

High Sensitivity Human IL-6 ELISA with an Expanded Dynamic Range:

Enhanced Performance using Sword™ Peroxidase Assay and the Tecan Infinite® M1000 Multimode Reader

Introduction

Interleukin 6

Interleukin 6 (IL-6) is a pleiotropic alpha-helical 22 - 28 kDa phosphorylated and variably glycosylated cytokine that plays important roles in acute phase reactions, inflammation, hematopoiesis, bone metabolism, and cancer progression. Mature human IL-6 is 183 amino acids (aa) in length and shares 41% aa sequence identity with mouse and rat IL-6. Alternate splicing generates several isoforms with internal deletions, some of which exhibit antagonistic properties. Cells known to express IL-6 include CD8+ T cells, fibroblasts, synoviocytes, adipocytes, osteoblasts, megakaryocytes, endothelial cells (under the influence of endothelins), sympathetic neurons, cerebral cortex neurons, adrenal medulla chromaffin cells, retinal pigment cells, mast cells, keratinocytes, Langerhans cells, fetal and adult astrocytes, neutrophils, monocytes, eosinophils, colonic epithelial cells, B1 B cells, and pancreatic islet beta cells. IL-6 production is generally correlated with cell activation and is normally kept in control by glucocorticoids, catecholamines and secondary sex steroids.

Normal human circulating IL-6 is in the 1 pg/mL range, with slight elevations during the menstrual cycle, modest elevations in certain cancers, and large elevations after surgery. IL-6, along with TNF-alpha and IL-1, drives the acute inflammatory response, is almost solely responsible for fever and the acute phase response in the liver, and is important in the transition from acute inflammation to either acquired immunity or chronic inflammatory disease. It contributes to chronic inflammation in conditions such as obesity, insulin resistance, inflammatory bowel disease, inflammatory arthritis, and sepsis when dysregulated, often involving IL-6 trans-signaling. It plays an important role in differentiation of naïve T cells to Th17 inflammatory cells in the presence of TGF-alpha. IL-6 modulates bone resorption and is a major effector in inflammatory joint destruction in rheumatoid arthritis through promoting Th17 T cell activity. It contributes to atherosclerotic plaque development and destabilization. IL-6 can also have anti-inflammatory effects, however, such as in skeletal muscle where it is secreted in response to exercise. It promotes hematopoiesis by being a growth factor for hematopoietic stem cells, induces B cell maturation to plasma cells, and perpetuates multiple myeloma. IL-6 also promotes, but probably does not initiate, other types of inflammation-associated carcinogenesis, such as colitis-associated cancer.

This description of the IL-6 analyte was adapted from the R&D Systems assay insert (1). Relevant references can be found therein.

Principle of the Assay

This assay employed the quantitative sandwich enzyme immunoassay technique. A mouse anti-human IL-6 antibody was coated onto a microplate. Standards and samples were subsequently pipetted into the wells, and any IL-6 present was bound by the immobilized antibody. After washing away any unbound substances, a second biotinylated goat anti-human IL-6 antibody was added. After washing, the biotinylated antibody was retained if any sample dependent IL-6 had been previously immobilized within the assay well. After removal of excess second antibody, Streptavidin-Peroxidase (enzyme conjugate) was added. This bound to the biotinylated antibody to completing the four-member sandwich. After a second incubation and washing to remove all the unbound enzyme, a substrate solution was added to detect the present of the bound Streptavidin-Peroxidase. The amount of signal generated by the enzyme conjugate was proportional to the amount of IL-6 in the original sample.

This technical note describes the performance of a Human IL-6 ELISA using the R&D Systems IL-6 DuoSet reagents on the Tecan Infinite M1000 multimode reader, using a sensitive reagent system developed by Sword™ Diagnostics.

Materials and Methods

Instrument:

Tecan Infinite® M1000 Multimode Reader (Tecan Austria, Austria)

Microplates:

Corning Costar 96 Well, flat bottom clear polystyrene plates (High Binding Stripwell Plates 2592, High Binding Solid Plate 9017).

Reagents:

- R&D Systems DuoSet Human IL-6 assay (R&D Systems Cat # DY206)
- Assay Diluent (1% BSA in PBS, pH 7.2 - 7.4) from Reagent Diluent Concentrate 2 (R&D Systems Cat # DY995).
- Sword Peroxidase Assay reagents (N818)
- PBS and PBS + 0.05 % Tween-20 (Wash Buffer) were prepared from ACS grade reagents.

Procedures:

The Human IL-6 Assay

The R&D Systems DuoSet Human IL-6 assay reagents were prepared as described in the assay insert (2). The assay was run as prescribed in the assay documentation up until the addition of TMB detection reagents.

Calibrators at 0.00, 0.59, 1.17, 2.34, 9.38, 37.50, 150, 600 and 5,000 pg/mL in replicates of three or more were run routinely, however more analyte levels were employed when the entire assay range was characterized. Eight replicates were used to establish the negative control variation used in the Limit of Detection calculation, and when Quantitation limits were estimated.

When TMB detection was used, the procedures prescribed in the assay documentation were utilized. When Sword Peroxidase Assay reagents were used, the following procedure was used.

Sword Peroxidase Assay

1. The Sword Peroxidase Assay Substrate / Peroxide Mixture and Development solutions were prepared from concentrated stock solutions as described in the peroxidase assay insert (3). The mixtures were stable at Room Temperature (20-25°C) for 8 hours when stored in the dark.
2. 150 µL of Sword Peroxidase Assay Substrate/Peroxide Mixture was added each assay well once the post conjugate incubation wash step of the ELISA assay was completed.
3. The microtiter plate was incubated at Room Temperature in the dark for 15 minutes.
4. 150 µL of Sword Peroxidase Assay Development solution was added to each well after the incubation step. To ensure adequate mixing, 50 µL was gently aspirated within each well twice. In this step, the solution in each well turned from a predevelopment yellow color to a light pink, confirming that the development process had started.
5. The microtiter plate was incubated at Room Temperature in the dark for 30 minutes.
6. The microtiter plate was read using the Tecan Infinite M1000 Multimode Reader using the settings provided in Table 1, within one hour of the development step completion.

Note: A brownish color was observed with the higher sample concentrations. This was both expected and normal, however this color is not what is being evaluated in this reaction.

High Sensitivity Human IL-6 ELISA with an Expanded Dynamic Range:

Enhanced Performance using Sword™ Peroxidase Assay and the Tecan Infinite® M1000 Multimode Reader

Table 1: Tecan Infinite M1000 Multimode Reader Settings

Parameter	M1000 Setting
Mode	Fluorescent Intensity
Reads	Top Read
Excitation Wave Length	Length 530 nm
Excitation bandwidth	20 nm
Emission Wave Length	700 nm
Emission bandwidth	20 nm
Flash Number (Frequency)	25 (100 Hz)
Integration	20 µsec
Gain	150 (manual)
Z Height (Top Read)	25,000 (manual)

Note: Auto settings of the Gain and Z height may also be used, although for more sensitive readings the Z height should be optimized to the microtiter plate and unit being used.

Data Analysis:

The experimental data, as well as the measurement parameters were loaded automatically by the i-Control software to Microsoft Excel for further analysis. The means of the sample replicates were calculated, after examining the data for obvious outliers.

Dose response curves were generated by plotting the sample means against IL-6 standard concentration on a semi-log plot (mean signal vs. log of standard concentration). The mean data points were fit to a four parameter logistic curve (4PLC) using the equation communicated by D. Rodbard (3)

Where:

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

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

The Analytical Limit of Detection (LOD) was defined as the concentration read from the fitted 4PLC at a response level equal to the mean Negative Control level plus two times the standard deviation estimated from the Negative Control population.

The Lower Limit of Quantitation (LLQ) was defined as the lowest calibrator concentration whose concentration dispersion (% CV of concentration values when read from the fitted 4PLC dose response curve) was 25 % or less.

The Upper Limit of Quantitation (ULQ) was defined as the highest calibrator concentration whose concentration dispersion (% CV of concentration values when read from the fitted 4PLC dose response curve) was 25 % or less.

Results

Table 2 and Figure 1 provides some representative Human IL-6 assay results when the Sword Peroxidase Assay reagents are incorporated into the assay. The assay was run and analyzed as described in the Material and Methods section. Representative TMB results are provided in Table 3.

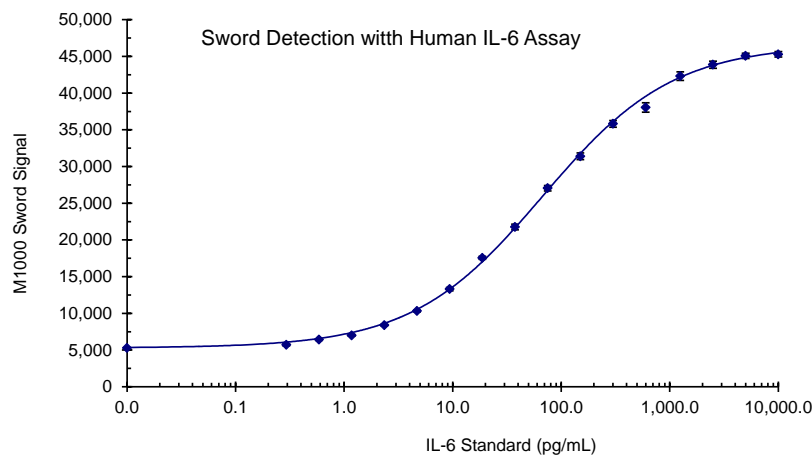
Table 2: Sword Peroxidase Assay dose response curve data covering the entire detection range

Standard Concentration (pg/mL)	Mean	SD	N	CV
0.00	5283	148	8	2.8 %
0.29	5707	262	4	4.6 %
0.59	6413	205	4	3.2 %
1.17	7001	216	4	3.1 %
2.34	8395	191	4	2.3 %
4.69	10319	241	4	2.3 %
9.38	13296	470	4	3.5 %
18.75	17560	417	4	2.4 %
37.50	21759	792	4	3.6 %
75	27048	751	4	2.8 %
150	31389	915	4	2.9 %
300	35,815	912	4	2.5 %
600	38,044	1261	4	3.3 %
1,250	42,306	1174	4	2.8 %
2,500	43,864	961	4	2.2 %
5,000	45,063	706	4	1.6 %
10,000	45,278	747	4	1.7 %

Note: In this study more calibrator levels were employed to fully characterize the entire detection range

Figure 1: Dose response curve covering the entire detection range

(Error bars = Standard error of means)



High Sensitivity Human IL-6 ELISA with an Expanded Dynamic Range:

Enhanced Performance using Sword™ Peroxidase Assay and the Tecan Infinite® M1000 Multimode Reader



Table 3: TMB dose response curve data covering the entire detection range

Standard Concentration (pg/mL)	Mean	SD	N	CV
0.00	0.02204	0.00348	7	15.8
0.29	0.03025	0.00385	4	12.7
0.59	0.03313	0.00963	4	29.1
1.17	0.03550	0.00186	4	5.2
2.34	0.04793	0.00421	4	8.8
9.38	0.08903	0.00303	4	3.4
37.5	0.23845	0.00503	4	2.1
75	0.41503	0.01057	4	2.5
150	0.73630	0.01690	4	2.3
600	2.11148	0.12287	4	5.8
5,000	3.74150	0.05027	3	1.3

Discussion

In this study, the Sword Peroxidase Assay reagent response imprecision (estimated as the %CV associated with the sample replicates) was significantly lower than that observed with the TMB reagents (Tables 2 and 3). This observation is consistent with similar observations made in the Human TNF-alpha (9), Mouse IL-6 (10) and Free Peroxidase (11) previously published studies performed on the Tecan Infinite M1000 Multimode Reader.

The mean Analytical Limit of Detection (LOD) was estimated from five individual determinations as 0.038 pg/mL (95 % confidence interval: 0.014 - 0.062 pg/mL). This minimum level of detection is significantly lower than that observed with similar ELISA methodologies (Table 4).

Table 4: Analytical Limit of Detection Values and Detection Ranges with Different ELISA Assays

Supplier	Assay Type	Assay Number	Analytical Limit of Detection	Recommended Calibrator Range
Sword Diagnostics	Sword Peroxidase Assay with R&D Systems DuoSet Reagents	Reagent Kit: DY206 ⁽²⁾	Mean: 0.038 pg/mL 95% CI: 0.014 - 0.062	Sword: 0.29 - 5,000 pg/mL DuoSet: 9.38 - 600 pg/mL
R&D Systems	QuantiGlo®	Q6000B ⁽⁵⁾ SQ6000B PQ6000B	Mean: 0.16 pg/mL Range 0.05 - 0.35	0.48 - 1,500 pg/mL
R&D Systems	Quantikine®	D6050 ⁽¹⁾ S6050 PD6050	Typically less than 0.70 pg/mL	3.12 - 100 pg/mL
R&D Systems	Quantikine® HS (High Sensitivity)	HS600B ⁽⁶⁾ SS600B PHS600B	Mean: 0.039 pg/mL Range: 0.016 - 0.110	0.156 - 10 pg/mL
Invitrogen	ELISA	KHC0061 ⁽⁷⁾ KHC0062 KHC0061C	Less than 2 pg/mL	7.8 - 500 pg/mL
Invitrogen	Ultrasensitive (High Sensitivity) ELISA	KHC0064 ⁽⁸⁾ KHC0063 KHC0064C	Less than 0.104 pg/mL	0.156 - 10 pg/mL

The range of detectable analyte was also expanded when Sword Peroxidase Assay reagents were adopted. A clear dose response was observed using calibrators between 0.29 and 5,000 pg/mL IL-6 (Table 2 and Figure 1). Use of these reagents has allowed the expansion of both the upper and lower detection limits beyond the ranges recommended by the ELISA suppliers listed in Table 3.

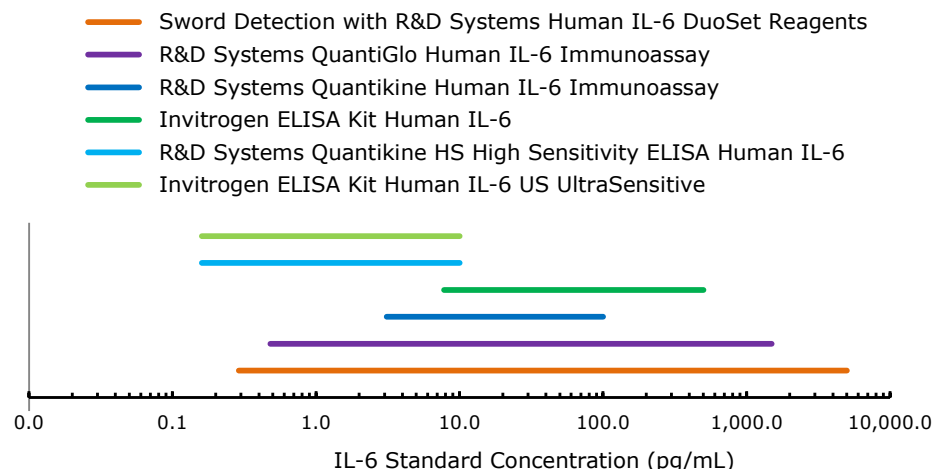
Using the Sword Peroxidase Assay reagents, the Human IL-6 assay Lower Limit of Quantitation (LLQ) was observed to be 4.69 pg/mL and the Upper Limit of Quantitation (ULQ) was observed to be 5,000 pg/mL. We were unable to verify the LLQ and ULQ values for the other assays listed.

The dynamic range data from Table 4 are graphically represented in figure 2 for ease of comparison.

High Sensitivity Human IL-6 ELISA with an Expanded Dynamic Range:

Enhanced Performance using Sword™ Peroxidase Assay and the Tecan Infinite® M1000 Multimode Reader

Figure 2: Comparison of Assay Dynamic Ranges



These results may be compared with the performance of Human IL-6 electrochemiluminescence assays (Meso Scale Discovery (MSD), Gaithersburg, Maryland) in Table 5. Here, the Sword Peroxidase Assay derived Analytical Limit of Detection values (LOD) were recalculated to use 2.5 SD which is the calculation used by MSD. When compared, the Sword assay derived LODs were significantly lower (4 – 26 fold lower depending upon which MSD assay data set is used) than the published electrochemiluminescence results. The assay dynamic ranges were comparable in magnitude with the electrochemiluminescence assays.

Table 5: Analytical Limit of Detection Values and Detection Ranges with Electrochemiluminescence Assays

Supplier	Assay Type	Assay Number (Data source)	Analytical Limit of Detection **	Calibrator Range
Sword Diagnostics	Sword Peroxidase Assay with R&D Systems DuoSet Reagents	Reagent Kit: DY206	Mean: 0.061 pg/mL 95% CI: 0.022 - 0.083	0.29 - 5,000 pg/mL
Meso Scale Discovery	MULTI-ARRAY™ 96 well Small Spot Plate	(MULTI-ARRAY 96 well Small Spot Plate Brochure)	0.7 - 1.6 pg/mL	0.01 - 40,000
Meso Scale Discovery	IL-6 QuickPLex	K15A06 (19511-V1.3-2007Jan)	0.81 pg/mL	0.61 - 10,000
Meso Scale Discovery	Human ProInflammatory II 4 plex Ultrasensitive	K15025C (17697-V1-2010Dec)	0.29 pg/mL	2.4 - 2,500
Meso Scale Discovery	Human ProInflammatory 9 plex Ultrasensitive assay	K15007C (17706-V1-2010Dec)	0.26 pg/mL	0.61 - 2,500

** The Analytical Limit of Detection (LOD) was defined here as the concentration read from the fitted 4PLC at a response level equal to the mean Negative Control level plus two and one-half times the standard deviations estimated from the Negative Control population.

The results demonstrate that the Human IL-6 Assay with Sword Peroxidase Assay reagents presented here has:

- Analytical sensitivity equal or potentially superior to other specialized High Sensitivity Microtiter plate ELISA assays
- Expanded dynamic range well beyond the range of specialized High Sensitivity Microtiter plate ELISA assays
- Analytical sensitivity superior to that seen with electrochemiluminescence assays with comparable dynamic ranges

The enhanced assay performance cited here was obtained by substituting in Sword detection chemistry without changing the assay architecture (capture or detection antibody, antibody conjugates or reporter enzymes), assay formulation or assay format. While these attributes are often targeted in sensitivity enhancement efforts, such modifications were not required to obtain the performance reported here. As such, these results were obtained quickly with a minimum of effort.

This work is intended to demonstrate the impact that adoption of Sword Peroxidase Assay reagents can have upon an assay, independently of assay optimization efforts. There remain several additional arenas for further optimization including modifications to the basic assay architecture, reagent, diluent and wash buffer reformulation and modification of the assay kinetics.

In summary, the use of the Sword Peroxidase Assay reagents in this Human IL-6 assay has resulted in a more precise assay with sensitivity equal or superior to other High Sensitivity IL-6 assays, without the truncated dynamic ranges these specialized ELISA assays often exhibit. This enhanced performance does not require changes to assay architecture, assay formulation or assay format and was obtained quickly with a minimum of effort.

Literature

- 1) Human IL-6 Immunoassay Reagent Kit Insert (Quantikine® Reagents), R&D Systems catalog D6050 (614 McKinley Place, NE, Minneapolis, MN 55413)
- 2) Human IL-6 DuoSet Reagent Kit Insert, R&D Systems catalog DY206 (614 McKinley Place, NE, Minneapolis, MN 55413)
- 3) Sword Diagnostics Peroxidase Reagent Kit Insert. Sword Catalog N818, (3440 S. Dearborn Street, Suite 260, Chicago, IL 60616)
- 4) Rodbard D. Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assays. Clin Chem. 1974 Oct; **20** (10):1255–1270
- 5) Human IL-6 Immunoassay Reagent Kit Insert (Quantiglo® Reagents), R&D Systems catalog Q6000B (614 McKinley Place, NE, Minneapolis, MN 55413)
- 6) Human IL-6 Immunoassay Reagent Kit Insert (Quantikine® HS Reagents), R&D Systems catalog HS600B (614 McKinley Place, NE, Minneapolis, MN 55413)
- 7) Human IL-6 ELISA Kit Insert, Invitrogen™ catalog KHC0061 (542 Flynn Road, Camarillo, CA 93012)
- 8) Human IL-6 Ultrasensitive ELISA Kit Insert, Invitrogen™ catalog KHC0064 (542 Flynn Road, Camarillo, CA 93012)

High Sensitivity Human IL-6 ELISA with an Expanded Dynamic Range:

Enhanced Performance using Sword™ Peroxidase Assay and the Tecan Infinite® M1000 Multimode Reader

- 9) Human TNF-alpha ELISA using Sword™ Peroxidase Reagents: Enhanced performance using the Tecan Infinite® M1000 multimode reader. Tecan Technical Note 396610 V1.0, 02-2011 (February 2011, Tecan Group Ltd, Sword Diagnostics Inc.)
- 10) An Optimized Mouse IL-6 ELISA using Sword Diagnostics Peroxidase Reagents: Enhanced Assay Performance using the Tecan Infinite® M1000 Multimode reader. Tecan Technical Note 396728 V 1.0. (August 2011, Tecan Group Ltd, Sword Diagnostics Inc.)
- 11) Peroxidase detection using Sword™ Peroxidase Reagents: Enhanced performance using the Tecan Infinite® M1000 multimode reader. Tecan Technical Note 396612 V1.0, 02-2011 (February 2011, Tecan Group Ltd, Sword Diagnostics Inc.)