Human IL-6 chemiluminescent ELISA using Sword™ Peroxidase Reagents

Sensitive detection using the Tecan Infinite® M200 multimode reader

Introduction

Human tumor necrosis factor alpha, Interleukin 6
Interleukin 6 (IL-6) is a multifunctional protein produced by lymphoid and non-lymphoid cells, and by both 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, and malignancies such as plasmacytomas, myelomas, lymphomas, leukemias and ovarian cancers.

Principle of the assay
This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-6 is pre-coated onto a microplate. Standards and samples are pipetted into the wells, and any IL-6 present is bound by 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 reagent, an enhanced luminol/peroxide substrate solution is added to the wells and light is produced in proportion to the amount of IL-6 bound in the initial step. A microplate luminometer is used to measure the intensity of the light emitted.

This technical note describes the performance of the R&D Systems IL-6 ELISA on the Tecan Infinite M1000 multimode reader, using the new and more sensitive reagent system developed by Sword Diagnostics.

Note that the descriptions of the IL-6 analyte and the R&D Systems IL-6 ELISA are taken from the R&D Systems assay insert[1].

Materials and methods

Instrument

Tecan Infinite M200 multimode reader

Microplates

Pre-coated 96-well, flat bottom, polystyrene microplates, supplied with the R&D Systems Human IL-6 ELISA

Reagents

- QuantiGlo® Human IL-6 Immunoassay (R&D Systems, MN)
- Sword Peroxidase Reagents (Sword Diagnostics, IL)

Assay procedures

The R&D Systems Human IL-6 immunoassay was run as described in the assay insert1 up until the addition of chromogen, when Sword Peroxidase Reagents were substituted for the vendor-supplied enhanced luminol / peroxide substrate (Glo Reagent). The Infinite M1000 instrument was set to read for 1.5 seconds, 5 – 6 minutes after the initiation of the Glo Reagent reaction.

Peroxidase reactions using Sword Peroxidase Reagents

1) Sword 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) Once the post conjugate incubation wash step of the ELISA was complete, 150 µl of Sword Substrate / Peroxide Mixture was added to each assay well.
3) The microplate was 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, the 50 µl of each well was gently aspirated twice. The solution in each well turns from a pre-development yellow color to a light pink, confirming that the development process has started.
5) The microplate was incubated at room temperature in the dark for 30 minutes.

Parameter	Setting
Mode	Luminiscence
Attenuation	NONE
Integration time	100 ms/well
Settle time	0 ms
Autogain	ON
Summation mode	ON

Table 1a Infinite M200 multimode reader measurement parameters (luminescence)

Parameter	Setting
Mode	Fluorescence intensity
Reads	Top read
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	150 (manual)

Table 1b Infinite M200 multimode reader measurement parameters (Sword Reagents)

Note: Auto settings of the gain may also be used.

Data analysis

The experimental data and measurement parameters were exported automatically by the i-control™ software into Microsoft Excel® 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 here 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 one-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 of the Sword Peroxidase Reagent dose response curve. 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 IL-6 ELISA was performed using the calibrator levels cited in the assay insert, plus one additional calibrator at 900 pg/ml. The assay was evaluated using both the Sword Peroxidase Reagents and the Glo Reagents supplied by the vendor. The signal generated using the Sword Peroxidase Reagents was detected using the fluorescence intensity detection channel, using the top read optics to collect data. These results are shown in Tables 2 and 3.

IL-6 STD (pg/ml)	Mean	SD	N	% CV	SE
0.00	728	167	6	23.0	68
0.48	8,346	21	2	0.3	15
2.4	7,767	369	3	4.8	213
12	48,281	670	3	1.4	387
60	241,357	15,751	3	6.5	9,094
300	1,334,606	31,276	3	2.3	18,057
900	3,621,092	61,071	2	1.7	43,184
1,500	4,930,689	41,971	2	0.9	29,678

Table 2 Response signal generated with Glo Reagents

IL-6 STD (pg/ml)	Mean	SD	N	% CV	SE
0.00	746	25	6	3.4	10
0.48	921	15	2	1.6	11
2.4	919	9	3	1.0	5
12	1,420	15	3	1.0	9
60	2,919	84	3	2.9	48
300	5,030	41	3	0.8	24
900	5,896	3	2	0.0	2
1,500	6,196	77	2	1.2	55

Table 3 Response signal generated with Sword Peroxidase Reagents

These results demonstrate that the Tecan Infinite M200 multimode reader is capable of measuring the Sword Peroxidase Reagents-generated response with a precision equal to or greater than that seen with the Glo-generated signal. The precision of the Glo signal observed here is consistent with that cited in the assay insert[1].

The mean response values were plotted against the IL-6 standard concentrations on semi-log plots. The data was then fitted to a 4PLC, using only the standard calibrator levels cited in the assay insert. These fitted cures were shown on the plots, with standard error bars, to demonstrate the relative fit of the observed data (Figure 1). The Sword- and Glo-derived results were drawn on the sample plot, scaling the response axis (Y axis) such that the negative control (0 pg/ml) and highest positive (1,500 pg/ml) values from each respective curve overlapped.

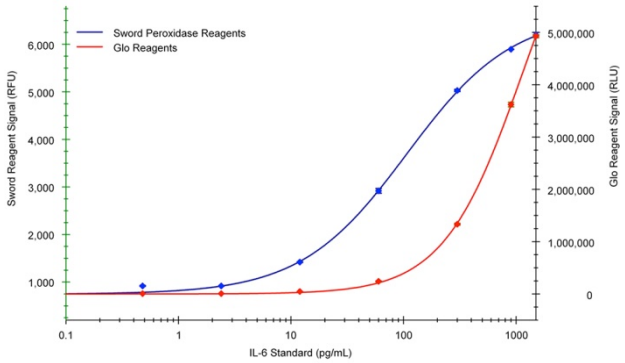

Figure 1 Sword Peroxidase Reagents and Glo Reagents dose response curves

The data obtained using both the Sword and Glo detection reagents yields smooth dose response curves that fit well to a 4PLC (with R^2 values of 0.9995 and 0.9999 for the Sword and Glo data respectively).

Of more importance is the relative position of these dose response curves. The Sword Peroxidase Reagent-derived dose response curves are shifted to a lower concentration relative to the Glo-derived curves. The magnitude of this shift is described by evaluating the concentration at ½ the maximum response of the highest calibrator ($Conc^{½\ Max}$).

This indicates that in the IL-6 assay, the Sword Peroxidase Reagents are capable of detecting nearly six fold lower sample concentrations when only half of the available signal is expended. Evaluating the plot in Figure 1 and the data in Table 4 indicates that when the Sword Peroxidase Reagents are applied to this assay, there is there is both greater signal strength at the lower concentrations (concentrations that are often of interest in assay), and improved ability to distinguish samples from negative control samples.

Detection system	Concentration ½ Max
Glo Reagents curve	559
Sword Peroxidase Reagents curve	68

Table 4 Concentration at ½ maximum signal

This shift offers assay developers more signal to use in their development effort. The signal response that is distinguishable from background becomes a type of 'currency' that the development effort can expend in the optimization effort. This currency is often spent obtaining acceptable balances between critical attributes such as sample volume, assay kinetics, assay sensitivity, assay specificity (non-specific background and cross reactivity), and assay stability.

Inevitably, assay development efforts find that improvements in critical attributes can only be attained at the expense of others. For example, higher assay sensitivity often comes at the expense of assay specificity, sample size or kinetics, all attributes critical to the customer. When more currency is available, this allows the optimization effort to attain better balance (more acceptable trade-off) between competing performance attributes. The shift of the response curve represents such an increase in the currency.

To demonstrate the power of this added optimization flexibility, a sample volume reduction study was performed. In this study, the original sample size (100 µl) was reduced by 50 % (50 µl) and 75 % (25 µl). The IL-6 assay was run as before, with these reduced sample volumes. The dose response curves from the reduced sample volume runs were compared to that of the normal Sword and Glo reagent runs (Figure 2).

As the sample size was reduced, the analyte dose response was maintained over the entire range. However, the observed shift using the Sword Peroxidase Reagents diminished with each volume reduction, becoming increasingly similar to the Glo curve. Here, the improved optimization flexibility (or currency) was used to obtain a significant reduction in the required sample size.

It should also be noted that each assay is different, and may react differently to changes and optimization. The factors driving performance changes are often dependent upon the biological reagents used, the assay format and the protocols adopted.

In the case of the R&D Systems IL-6 ELISA, the analytical LOD was estimated to be equivalent for the two detection systems. Specifically, IL-6 LODs of 0.33 and 0.25 pg/ml were observed for the Sword Peroxidase Reagents and Glo reagents respectively. These LOD values are consistent with that cited in the IL-6 ELISA insert[1]. Note that the

reproducibility of the LOD determinations has not been characterized with this assay.

The equivalent LOD for the detection systems, accompanied by the significant curve shift observed when using Sword reagents, indicates that there is room for improvement to the assay at lower concentrations. In this case, these reagents were simply introduced into a commercial assay, (optimized for Glo detection) without any additional optimization. It is expected that performance could be further improved by optimizing the assay for Sword Peroxidase Reagents. However, demonstration of such improvements without access to the assay formulation or biological reagents was not feasible with this commercial assay.

Conclusion

This technical note describes the successful performance of the R&D Systems Human IL-6 ELISA on the Tecan Infinite M200 multimode reader using the Sword Peroxidase Reagents.

The Sword Peroxidase Reagents were easily introduced into this Glo-optimized assay without any modifications to the IL-6 ELISA or the Tecan Infinite M200 multimode reader. The signal from the Sword Peroxidase Reagents could be detected by the Infinite M200 using the top fluorescence optics.

Use of the Sword Peroxidase Reagents results in a shift in the dose response curve to a lower concentration than the typical Glo curve. This shift gives assay developers more flexibility in their optimization efforts, allowing more acceptable trade-offs between competing critical performance attributes. An example was provided as to how the flexibility provided by the Sword Peroxidase Reagents allows a 75% reduction in sample volume for the IL-6 ELISA.

The described applications are intended for research use only.

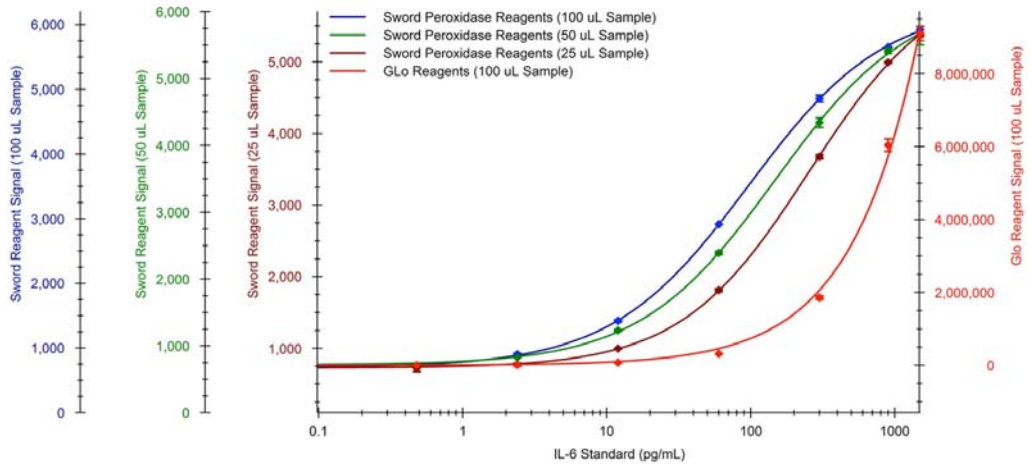

Figure 2 Dose response curves obtained in sample volume reduction

Literature

1) QuantiGlo Human IL-6 Immunoassay Reagent Kit Insert, R&D Systems catalog Q6000B (614 McKinley Place, NE, Minneapolis, MN 55413)
2) Sword Diagnostics Peroxidase Reagent Kit Insert. Sword Catalog N818, (3440 S. Dearborn Street, Suite 260, Chicago, IL 60616)
3) Rodbard D. Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assays. Clin Chem. 1974 Oct; 20 (10):1255–1270

Contact

For product support inquiries contact
Sword Diagnostics
3440 South Dearborn Street
Suite 260, Chicago, IL 60616
+1 312 235 3660
info@sworddiagnostics.com

Austria +43 62 46 89 33 **Belgium** +32 15 42 13 19 **China** +86 21 2898 6333 **Denmark** +45 70 23 44 50 **France** +33 4 72 76 04 80 **Germany** +49 79 51 94 170
Italy +39 02 92 44 790 **Japan** +81 44 556 73 11 **Netherlands** +31 18 34 48 **Singapore** +65 644 41 886 **Spain** +34 93 490 01 74 **Sweden** +46 31 75 44 000
Switzerland +41 44 922 89 22 **UK** +44 118 9300 300 **USA** +1 919 361 5200 **Other countries** +41 44 922 8125

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