Human TNF-α ELISA using Sword™ Peroxidase Reagents

Enhanced performance using the Tecan Infinite® M200 multimode reader

Introduction

Human tumor necrosis factor-alpha
Human tumor necrosis factor-alpha (Hu TNF-α), also called cachectin, is a 157 amino acid non-glycosylated cytokine mainly produced by activated macrophages.

Lipopolysaccharide (LPS) is a potent stimulus for TNF-α production in macrophages. TNF-α is an important mediator of the *in vivo* effects of LPS, such as tumor hemorrhagic necrosis, fever, shock and neutrophil activation.

The various biological activities of TNF-α may be classified as:

- Antitumor and growth regulatory activities: TNF-α displays a selective toxicity for tumor and virus-infected cells.
- Immunomodulating and proinflammatory activities: TNF-α regulates the production of antibodies by B-cells and stimulates cytotoxic T-cells.
- Metabolic activities: TNF-α strongly inhibits lipoprotein lipase and adipocyte gene expression.

TNF-α has a major pathogenic role in cachexia associated with chronic infectious or malignant diseases, septic shock, graft rejection, graft-versus-host disease and parasitic infections. TNF-α, along with other cytokines, has been reported to be involved in several autoimmune diseases and in the pathogenesis of arteriosclerosis.

Principle of the assay
The Invitrogen® Hu TNF-α kit is a solid phase sandwich enzyme-linkedimmunosorbent assay (ELISA). A monoclonal antibody specific for Hu TNF-α has been coated onto the wells of the microtiter strips provided. Samples, including standards of known Hu TNF-α content and control specimens, are pipetted into these wells.

During the first incubation, the Hu TNF-α antigen binds to the immobilized (capture) antibody on one site. After washing, a biotinylated monoclonal antibody specific for Hu TNF-α is added. During the second incubation, this antibody binds to the immobilized Hu TNF-α captured during the first incubation

After removal of excess second antibody, streptavidin-peroxidase (enzyme) is added. This binds to the biotinylated antibody to complete the four member sandwich.

After a third incubation and washing to remove all the unbound enzyme, a substrate solution is added which is acted upon by the bound enzyme to produce a signal. The magnitude of this signal is directly proportional to the concentration of Hu TNF-α present in the original specimen.

The Hu TNF-α ELISA is intended for the *in vitro* quantitative determination of Hu TNF-α in human serum, plasma, buffered solutions, or cell culture medium. The assay recognizes both natural and recombinant forms of TNF-α. This technical note describes the performance of the Hu TNF-α 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 TNF-α analyte and of the HuTNF-α ELISA are taken from the Invitrogen Hu TNF-α ELISA insert[1].

Materials and methods

Instrument

Tecan Infinite M200 multimode reader

Microplates

Pre-coated 96-well, flat bottom, polystyrene microplates, supplied with the Invitrogen Hu TNF-α ELISA

Reagents

- Hu TNF-α ELISA (Invitrogen, CA)
- Sword Peroxidase Reagents (Sword Diagnostics, IL)

Assay procedures: Hu TNF-α ELISA

The Hu TNF-α ELISA was run as described in the assay insert[1] up until the addition of chromogen. At this point the Sword Peroxidase Reagents were substituted for the vendor-supplied tetramethylbenzidine chromogen (TMB). When used, the TMB derived signals (absorbance at 450 nm) were corrected using an absorbance at 540 nm, rather than subtracting the reagent blank signal.

Peroxidase reactions using Sword Peroxidase Reagents

1) Sword Peroxidase Substrate / Peroxide Mixture and Sword 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	Fluorescence intensity
Reads	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 experimental data and the measurement parameters were exported automatically by the i-control™ software to Microsoft Excel® for further analysis. TNF-α 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 of 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 Reagents dose response curve. This value is related to the shift of the curve; the lower the value, the greater the shift of the curve.

Results and discussion

The Hu TNF-α ELISA was performed using the calibrator levels cited in the assay insert, plus several levels below the lowest calibrator levels cited (15.63 pg/ml), as the samples. The assay was evaluated using both the Sword Peroxidase Reagents and the TMB 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. The results are shown in Tables 2 and 3.

TNF-α STD (pg/ml)	Mean	SD	N	% CV	SE
0.000	0.0049	0.0024	8	48.6	0.0008
0.975	0.0062	0.0005	4	8.8	0.0003
1.95	0.0121	0.0017	4	13.9	0.0008
3.91	0.0148	0.0010	4	6.9	0.0005
7.81	0.0280	0.0023	4	8.2	0.0011
15.63	0.0460	0.0033	4	7.1	0.0016
31.25	0.0964	0.0095	4	9.9	0.0048
62.50	0.1715	0.0129	4	7.5	0.0064
125	0.3080	0.0130	4	4.2	0.0065
250	0.6564	0.0002	2	0.0	0.0001
500	1.1664	0.0180	2	1.5	0.0127
1,000	1.8896	0.0257	2	1.4	0.0182

Table 2 Response signal generated with TMB reagents

TNF-α STD (pg/ml)	Mean	SD	N	% CV	SE
0.000	1,255	37	8	3.0	13
0.975	1,324	15	4	1.1	7
1.95	1,338	47	4	3.5	24
3.91	1,361	13	4	0.9	6
7.81	1,526	46	4	3.0	23
15.63	1,599	45	4	2.8	23
31.25	1,917	59	4	3.1	30
62.50	2,207	78	4	3.5	39
125	2,871	97	4	3.4	49
250	3,267	13	4	0.4	9
500	3,972	60	4	1.5	43
1,000	4,813	93	8	1.9	66

Table 3 Response signal generated with Sword Peroxidase Reagents

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

The mean response values were plotted against the TNF-α 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 absorbance at 450 nm, with a correction at 540 nm. The data was then fitted to a 4PLC, using only the standard calibrator levels cited in the assay insert. These fitted curves were shown on the

plots, with standard error bars, to demonstrate the relative fit of the observed data (Figures 1 and 2). The Sword- and TMB-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 (1,000 pg/ml) values from each respective curve overlapped.

The data obtained using both the Sword and TMB detection reagents yields smooth dose response curves that fit well to a 4PLC (with R^2 values of 0.9991 and 1.000 for the Sword and TMB data respectively). Also note how well the low concentration standards (below 15.63 pg/ml) align with the fitted curve.

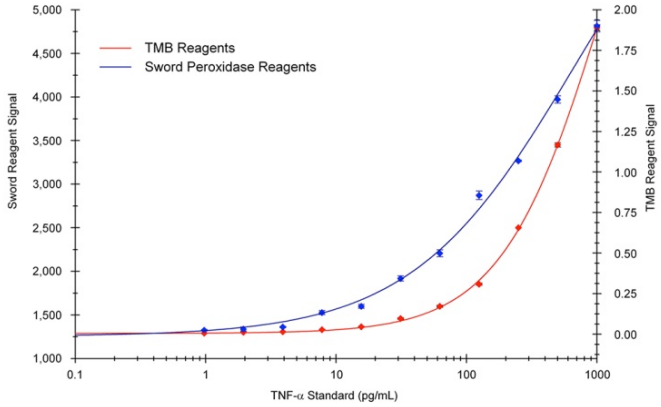

Figure 1 Sword Peroxidase Reagent and TMB reagent dose response curves

Of more importance is the relative position of these dose response curves. The Sword Peroxidase Reagents-derived dose response curves are shifted to a lower concentration relative to the TMB-derived curves. The magnitude of this shift is described by evaluating the concentration at ½ the maximum response of the highest calibrator ($Conc^{1/2\ Max}$), see Table 4.

Detection system	Concentration ½ Max
TMB Reagent curve	386
Sword Peroxidase Reagent curve	79

Table 4 Concentration at ½ maximum signal

This indicates that, in the Hu TNF-α ELISA, the Sword Peroxidase Reagents are capable of detecting more than five fold lower sample concentrations when only half of the available signal is expended. Evaluation of 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 both greater signal strength at the lower concentrations (concentrations that are often of interest in the assay), and improved ability to distinguish samples from negative controls.

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 this currency.

To demonstrate the power of this added optimization flexibility, a sample volume reduction study was performed. In this study, the original sample volume (100 µl) was reduced by 50 % (50 µl) and 75% (25 µl). The Hu TNF-α ELISA 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 TMB 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 TMB curve.

Here the additional optimization flexibility (or currency) was used to obtain a significant reduction in the required sample volume.

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 protocols adopted.

In the case of the Hu TNF-α ELISA, an improvement in the estimated analytical LOD was observed. Specifically, TNF-α LODs of 1.32 and 2.37 pg/mlwere observed for the Sword Peroxidase Reagents and TMB reagents respectively. The TMB-derived LOD was consistent with that cited in the TNF-α ELISA insert[1].

The observed LOD shift demonstrates some of the assay performance improvements that might be attained by adopting the Sword Peroxidase Reagents. In this case, these reagents were simply introduced into a commercial assay (optimized for TMB detection), without any additional optimization. It is expected that additional performance improvements could be attained with further assay optimization to the Sword Peroxidase Reagents. However, demonstration of such improvements without access to the assay formulation or biological reagents was not feasible with this commercial assay.

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

Conclusion

This technical note describes the successful performance of the Invitrogen Human TNF-α ELISA on the Infinite M200 multimode reader using Sword Peroxidase Reagents.

Sword Peroxidase Reagents were easily introduced into this TMB-optimized assay, without any modifications to the Hu TNF-α ELISA or to the 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 TMB 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 Hu TNF-α ELISA.

An improvement in the estimated TNF-α analytical limit of detection was also observed using the Sword Peroxidase Reagents.

The described applications are intended for research use only.

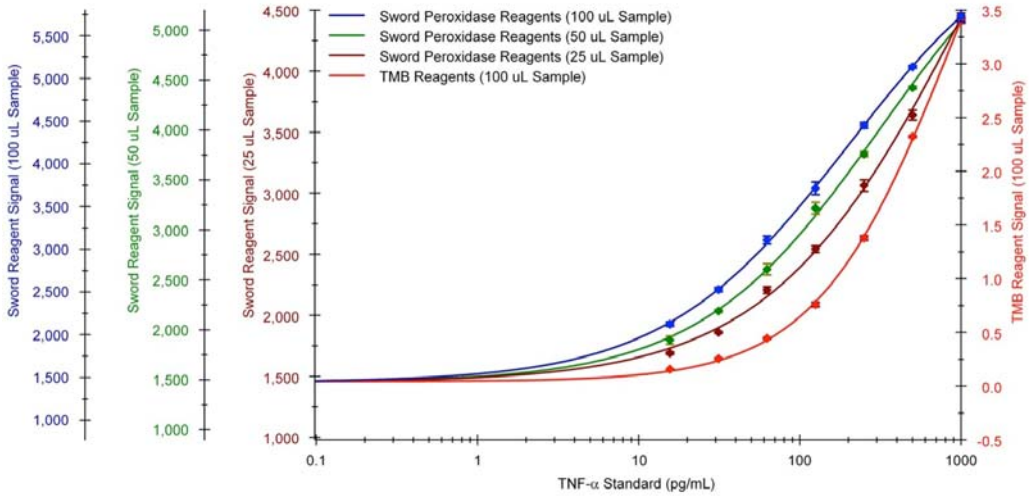

Figure 2 Dose response curves obtained in sample volume reduction

Literature

1) Invitrogen HuTNF-α ELISA Reagent Kit Insert, Invitrogen catalog KHC3012 (542 Flynn Rd, Camarillo, CA 93012)
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

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