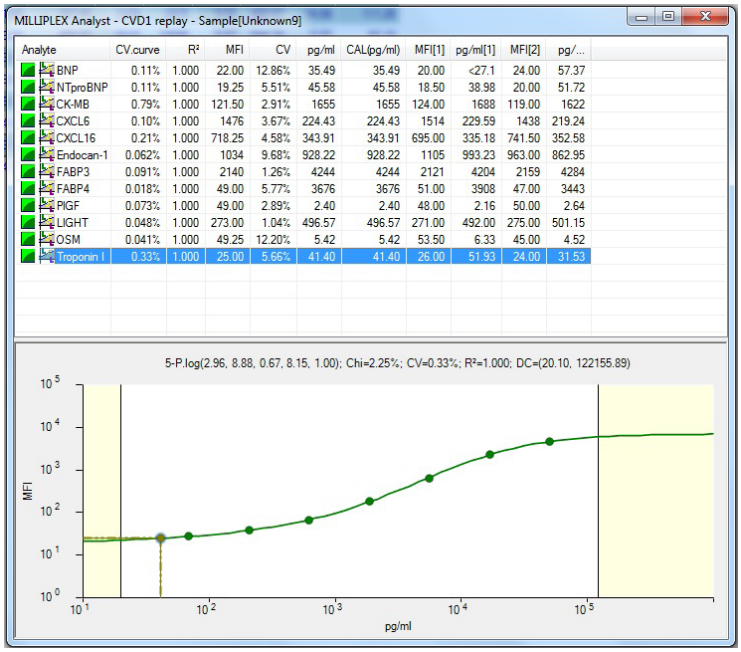
	MILLIPLEX® Analyst 5.1*	StatLIA®	Bio-Plex®
Standard1	134.2	127.0	127.3
Standard2	470.5	503.0	502.8
Standard3	2099.0	2073.0	2072.8
Standard4	8047.0	7887.0	7887.9
Standard5	32635.0	34418.0	34409.9
Standard6	129603.0	121675.0	121695.5
Unknown1	**113.5**	**99.0**	**OOR <**
Unknown2	2744.0	2684.0	2684.0
Unknown3	2566.0	2514.0	2514.5
Unknown4	10125.0	10018.0	10018.6
Unknown5	**107.0**	**90.0**	**OOR <**
Unknown6	**126.3**	**117.0**	**OOR <**
Unknown7	**93.6**	**70.0**	**OOR <**
Unknown8	**93.6**	**70.0**	**OOR <**
Unknown9	**107.0**	**90.0**	**OOR <**
Unknown10	**126.3**	**117.0**	**OOR <**
Unknown11	**105.3**	**87.0**	**OOR <**
Unknown12	205.4	215.0	215.3
Unknown13	160.2	161.0	160.6
Unknown14	**121.6**	**110.0**	**OOR <**
Unknown15	**118.4**	**106.0**	**OOR <**
Unknown16	**121.6**	**110.0**	**OOR <**
Unknown17	**123.2**	**113.0**	**OOR <**
Unknown18	142.0	138.0	137.4
Unknown19	**98.7**	**78.0**	**OOR <**
Unknown20	**102.0**	**83.0**	**OOR <**
Unknown21	**107.0**	**90.0**	**OOR <**
Unknown22	**118.4**	**106.0**	**OOR <**
Unknown23	**98.7**	**78.0**	**OOR <**
Unknown24	**120.0**	**108.0**	**OOR <**

Table 1.
Significantly more IL-28 concentrations could be calculated at the low end of the curve in the Mouse TH17 Magnetic Bead Panel by MILLIPLEX® Analyst 5.1 software compared to StatLIA® (extrapolated values) and Bio-Plex® software packages.

Green: Extrapolated value
OOR<: Out of Range Below
*Best Fitting, 5P lin weighted

Quantification of Troponin I using the Human CVD Panel. MILLIPLEX® Analyst 5.1 software calculated Troponin I concentrations more precisely at the low end of the standard curve compared to Bio-Plex® and StatLIA® software (Figure 3 and Table 2). MILLIPLEX® Analyst 5.1 software results from the Best Fitting option and the 5P Log option are shown. In this case, the Best Fitting option used a 5P Log model.

Figure 3.
Precise quantification of Troponin 1 in Sample Unknown 9 using the human CVD panel as calculated by MILLIPLEX® Analyst 5.1 software, using 5P Log curve fit with Best Fitting option.



Analyte: Troponin I, Kit: Human CVD
Units: pg/mL

	MILLIPLEX® Analyst 5.1*	MILLIPLEX® Analyst 5.1**	StatLIA®	Bio-Plex®
Standard1	68.7	70.0	54.0	54.3
Standard2	204.2	200.8	227.0	226.6
Standard3	619.0	611.6	620.0	619.7
Standard4	1905.0	1910.0	1828.0	1827.8
Standard5	5308.0	5375.0	5544.0	5544.0
Standard6	17085.0	17260.0	16729.0	16729.1
Standard7	49767.0	48665.0	49912.0	49911.6
Unknown1	700.8	693.5	693.0	693.4
Unknown2	6253.0	6337.0	6611.0	6611.2
Unknown3	111.3	110.7	117.0	117.4
Unknown4	**60.2**	**61.9**	**39.0**	**39.0**
Unknown5	**44.0**	**46.5**	**1.0**	**1.3**
Unknown6	**31.5**	**34.7**	**OOR<**	**OOR <**
Unknown7	**51.9**	**54.1**	**22.0**	**22.2**
Unknown8	**31.5**	**34.7**	**OOR<**	**OOR <**
Unknown9	**41.4**	**44.1**	**OOR<**	**OOR <**
Unknown10	214.5	210.8	238.0	237.6

Table 2.
Using both curve-fitting models, MILLIPLEX® Analyst 5.1 demonstrated significantly greater ability than StatLIA® (extrapolated values) and Bio-Plex® software to accurately quantify low levels of Troponin I in samples assayed with the Human CVD Magnetic Bead Panel.

Green: Extrapolated value
Yellow: Extrapolated value
OOR<: Out of Range Below
*Best Fitting, 5P Log
**5P Log

Percent Recovery for Troponin I. Recovery used to assess how accurately an algorithm is able to calculate samples with known concentrations. As seen in Table 3, MILLIPLEX® Analyst 5.1 is able to precisely determine (close to 100%) even the lowest actual concentration values in the Standard Curve. Typically, any value between 90% and 110% is considered a reliable recovery.

Recovery* Troponin I

	MILLIPLEX® Analyst 5.1	StatLIA®	Bio-Plex®
Standard1	**100.18%**	**78.7%**	**79%**
Standard2	99.26%	110.3%	110%
Standard3	100.27%	100.4%	100%
Standard4	102.88%	98.7%	99%
Standard5	95.55%	99.8%	100%
Standard6	102.51%	100.4%	100%
Standard7	99.54%	99.8%	100%

Table 3.
Recovery for Troponin I. MILLIPLEX® Analyst 5.1 software calculated Troponin I recoveries close to 100% at all concentrations.

*Recovery is a parameter to assess the quality of a curve fit
Recovery =(Observed concentration / Expected concentration) X 100%

Quantification of IL-6 using the Mouse TH17 Magnetic Bead Panel. MILLIPLEX® Analyst 5.1 software calculated concentrations more precisely at the high end of the standard curve compared to Bio-Plex® software. MILLIPLEX® Analyst 5.1 software generated similar results to StatLIA® software (Table 4).

Analyte: IL-6, Kit: Mouse TH17
Units: pg/mL

	MILLIPLEX® Analyst 5.1*	StatLIA®	Bio-Plex®
Standard1	7.8	7.8	7.8
Standard2	31.2	31.0	31.0
Standard3	125.2	125.9	125.9
Standard4	499.9	498.4	498.3
Standard5	2000.0	2000.9	2001.3
Standard6	8000.0	8000.0	8000.0
Unknown1	16.5	16.3	16.3
Unknown2	141.1	142.0	141.9
Unknown3	125.3	126.1	126.0
Unknown4	560.9	558.8	558.7
Unknown5	9.9	9.9	9.9
Unknown6	**7.3**	**7.4**	**OOR <**
Unknown7	136.0	136.9	136.8
Unknown8	2076.0	2076.7	2077.1
Unknown9	**14289.0**	**>1000000**	**OOR >**
Unknown10	3292.0	3218.9	3222.1
Unknown11	>15808 ↑	>1000000	OOR >
Unknown12	4646.0	4287.1	4309.2
Unknown13	>15808 ↑	>1000000	OOR >
Unknown14	>15808 ↑	>1000000	OOR >
Unknown15	>15808 ↑	>1000000	OOR >
Unknown16	>15808 ↑	>1000000	OOR >
Unknown17	>15808 ↑	>1000000	OOR >
Unknown18	>15808 ↑	>1000000	OOR >
Unknown19	3623.0	3501.1	3506.6
Unknown20	**15442.0**	**>1000000**	**OOR >**
Unknown21	3453.0	3357.9	3362.1
Unknown22	>15808 ↑	>1000000	OOR >
Unknown23	49.6	49.6	49.6
Unknown24	**11040.0**	**>1000000**	**OOR >**

Table 4.
Greater sample detectability is evident at both the high and low ends of the curve in the Mouse TH17 Magnetic Bead Panel by MILLIPLEX® Analyst 5.1 software compared to StatLIA® and Bio-Plex® software packages.

Green:	Extrapolated value
OOR>:	Out of Range Above
OOR<:	Out of Range Below
>1000000:	Out of Range Above
>15808 ↑ :	Out of Range Above
*Best Fitting, 5P lin weighted	

Quantification of IL-23 and IL-33 using the Mouse TH17 Magnetic Bead Panel. MILLIPLEX® Analyst 5.1 software exhibited greater sensitivity at the low end of the standard curves than did Bio-Plex® software. MILLIPLEX® Analyst 5.1 software generated results similar to StatLIA® extrapolated values (Tables 5 and 6).

Table 5.
Significantly more IL-23 concentrations could be calculated at the low end of the curve in the Mouse TH17 Magnetic Bead Panel by MILLIPLEX® Analyst 5.1 software compared to the Bio-Plex® software.

Analyte: IL-23, Kit: Mouse TH17
Units: pg/mL

	MILLIPLEX® Analyst 5.1*	StatLIA®	Bio-Plex®
Standard1	346.1	355.0	345.4
Standard2	1320.0	1273.0	1335.5
Standard3	5648.0	5665.0	5634.3
Standard4	21603.0	22551.0	21472.8
Standard5	87650.0	85871.0	88125.6
Standard6	349975.0	350013.0	349463.5
Unknown1	**150.1**	**180.0**	**OOR <**
Unknown2	5815.0	5838.0	5799.4
Unknown3	5216.0	5216.0	5207.0
Unknown4	22490.0	23486.0	22357.6
Unknown5	**156.0**	**185.0**	**OOR <**
Unknown6	**144.2**	**175.0**	**OOR <**
Unknown7	**126.5**	**159.0**	**OOR <**
Unknown8	**262.7**	**280.0**	**OOR <**
Unknown9	387.8	392.0	388.7
Unknown10	**250.8**	**269.0**	**OOR <**
Unknown11	633.4	619.0	640.9
Unknown12	477.5	474.0	481.2
Unknown13	753.5	731.0	763.2
Unknown14	358.0	365.0	357.8
Unknown15	651.4	636.0	659.3
Unknown16	813.7	788.0	824.3
Unknown17	585.4	574.0	591.8
Unknown18	825.7	799.0	836.5
Unknown19	**286.5**	**301.0**	**OOR <**
Unknown20	723.5	703.0	732.7
Unknown21	**114.7**	**149.0**	**OOR <**
Unknown22	358.0	365.0	357.8
Unknown23	**85.4**	**123.0**	**OOR <**
Unknown24	**334.1**	**344.0**	**OOR <**

Green: Extrapolated value
OOR<: Out of Range Below
*Best Fitting, 5P lin weighted

Analyte: IL-33, Kit: Mouse TH17
Units: pg/mL

	MILLIPLEX® Analyst 5.1*	StatLIA®	Bio-Plex®
Standard1	78.4	79.0	79.1
Standard2	304.5	305.0	304.5
Standard3	1307.0	1297.0	1297.0
Standard4	4824.0	4833.0	4834.5
Standard5	20149.0	20674.0	20662.5
Standard6	79956.0	78169.0	78193.2
Unknown1	**35.5**	**35.0**	**OOR <**
Unknown2	1520.0	1509.0	1508.7
Unknown3	1450.0	1439.0	1439.0
Unknown4	5905.0	5935.0	5936.6
Unknown5	**37.7**	**37.0**	**OOR <**
Unknown6	<4.34 ↓	1.0	OOR <
Unknown7	**22.3**	**21.0**	**OOR <**
Unknown8	**15.7**	**15.0**	**OOR <**
Unknown9	250.2	251.0	250.6
Unknown10	**12.4**	**11.0**	**OOR <**
Unknown11	**29.4**	**29.0**	**OOR <**
Unknown12	1547.0	1535.0	1534.6
Unknown13	**31.1**	**31.0**	**OOR <**
Unknown14	**4.7**	**3.0**	**OOR <**
Unknown15	**11.8**	**11.0**	**OOR <**
Unknown16	110.5	111.0	111.4
Unknown17	**15.7**	**15.0**	**OOR <**
Unknown18	**21.7**	**21.0**	**OOR <**
Unknown19	**17.3**	**16.0**	**OOR <**
Unknown20	**11.8**	**11.0**	**OOR <**
Unknown21	**4.7**	**3.0**	**OOR <**
Unknown22	**<4.34 ↓**	**0.0**	**OOR <**
Unknown23	139.4	140.0	140.3
Unknown24	**8.0**	**6.0**	**OOR <**

Table 6.
Significantly more IL-33 concentrations could be calculated at the low end of the curve in the Mouse TH17 Magnetic Bead Panel by MILLIPLEX® Analyst 5.1 software compared to the Bio-Plex® software.

Green: Extrapolated value
OOR<: Out of Range Below
<4.34 ↓ : Out of Range Below
*Best Fitting, 5P lin weighted

Quantification of analytes GM-CSF, IL-12p70, and IFNγ using the Rat Cytokine/Chemokine Magnetic Bead Panel. MILLIPLEX® Analyst 5.1 software showed greater sensitivity than did Bio-Plex® and StatLIA® software packages for multiple analytes detected by the Rat Cytokine/Chemokine Panel (Tables 7, 8, 10). Many "Unknown" samples contained very low levels of other analytes (IL-23, IL-17a, IL-5, IL-13, IL-18, MCP-1, IP-10, GRO/KC, VEGF, and Fractalkine), and these analyte concentrations on the low end of their respective curves were successfully calculated with MILLIPLEX® Analyst 5.1 software but were not reliably quantified with the other software packages (data not shown).

Table 7.
Significantly more GM-CSF concentrations could be calculated at the low end of the curve in the Rat Cytokine/Chemokine Magnetic Bead Panel by MILLIPLEX® Analyst 5.1 software compared to the Bio-Plex® software.

Analyte: GM-CSF, Kit: Rat Cytokine/Chemokine
Units: pg/mL

	MILLIPLEX® Analyst 5.1*	StatLIA®	Bio-Plex®
Standard1	4.9	4.9	4.9
Standard2	19.3	19.4	19.4
Standard3	78.8	78.8	78.8
Standard4	311.7	311.1	311.1
Standard5	1250.0	1250.9	1250.6
Standard6	5001.0	5004.8	5006.9
Standard7	19994.0	19966.9	19957.7
Unknown1	<0.75 ↓	<1.0e-07	OOR <
Unknown2	**4.7**	**4.7**	**OOR <**
Unknown3	19.8	19.9	19.9
Unknown4	77.1	77.1	77.2
Unknown5	305.2	304.6	304.6
Unknown6	1156.0	1155.8	1155.5
Unknown7	4932.0	4936.8	4938.9
Unknown8	19255.0	19218.7	19211.6
Unknown9	<0.75 ↓	<1.0e-07	OOR <
Unknown10	<0.75 ↓	<1.0e-07	OOR <
Unknown11	<0.75 ↓	<1.0e-07	OOR <
Unknown12	<0.75 ↓	<1.0e-07	OOR <
Unknown13	**<0.75 ↓**	**0.0**	**OOR <**
Unknown14	<0.75 ↓	<1.0e-07	OOR <
Unknown15	<0.75 ↓	<1.0e-07	OOR <
Unknown16	**2.0**	**2.0**	**OOR <**
Unknown17	**1.4**	**1.4**	**OOR <**
Unknown18	**1.9**	**1.9**	**OOR <**
Unknown19	<0.75 ↓	<1.0e-07	OOR <
Unknown20	<0.75 ↓	<1.0e-07	OOR <
Unknown21	<0.75 ↓	<1.0e-07	OOR <
Unknown22	<0.75 ↓	<1.0e-07	OOR <
Unknown23	<0.75 ↓	<1.0e-07	OOR <
Unknown24	<0.75 ↓	<1.0e-07	OOR <
Unknown25	<0.75 ↓	<1.0e-07	OOR <
Unknown26	<0.75 ↓	<1.0e-07	OOR <
Unknown27	<0.75 ↓	<1.0e-07	OOR <
Unknown28	<0.75 ↓	0.6	OOR <
Unknown29	<0.75 ↓	0.0	OOR <
Unknown30	**0.8**	**0.7**	**OOR <**
Unknown31	<0.75 ↓	<1.0e-07	OOR <
Unknown32	<0.75 ↓	<1.0e-07	OOR <

Green: Extrapolated value
OOR<: Out of Range Below
<0.75 ↓ : Out of Range Below
<1.0e-07: Out of Range Below
*Best Fitting, 5P Log

Analyte: IL-12p70, Kit: Rat Cytokine/Chemokine
Units: pg/mL

	MILLIPLEX® Analyst 5.1*	StatLIA®	Bio-Plex®
Standard1	12.2	10.0	10.5
Standard2	49.6	55.0	54.9
Standard3	193.0	192.0	192.0
Standard4	764.4	744.0	743.9
Standard5	3269.0	3319.0	3318.8
Standard6	12150.0	12177.0	12176.6
Standard7	50276.0	50457.0	50457.3
Unknown1	**3.5**	**<1.0e-07**	**OOR <**
Unknown2	**7.1**	**1.0**	**OOR <**
Unknown3	25.8	29.0	28.7
Unknown4	110.6	115.0	114.6
Unknown5	527.7	511.0	510.8
Unknown6	2903.0	2941.0	2941.0
Unknown7	10563.0	10630.0	10630.0
Unknown8	44341.0	44059.0	44059.3
Unknown9	**4.6**	**<1.0e-07**	**OOR <**
Unknown10	**3.7**	**<1.0e-07**	**OOR <**
Unknown11	**6.8**	**0.0**	**OOR <**
Unknown12	**6.8**	**0.0**	**OOR <**
Unknown13	**10.3**	**7.0**	**OOR <**
Unknown14	**10.7**	**8.0**	**OOR <**
Unknown15	**11.0**	**9.0**	**OOR <**
Unknown16	60.2	66.0	65.7
Unknown17	42.3	47.0	47.2
Unknown18	50.0	55.0	55.4
Unknown19	<2.93 ↓	<1.0e-07	OOR <
Unknown20	**3.5**	**<1.0e-07**	**OOR <**
Unknown21	**4.9**	**<1.0e-07**	**OOR <**
Unknown22	**4.0**	**<1.0e-07**	**OOR <**
Unknown23	<2.93 ↓	<1.0e-07	OOR <
Unknown24	<2.93 ↓	<1.0e-07	OOR <
Unknown25	**9.2**	**5.0**	**OOR <**
Unknown26	**9.2**	**5.0**	**OOR <**
Unknown27	**8.5**	**4.0**	**OOR <**
Unknown28	18.5	20.0	19.6
Unknown29	14.5	14.0	14.0
Unknown30	19.0	20.0	20.2
Unknown31	<2.93 ↓	<1.0e-07	OOR <
Unknown32	<2.93 ↓	<1.0e-07	OOR <

Table 8.
Significantly more IL-12p70 concentrations could be calculated at the low end of the curve in the Rat Cytokine/Chemokine Magnetic Bead Panel by MILLIPLEX® Analyst 5.1 software compared to the Bio-Plex® and StatLIA® software packages.

Green: Extrapolated value
OOR<: Out of Range Below
<2.93 ↓ : Out of Range Below
<1.0e-07: Out of Range Below
*Best Fitting, 5P Log

Recovery for IL-12p70 and IFNγ. As illustrated in Tables 9 and 11, MILLIPLEX® Analyst 5.1 software calculated consistently better recoveries at the lower concentrations of the Standard Curves for both IL-12p70 and IFNγ. These better recoveries were also reflected in the ability to accurately calculate lower concentrations of Unknown samples compared to other tested software packages, which were not able to determine a value.

Recovery* IL-12p70

	MILLIPLEX® Analyst 5.1	StatLIA®	Bio-Plex®
Standard1	99.62%	81.9%	86%
Standard2	101.54%	112.6%	112%
Standard3	98.82%	98.3%	98%
Standard4	97.85%	95.2%	95%
Standard5	104.61%	106.2%	106%
Standard6	97.2%	97.4%	97%
Standard7	100.55%	100.9%	101%

*Recovery is a parameter to assess the quality of a curve fit
Recovery =(Observed concentration / Expected concentration) X 100%

Table 9.
Recovery for IL-12p70. MILLIPLEX® Analyst 5.1 software calculated IL-12p70 recoveries close to 100% at all concentrations.

Analyte: IFNγ, Kit: Rat Cytokine/Chemokine
Units: pg/mL

Table 10.
Significantly more IFNγ concentrations could be calculated at the low end of the curve in the Rat Cytokine/Chemokine Magnetic Bead Panel by MILLIPLEX® Analyst 5.1 software compared to the Bio-Plex® and StatLIA® software packages.

	MILLIPLEX® Analyst 5.1*	StatLIA®	Bio-Plex®
Standard1	14.7	3.0	
Standard2	57.4	67.0	53.7
Standard3	241.6	245.0	248.6
Standard4	932.1	897.0	908.1
Standard5	3683.0	3858.0	3820.0
Standard6	15184.0	14769.0	14824.5
Standard7	59874.0	61392.0	60975.2
Unknown1	<3.40 ↓	<1.0e-07	OOR <
Unknown2	**16.8**	**8.0**	**OOR <**
Unknown3	51.3	60.0	45.0
Unknown4	197.1	205.0	205.8
Unknown5	844.1	809.0	821.3
Unknown6	3412.0	3564.0	3531.3
Unknown7	14639.0	14296.0	14339.3
Unknown8	70718.0	82002.0	78697.5
Unknown9	<3.40 ↓	<1.0e-07	OOR <
Unknown10	<3.40 ↓	<1.0e-07	OOR <
Unknown11	<3.40 ↓	<1.0e-07	OOR <
Unknown12	**3.7**	**<1.0e-07**	**OOR <**
Unknown13	<3.40 ↓	<1.0e-07	OOR <
Unknown14	<3.40 ↓	<1.0e-07	OOR <
Unknown15	**5.0**	**<1.0e-07**	**OOR <**
Unknown16	**16.8**	**8.0**	**OOR <**
Unknown17	**24.0**	**22.0**	**OOR <**
Unknown18	**28.6**	**29.0**	**1.1**
Unknown19	<3.40 ↓	<1.0e-07	OOR <
Unknown20	<3.40 ↓	<1.0e-07	OOR <
Unknown21	<3.40 ↓	<1.0e-07	OOR <
Unknown22	**14.7**	**3.0**	**OOR <**
Unknown23	<3.40 ↓	<1.0e-07	OOR <
Unknown24	<3.40 ↓	<1.0e-07	OOR <
Unknown25	6.8	<1.0e-07	OOR <
Unknown26	<3.40 ↓	<1.0e-07	OOR <
Unknown27	<3.40 ↓	<1.0e-07	OOR <
Unknown28	**5.9**	**<1.0e-07**	**OOR <**
Unknown29	**4.5**	**<1.0e-07**	**OOR <**
Unknown30	**8.2**	**<1.0e-07**	**OOR <**
Unknown31	<3.40 ↓	<1.0e-07	OOR <
Unknown32	<3.40 ↓	<1.0e-07	OOR <

Green:	Extrapolated value
Orange:	Extrapolated value
OOR<:	Out of Range Below
<3.40 ↓ :	Out of Range Below
<1.0e-07:	Out of Range Below
*Best Fitting, 5P Log	

Recovery* IFNγ

Table 11.
Recovery for IFNγ. MILLIPLEX® Analyst 5.1 software calculated IFNγ recoveries close to 100% at all concentrations.

	MILLIPLEX® Analyst 5.1	StatLIA®	Bio-Plex®
Standard1	100.4%	20.5%	excluded
Standard2	97.96%	114.3%	92
Standard3	103.08%	104.5%	106
Standard4	99.42%	95.7%	97
Standard5	98.21%	102.9%	102
Standard6	101.23%	98.5%	99
Standard7	99.79%	102.3%	102

*Recovery is a parameter to assess the quality of a curve fit
Recovery =(Observed concentration / Expected concentration) X 100%

Quantification of EGF using the Rat Kidney Toxicity Panel 2. MILLIPLEX® Analyst 5.1 software calculated EGF concentrations more precisely at the high end of the standard curve compared to Bio-Plex® and StatLIA® software (Table 12).

Analyte: EGF, Kit: Rat Kidney Toxicity Panel 2
Units: pg/mL

	MILLIPLEX® Analyst 5.1*	StatLIA®	Bio-Plex®
Standard1	0.00	0.00	0.00
Standard2	0.01	0.01	0.01
Standard3	0.03	0.03	0.03
Standard4	0.07	0.07	0.07
Standard5	0.21	0.23	0.23
Standard6	0.76	0.67	0.67
Standard7	1.91	1.99	2.00
Unknown1	0.03	0.03	0.03
Unknown2	0.12	0.14	0.14
Unknown3	1.38	0.96	0.96
Unknown4	1.25	0.90	0.90
Unknown5	**1.98**	**>1000000**	**OOR >**
Unknown6	**2.15**	**>1000000**	**OOR >**
Unknown7	1.58	1.05	1.04
Unknown8	0.71	0.64	0.64
Unknown9	1.52	1.02	1.02
Unknown10	**2.19**	**>1000000**	**OOR >**
Unknown11	1.46	0.99	0.99
Unknown12	1.00	0.79	0.79
Unknown13	0.93	0.76	0.76
Unknown14	1.65	1.09	1.08
Unknown15	0.98	0.78	0.78
Unknown16	1.28	0.92	0.91
Unknown17	1.66	1.09	1.09
Unknown18	1.86	1.30	1.30
Unknown19	1.78	1.18	1.18
Unknown20	**1.99**	**>1000000**	**OOR >**
Unknown21	0.73	0.65	0.65
Unknown22	1.75	1.15	1.15
Unknown23	0.95	0.77	0.77
Unknown24	1.41	0.97	0.97
Unknown25	0.86	0.72	0.72
Unknown26	1.42	0.97	0.97
Unknown27	1.31	0.93	0.92
Unknown28	0.80	0.69	0.69
Unknown29	0.59	0.57	0.56
Unknown30	0.64	0.60	0.59
Unknown31	1.70	1.12	1.12
Unknown32	0.75	0.67	0.66
Unknown33	1.11	0.84	0.84
Unknown34	1.46	0.99	0.99
Unknown35	0.76	0.67	0.67
Unknown36	1.50	1.01	1.01
Unknown37	1.31	0.93	0.93
Unknown38	1.65	1.08	1.08
Unknown39	0.73	0.65	0.65
Unknown40	1.46	0.99	0.99

Table 12.
Significantly more EGF concentrations could be calculated at the high end of the curve in the Rat Kidney Toxicity Magnetic Bead Panel by MILLIPLEX® Analyst 5.1 software compared to the Bio-Plex® and StatLIA® software packages.

OOR>: Out of Range Above
>1000000: Out of Range Above
*Best Fitting, 5P Log

Discussion

Typically, data calculations from biomarker immunoassays are more reliable in the linear portion of a sigmoidal curve. Most multiplex data analysis tools can accurately calculate concentrations in the middle of the curve, but may struggle with the data from the plateaus. In developing MILLIPLEX® Analyst 5.1 software, special attention was paid to improving curve fit quality in these two regions, by refining curve-fitting algorithms and weighting methods. This enhancement typically resulted in the software's ability to generate more, reliable results at high and low concentration extremes for many biologically important analytes, as illustrated in this study. The Five-parameter equations used in MILLIPLEX® Analyst 5.1 software are actually Flexible Five-parameter (linear scale) and a Flexible Five-parameter (log scale) curve-fitting models. MILLIPLEX® Analyst 5.1 software does not just perform a straightforward statistical analysis—the software's algorithms, based on real world data, strengthen/hone/enrich the basic algorithms to produce the most reliable data from a wide variety of standard curves, including "poor" standard curves. For some of these data points, the other data analysis programs either extrapolated these values or reported them as being out of the detectable range of the assay.

To obtain an accurate and meaningful curve fit for the widest variety of analyte data, it is very important to start with a good set of initial parameters. This will guarantee a rapid convergence to the global minimum value. MILLIPLEX® Analyst 5.1 software is able to intelligently set the proper initial parameters based on the data input and the curve-fitting model. This has led to a significant increase in the amount of reliable data generated. In addition, MILLIPLEX® Analyst 5.1 software is designed to prevent overfitting of curves and to strike a balance between minimizing errors and having the most meaningful fitting curve. This balance has resulted in MILLIPLEX® Analyst 5.1 software being the most robust data analysis tool available today for Luminex® platform-based multiplexed immunoassays.

MILLIPLEX Analyst 5.1 Software Ordering Information

Description	Qty	Catalogue No.
MILLIPLEX® Analyst 5.1 Software	1 seat license	40-086
MILLIPLEX® Analyst 5.1 Software	5 seat license	40-087

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