An Optimized Mouse IL-6 ELISA using Sword Diagnostics Peroxidase Reagents

Enhanced Assay Performance using the Tecan Infinite® M200 Multimode Reader

Introduction

Interleukin 6

Interleukin 6 (IL-6) is a multifunctional protein produced by normal and transformed cells, including T cells, monocytes / macrophages, fibroblasts, hepatocytes, vascular endothelial cells, cardiac myxomas, bladder cell carcinomas, myelomas, astrogliomas and glioblastomas. The production of IL-6 in these cells is regulated, either positively or negatively, by a variety of signals including mitogens, antigenic stimulation, lipopolysaccharides, IL-1, TNF, PDGF and viruses. On the basis of its various activities, IL-6 has also been called interferon-β2 (IFN-β2), 26 kDa protein, B-cell stimulatory factor-2 (BSF-2), hybridoma / plasmacytoma growth factor, hepatocyte stimulating factor, cytotoxic T cell differentiation factor, and macrophage-granulocyte inducing factor 2A (MGI-2A).

The various activities of IL-6 suggest that this factor will have a major role in the mediation of the inflammatory and immune responses initiated by infection or injury. Although the exact functions of IL-6 in vivo are not known, elevated IL-6 levels have been reported to be associated with a variety of diseases, including autoimmune diseases such as arthritis and Castleman's disease, mesangial proliferative glomerulonephritis, psoriasis, inflammatory bowel disease, ovarian cancers and malignancies such as plasmacytomas, myelomas, lymphomas and leukemias.

Principle of the ELISA

This assay employs the quantitative sandwich enzyme immunoassay technique. A purified antibody specific for IL-6 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IL-6 present is bound to the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IL-6 is added to the wells. Following a wash to remove any unbound antibody-enzyme conjugate, a mixture of biotinylated anti-mouse IL-6 detection antibody and Streptavidin-horseradish peroxidase conjugate solution is added to the wells. After washing away the unbound reagents, a substrate solution is added, which is catalyzed by the bound enzyme to produce a signal.

The magnitude of this signal is directly proportional to the concentration of mouse IL-6 present in the original specimens. The BD Biosciences mouse IL-6 ELISA is used for the in vitro quantitative determination of mouse IL-6 in serum, plasma, buffered solutions and cell culture media. The assay recognizes both natural and recombinant forms of IL-6.
In this technical note, we describe the steps taken to improve the performance of the BD Biosciences mouse IL-6 ELISA using a new and more sensitive reagent system developed by Sword Diagnostics, on the Tecan Infinite M200 multimode reader.
Note that the description of the mouse IL-6 analyte and of the BD Bioscience IL-6 ELISA are taken from the BD Bioscience IL-6 OptEIA™ ELISA insert (1).

Materials and Methods

Instrument
Tecan Infinite M200 Multimode Reader (Tecan, Austria)

Microplates
- Corning Costar® medium binding Stripwell™ microplates #2693
- Corning Costar high binding Stripwell microplates # 2692

Reagents
The following reagents were obtained from BD Biosciences (Franklin Lakes, NJ) as an OptEIA reagent set (cat # 555240, lot 78698). This set contains:
- Recombinant mouse IL-6 lyophilized standard (#51-26536E)
- Capture antibody – Purified anti-mouse IL-6 (# 51-26531E)
- Detection antibody – Biotinylated anti-mouse IL-6 (# 51-26532E)
- Enzyme Reagent – Streptavidin-horseradish peroxidase conjugate (# 51-9002813)

Additional assay reagents were obtained from BD Biosciences as part of the OptEIA Reagent Set B (Cat. No 550534), including:
- Coating buffer – 0.1 M Sodium carbonate, pH 9.5
- Assay diluent – PBS (phosphate buffered saline) with 10 % FBS (fetal bovine serum), pH 7.0 (This was also used for blocking microplates and as the IL-6 standard diluent)
- Wash buffer – PBS with 0.05 % Tween 20.
- Substrate solution – Tetramethylbenzidine (TMB) and hydrogen peroxide
- Stop solution – 2N H_2SO_4.

The Sword Diagnostics Peroxidase Reagents were obtained from Sword Diagnostics (Chicago, IL), catalog number N818.

Assay Procedures: BD Mouse IL-6 Assay
The BD OptEIA mouse IL-6 ELISA was run as recommended in the package insert (1) up until the addition of chromogen is called for. At this point the Sword Diagnostics Peroxidase Reagents were substituted for the vendor supplied tetramethylbenzidine (TMB) chromogen. When used, the TMB-derived signals (absorbance at 450 nm) were corrected using an absorbance at 570 nm, rather than subtracting the reagent blank signal.

Peroxidase Reactions Using Sword Diagnostics Peroxidase Reagents
1) Sword Diagnostics Peroxidase Substrate / Peroxide Mixture and Development Solution were prepared from concentrated stock solutions as described in the peroxidase assay insert (2). The mixtures were stable at room temperature (15 – 30 °C) for 8 hours.
2) After the post working detector incubation wash step of the ELISA assay was completed, 150 µL of Sword Substrate / Peroxide Mixture was added to each assay well.
3) The microplate was then incubated at room temperature in the dark for 15 minutes.
4) After this incubation, 150 µL of Sword Development Solution was added to each well. To ensure adequate mixing, 50 µL of each well was gently aspirated and dispensed twice, turning the solution from yellow to light pink and confirming that the development process has started.
5) The microplate was then incubated at room temperature in the dark for 30 minutes.

Detection of the Sword Peroxidase Reagent-generated signal was achieved using the fluorescence intensity detection channel and the top read optics of the Infinite M200 multimode reader with the settings in Table 1.

Table 1

Parameter	Setting
Mode	Fluorescent Intensity
Read	Top
Excitation Wavelength	530 nm
Excitation Bandwidth	9 nm
Emission Wavelength	700 nm
Emission Bandwidth	20 nm
Flash Number (Frequency)	25 (100 Hz)
Integration	20 µsec
Gain	170 (manual)

Table 1 Infinite M200 Multimode Reader Measurement Parameters
Note: Auto settings of the gain may also be used.

Data Analysis

The measurement parameters and experimental data was automatically exported from the i-control™ software to a Microsoft Excel® worksheet for further analysis. IL-6 dose response curves were fitted to a four parameter logistic curve (4PLC) using the equation communicated by D. Rodbard (3).

$$Y = D + \frac{(A - D)}{\left[1 + \left(\frac{X}{C}\right)^{B}\right]}$$

Where:
X = Assay response
Y = Concentration
A,B,C,D = Equation parameters.

The analytical limit of detection (LOD) is defined as the concentration read from the fitted 4PLC at a response level equal to the mean negative control level plus twice the standard deviation estimated from the negative control population.

The standard concentration read from the fitted 4PLC at a response level equal to half the response of the highest calibrator run was also determined. This value (Conc.$^{1/2\ Max}$) was used as a metric to describe the observed shift in the Sword Peroxidase Reagent dose response curve toward lower concentrations. This value is related to the shift of the curve: the lower this value, the greater the shift of the curve.

Results and Discussion

The BD Bioscience mouse IL-6 OptEIA ELISA was initially performed as suggested by the vendor. Calibration levels were extended below the lowest recommended calibrator levels cited in the assay insert (15.63 pg/mL) to assess the detection improvements.

This “baseline” assay comprised the following steps:

1) 100 µL of 1:250 dilution of concentrated coating antibody was incubated in a 96-well, medium binding flat bottom clear polystyrene microplate at 2 – 8 °C overnight. All referenced reagents were supplied by the vendor unless stated otherwise.
2) The antibody solution was aspirated and the wells were washed with three 300 µL aliquots of wash buffer.
3) The wells were blocked with 200 µL assay buffer (PBS with 10 % FBS) for one hour at room temperature.
4) Wells were aspirated and washed as described in step 2.
5) 100 µL samples (previously diluted in assay diluent) were added to the appropriate wells and were incubated for two hours at room temperature.
6) The samples were aspirated and the wells were washed with five 300 µL aliquots of wash buffer.
7) 100 µL of working detector solution (a mixture containing a 1:1,000 dilution of biotinylated antibody and 1:250 dilution of streptavidin HRPO conjugate) was added to each well and incubated for one hour at room temperature.
8) The samples were then aspirated, and the wells were washed with seven 300 µL aliquots of wash buffer.
9) The analyte-dependent signal from the washed wells was detected using either the Sword Peroxidase Reagents or the vendor supplied TMB reagents.

These conditions, and many of the subsequent steps modified in this investigation, are summarized in Table 4. The results using the baseline assay procedure are shown in Tables 2 and 3.

Table 2

IL-6 STD (pg/mL)	Mean	SD	N	CV %	SE
0.00	0.03174	0.00337	8	10.6	0.00119
3.91	0.04240	0.00369	4	8.7	0.00184
7.81	0.05270	0.00190	4	3.6	0.00095
15.63	0.08023	0.00588	4	7.3	0.00294
31.25	0.12390	0.00337	4	2.7	0.00168
62.50	0.21745	0.01536	4	7.1	0.00768
125	0.36823	0.00779	4	2.1	0.00390
250	0.71638	0.01821	4	2.5	0.00910
500	1.29455	0.04021	4	3.1	0.02011
1,000	2.21347	0.11302	4	5.1	0.05651
2,000	3.70552	0.10140	4	2.7	0.05070

Table 2 Response Signal Generated With TMB Reagents

Table 3

IL-6 STD (pg/mL)	Mean	SD	N	CV %	SE
0.00	11,138	531	8	4.8	188
3.91	11,168	968	4	8.7	484
7.81	11,522	528	4	4.6	264
15.63	12,761	378	4	3.0	189
31.25	14,840	864	4	5.8	432
62.50	17,090	657	4	3.8	329
125	21,447	484	4	2.3	242
250	26,793	399	4	1.5	199
500	31,957	600	4	1.9	300
1,000	38,294	723	4	1.9	361
2,000	43,315	104	4	0.9	201

table 3 Response Signal Generated With Sword Peroxidase Reagents

Table 4

Study	Plate Binding Capacity	Coating Antibody	Coating Reaction	Blocking Buffer	Sample Incubation	Biotinylated Antibody	Streptavidin HRPO Conjugate
Baseline Reaction	Medium binding	250 x	Overnight at 2 – 8 °C	FBS-based	Two hours at 25 °C	1,000 x	250 x
1	Medium binding	250 x	Overnight at 2 – 8 °C	Blocking solutions	Two hours at 25 °C	1,000 x	250 x
2	Medium binding	Response surface DOE	Overnight at 2 – 8 °C	FBS-based	Two hours at 25 °C	Response surface DOE	250 x
3	Medium binding	250 x	Overnight at 2 – 8 °C	FBS-based	Overnight at 2 – 8 °C	1,000 x	250 x
4	High binding	250 x	Overnight at 2 – 8 °C	FBS-based	Two hours at 25 °C	1,000 x	250 x
5	High binding	Varied concentrations	Overnight at 2 – 8 °C	FBS-based	Two hours at 25 °C	Varied concentrations	Varied concentrations
6	High binding	83 x	Overnight at 2 – 8 °C	FBS-based	Overnight at 2 – 8 °C	333 x	125 x

Table 4 Summary of Assay Conditions

The coating antibody, biotinylated antibody and streptavidin HRPO conjugate concentrations are shown as dilutions from stock reagents, where 250 X implies a 250-fold dilution from the stock solution supplied by the kit manufacturer. A change from a 250 X to a 125 X dilution represents a two-fold increase in reagent concentration. Conditions in red represent areas where reaction conditions were modified from the baseline procedure.

The results in Tables 2 and 3 demonstrate that the Tecan Infinite M200 microplate reader is capable of measuring the Sword Peroxidase Reagent-generated response with a precision (0.9 – 8.7 % CV) equal to or greater than that seen with the TMB-generated signal (2.1 – 10.6 % CV).

The mean response values were plotted against the IL-6 standard concentrations on semi-log plots. In these plots, the Sword signal was measured as a relative fluorescence unit (RFU), while the TMB signal was measured as an absorbance at 450 nm, with a correction at 570 nm. The data was then fitted to a 4PLC using only the standard calibrator levels cited in the assay insert, and the fitted curves were plotted to demonstrate the relative fit of the observed data. These plots are provided in Figure 1 (with response standard error bars). In these plots, the Sword and TMB-derived results were drawn as sample plots, scaling the response axis (Y axis) so that the

negative control (0 pg/mL) and highest positive (1,000 pg/mL) values from each respective curve overlapped.
The data obtained using both the Sword and TMB detection reagents yielded smooth dose response curves that fitted well to a 4PLC curve (with R^2 values of 0.9984 and 0.9999 for the Sword and TMB data respectively). The LOD was established from each of the fitted curves, with observed LOD values of 7.80 and 1.86 pg/mL using the Sword and TMB detection systems respectively (Table 5).

More importantly, the Sword Peroxidase Reagent-derived dose response curves are shifted to the left (toward zero dose), relative to the TMB-derived curves, indicating an improved signal-to-background measurement for the Sword Reagent. The magnitude of this shift is described by the Conc.$^{1/2\ Max}$ values shown in Table 5.

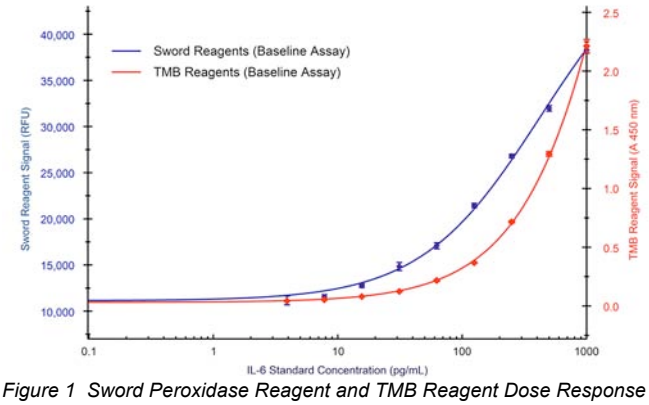

Figure 1 Sword Peroxidase Reagent and TMB Reagent Dose Response Curves

Table 5

Detection System	LOD	Conc. $^{½\ Max}$
TMB Reagent	1.86 pg/mL	789 pg/mL
Sword Peroxidase Reagents	7.80 pg/mL	134 pg/mL

Table 5 Mouse IL-6 Assay Performance

Typically, when the Sword Detection Reagents are incorporated into peroxidase-based assays without further assay optimization, the observed Sword Peroxidase Reagent dose response curves are shifted to a lower concentration relative to TMB curves. This shift generally results in lower or equivalent LOD values compared with the TMB reagents (4,5,6).

However, the use of Sword Detection Reagents without further optimization occasionally results in an LOD higher than the TMB-derived curve, as is the case with the mouse IL-6 assay described here. Adoption of the Sword Detection System with no optimization in this assay yielded a shift in the dose response curve to a lower concentration (Figure 1), but with a LOD higher than the TMB-derived value (Table 5).
An assay with this performance profile was specifically selected to demonstrate how, with relatively simple assay optimization, performance can be further enhanced with the Sword Detection Reagents.

Assay Optimization

Typically, the first step towards optimization is to examine if the background signal (represented by the 0 pg/mL IL-6 sample) can be reduced without sacrificing too much of the analyte-dependent signal. A series of blocking solutions containing different protein sources were examined. These solutions were as follows:

1) 10 % FBS in PBS (BD Biosciences, Part # 51-2641KC), with and without 0.05 % Tween 20
2) Thermo Scientific StartingBlock™ blocking buffer (Part # 37538), with and without 0.05 % Tween 20
3) Thermo Scientific ELISA blocking buffer (Part # N505), with and without 0.05 % Tween 20
4) KPL 1 % BSA diluent / blocking buffer (Part # 50-61-10), with and without 0.05 % Tween 20
5) 10 % FBS (GIBCO 10082-139) in PBS, pH 7.4 (Sigma P4417), with and without 0.05 % Tween 20

These blocking solutions were substituted into the baseline mouse IL-6 assay, and the sample / negative response ratios (S/N ratios) using the Sword Reagents were examined using 7.81, 31.25, 125 and 1,000 pg/mL IL-6 samples (Study 1 in Table 4). These samples were thought to sufficiently represent the dynamic range of this assay. Using alternate blocking solutions did not consistently result in improved S/N ratios (data not shown).

The next step was to optimize the concentrations of the coating and biotinylated antibodies used in the assay. This was accomplished using statistically designed experimentation (DOE) to capture both the main reagent effects and any significant ($P \leq 0.05$) reagent interactions.

A face-centered central composite design was used, varying the reagents over three-fold concentrations (3 X).
The streptavidin HRPO conjugate concentration was held constant, and this study (Study 2 in Table 4) was analyzed using JMP® Statistical Discovery Software, version 6.0 (SAS, Cary, NC, USA) to examine both the level and variability of the sample response and the S/N ratios. Both the reagent main effects, and the coating antibody - biotinylated antibody interaction were shown to be significant (as expected), but no new reagent concentrations or combinations were shown to be more advantageous that those used in the baseline assay (data not shown).

The sample incubation was then increased from two hours at room temperature to an overnight incubation at 2 – 8 °C, to encourage the immobilization of more sample analyte to the wells. Several "high sensitivity" assays have successfully employed this approach in their development. All other conditions were held constant in the study (Study 3 in Table 4).

The results using the increased sample incubations are shown in Tables 6 and 7.

Table 6

IL-6 STD (pg/mL)	Mean	SD	N	CV %	SE
0.00	0.02314	0.00207	8	9.0	0.00073
1.95	0.03258	0.00609	4	18.7	0.00304
3.91	0.04272	0.00480	4	11.2	0.00240
7.81	0.05327	0.00355	4	6.7	0.00178
15.63	0.08018	0.00421	4	5.3	0.00211
31.25	0.13738	0.00599	4	4.4	0.00300
62.50	0.25560	0.01872	4	7.3	0.00936
125	0.51437	0.00321	4	0.6	0.00161
250	0.96275	0.00786	4	0.8	0.00393
500	1.78433	0.04814	4	2.7	0.02407
1,000	2.80668	0.10354	4	3.7	0.05177

Table 6 Increased Sample Incubation – Study 3 TMB Reagent Response Signal

Table 7

IL-6 STD (pg/mL)	Mean	SD	N	CV %	SE
0.00	11,582	382	8	3.3	135
1.95	12,325	325	4	2.6	163
3.91	12,513	474	3	3.8	273
7.81	14,014	404	4	2.9	202
15.63	15,733	601	4	3.8	300
31.25	16,360	814	4	5.0	407
62.50	21,025	325	4	1.5	163
125	26,906	240	4	0.9	120
250	33,859	327	4	1.0	163
500	39,189	1647	4	4.2	823
1,000	45,984	1085	4	2.4	543

Table 7 Increased Sample Incubation – Study 3 Sword Reagent Response Signal

Table 8

Detection System	LOD	Conc. ½ Max
TMB Reagent	1.73 pg/mL	375 pg/mL
Sword Peroxidase Reagents	2.63 pg/mL	78 pg/mL

Table 8 Assay Performance with Increased Sample Incubation

This study resulted in a significant decrease in the Sword Reagent-derived LOD (from 7.80 to 2.63 pg/mL), and a further leftward shift in the dose response curve (Figure 2). There was also a small decrease in the TMB-derived LOD (from 1.86 to 1.73), but not to the extent observed with the Sword Reagents. Note that there was no impact on the response variation (% CVs) caused by the increased incubation times.

The sample incubation studies suggest that when more analyte is captured from low concentration samples, the Sword-derived LOD is improved. To leverage this effect, microplates with an increased binding capacity were used. Concurrent with these studies, the coating antibody, biotinylated antibody and streptavidin HRPO conjugate concentrations were all re-evaluated (Studies 4 and 5 in Table 4). In these studies, the original sample incubation of two hours at room temperature was retained.

An assay configuration, which included using a high binding capacity 96-well microplate, three times more coating antibody, three times more biotinylated antibody and two times more streptavidin HRPO conjugate appears to yield the best performance.

Table 9

Detection System	LOD	Conc. ½ Max
TMB Reagent	1.34 pg/mL	221 pg/mL
Sword Peroxidase Reagents	0.48 pg/mL	47 pg/mL

Table 9 Assay Performance with Modified Assay Configuration

This configuration results in an assay with markedly improved performance over the original baseline assay, with a Sword-derived LOD of 0.48 pg/mL and a further leftward shift in the curve (as indicated by the lower Conc.$^{1/2\ Max}$ values in Table 9 and in Figures 2 and 3). While the TMB curves also show a leftward shift, an improved LOD is not observed under these conditions.

Finally, the increased sample incubation was coupled with the improved assay configuration (Study 6 in Table 4), however increasing incubation time did not significantly improve assay performance in the re-optimized assay configuration.

Table 10

Detection System	LOD	Conc. ½ Max
TMB Reagent	1.42 pg/mL	268 pg/mL
Sword Peroxidase Reagents	0.317 pg/mL	45 pg/mL

Table 10 Assay Performance with Modified Assay Configuration and Increased Incubation Time

The Sword and TMB Reagent dose response curves under these conditions are provided in Figures 2 and 3. The leftward shift in the Sword curves are clearly shown in Figure 2. The TMB curves also shift left to a small extent (Figure 3), but there is no change at the lower concentrations, yielding an unchanged TMB-derived LOD value.

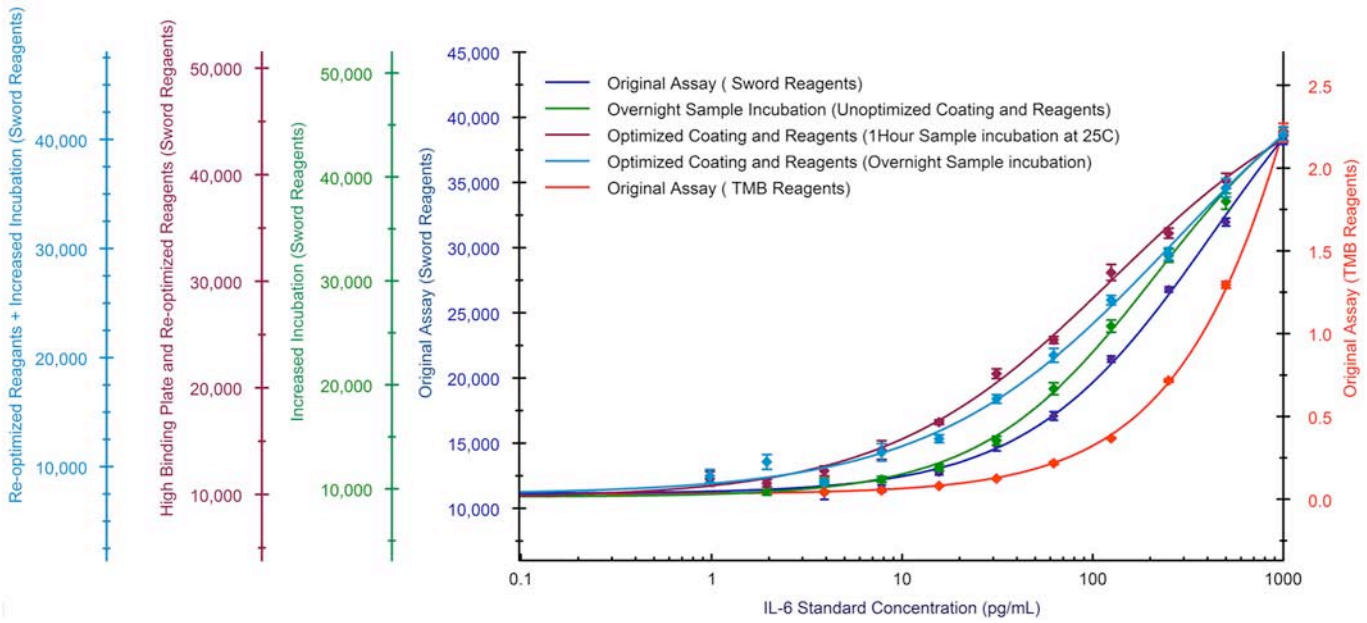

Figure 2 Sword Peroxidase Reagent Mouse IL-6 Dose Response Curves

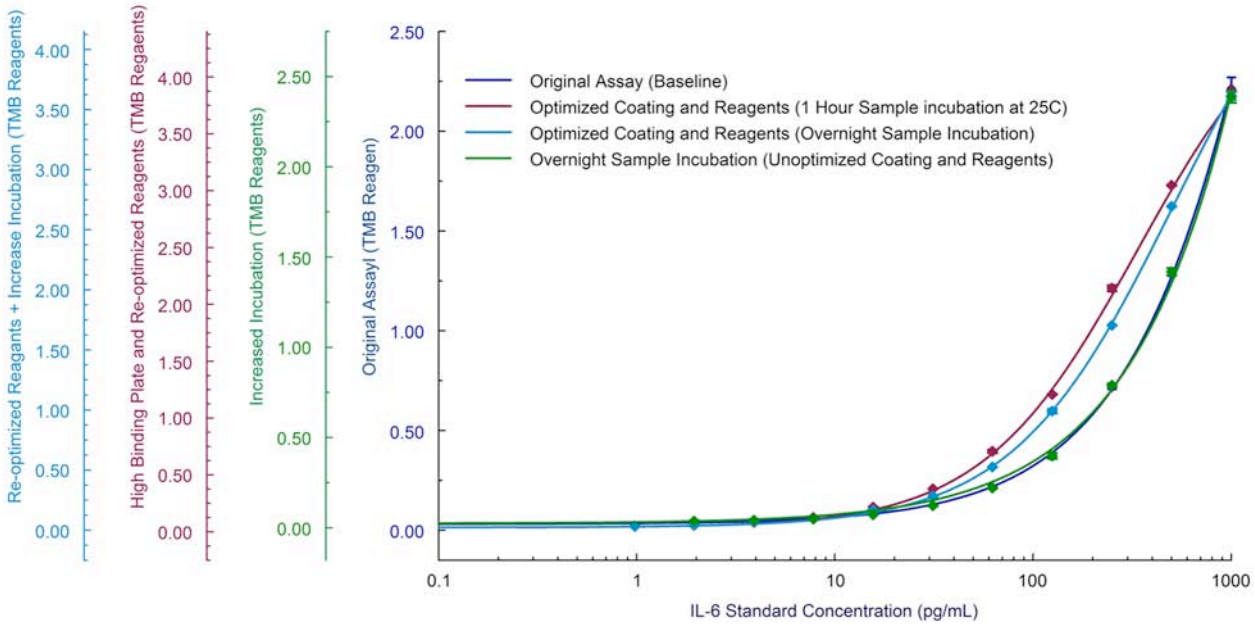

Figure 3 TMB Reagent Mouse IL-6 Dose Response Curves

Conclusion

This study confirms that the use of Sword Detection Reagents in ELISAs typically result in dose response curves that are shifted toward lower analyte concentrations relative to TMB curves. These shifted curves often result in lower sensitivity (LOD) than that observed with the TMB reagents. In this instance, we demonstrated that simple optimization studies could improve the ELISA performance substantially more with the Sword Peroxidase Detection reagents than with the native TMB detection reagents.

Regarding sensitivity improvement, this technical note describes an improved LOD and precision using Sword Diagnostics Peroxidase Reagents in the mouse IL-6 ELISA. The sensitivity is improved 16 – 17 from the original assay, with a 6 – 7-fold improvement in the Sword Peroxidase Reagent assay sensitivity over that originally observed with the TMB detection system (Figure 4).

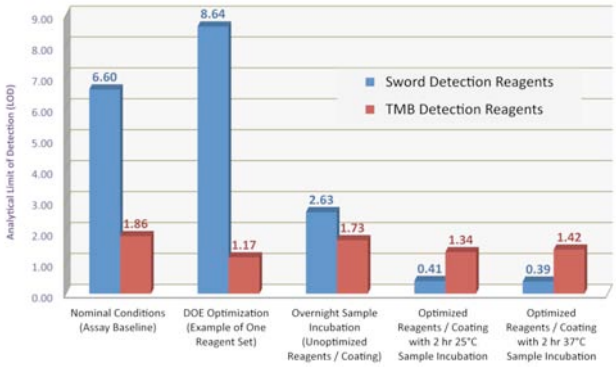

Figure 4 LOD Values During Optimization

This work is a demonstration of the concept of re-optimizing ELISAs once a new detection system is adopted. In this instance, assay re-optimization after the introduction of the Sword Peroxidase Detection System yielded a substantial improvement in performance, though this may not represent the ultimate results of a complete optimization effort, as several additional technical areas for further optimization remain, including diluent and wash buffer reformulation and kinetic studies.

Literature

1) BD BiosScience, San Diego, CA, Mouse IL-6 OptEiA Cat No. 55240 (Lot No. 78698) Instruction Manual and Certificate
2) Sword Diagnostics Peroxidase Reagent Kit Insert. Sword Catalog N818, (3440 S. Dearborn Street, Suite 260, Chicago, IL 60616)
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6) Human IL-6 chemiluminescent ELISA using Sword™ Peroxidase Reagents: Sensitive detection using the Tecan Infinite® M200 multimode reader. Tecan Technical Note 396609 V1.0, 02-2011 (February 2011, Tecan Group Ltd, Sword Diagnostics Inc.)

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