Human C-reactive protein ELISA using Sword™ Peroxidase Reagents

Enhanced performance using the Tecan Infinite® M200 multimode reader

Introduction

Human C-reactive protein

C-reactive protein (CRP) is a homopentameric protein belonging to the pentraxin family, with a molecular weight of about 115 kDa. CRP is an acute phase protein produced by hepatocytes in response to circulating IL-6, IL-1, and TNF-α. It plays an important role in host defense, as a pro-inflammatory mediator and activator of the complement pathway. Although normal circulation levels are low, they rise sharply within 48 hours of disease or trauma, and stay elevated until resolution of the inflammation. Elevated levels of CRP are associated with many pathological states, including rheumatoid arthritis, tissue trauma, viral or bacterial infection, hepatitis and some autoimmune conditions. CRP has also been shown to be a reliable indicator of increased risk of cardiovascular disease and post-operative complications.

Principle of the ELISA

This assay employs the quantitative sandwich enzyme immunoassay technique. Human CRP present in the samples and standards binds to the anti-human CRP antibodies adsorbed to the plate. Following incubation, a wash procedure is performed to remove any unbound material and an HRP-conjugated antibody against a distinct epitope of human CRP is added to the microwell plate. After a second incubation period and wash, a peroxidase substrate is added. The development of a blue color is proportional to the amount of soluble CRP present in the sample. The reaction is stopped with an acid solution that turns the liquid from blue to yellow. Color intensity is then determined reading the absorbance at 450 nm. A dilution series of standards at known concentrations is used to plot a curve and quantify the amount of human CRP present in the samples.

Note that the descriptions of the CRP analyte and of the CRP assay are taken from the eBioscience® Human C-Reactive Protein (CRP) ELISA Kit insert[1].

Materials and methods

Instrument

Tecan Infinite M200 multimode reader

Microplates

Pre-coated 96-well, flat bottom, polystyrene microplates, supplied with the eBioscience Human C-Reactive Protein (CRP) ELISA Assay

Reagents

- Human C-Reactive Protein (CRP) ELISA Kit, (eBioscience, CA)
- Sword Peroxidase Reagents (Sword Diagnostics, IL)

Assay Procedures: CRP ELISA

The eBioscience CRP ELISA was run as described in the assay insert[1] up until the addition of chromogen is called for. At this point the Sword Peroxidase Reagents were substituted for the vendor-supplied tetramethylbenzidine (TMB) chromogen.

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 for 8 hours.
2) Once the post conjugate incubation wash step of the ELISA was completed, 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	150 (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. CRP 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 this value, the greater the shift of the curve.

Results and discussion

The eBioscience CRP ELISA was performed using the calibrator levels cited in the assay insert, plus several levels below the lowest calibrator levels cited (156.25 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 top read optics to collect data. The results are shown in Tables 2 – 3.

CRP STD (pg/ml)	Mean	SD	N	% CV	SE
0.00	0.06383	0.00751	8	11.8	0.00266
39.06	0.09223	0.00545	4	5.9	0.00272
78.13	0.11504	0.00385	8	3.3	0.00136
156.25	0.16735	0.00309	4	1.8	0.00154
312.5	0.29970	0.01492	4	5.0	0.00746
625	0.48438	0.02067	4	4.3	0.01034
1,250	0.86697	0.02896	4	3.3	0.01448
2,500	1.39350	0.02339	4	1.7	0.01169
5,000	2.23890	0.03790	4	1.7	0.01895
10,000	3.01955	0.11589	4	3.8	0.05795

Table 2 Response signal generated with TMB reagents

CRP STD (pg/ml)	Mean	SD	N	% CV	SE
0.00	1,128	32	7	2.9	12
39.06	1,172	56	4	4.8	28
78.13	1,333	60	8	4.5	21
156.25	1,503	52	4	3.5	26
312.5	1,963	133	4	6.8	66
625	2,252	59	4	2.6	30
1,250	3,113	40	4	1.3	20
2,500	3,627	79	4	2.2	40
5,000	4,106	59	4	1.4	29
10,000	4,517	113	4	2.5	56

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 generated with 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 CRP 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 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 (10,000 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 0.9997 and 0.9973 for the Sword and TMB data respectively). Also note how well the low concentration standards (below 156.25 pg/ml) align with the fitted curve.

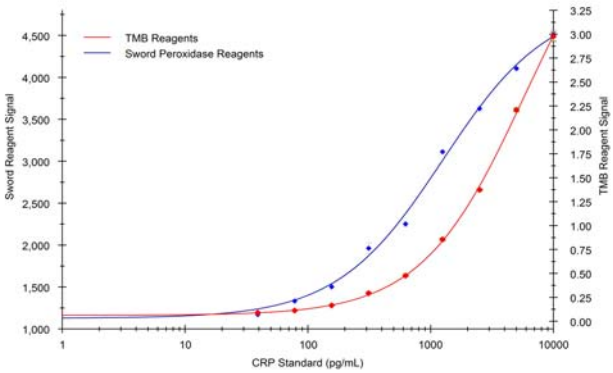

Figure 1 Sword Peroxidase Reagents and TMB reagent dose response curves

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	2,707
Sword Peroxidase Reagents curve	531

Table 4 Concentration at ½ maximum signal

This indicates that, in the CRP ELISA, the Sword Peroxidase Reagents are capable of detecting more than six 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 the 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 size (100 µl) was reduced by 50% (50 µl) and 75% (25 µl). The CRP 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.

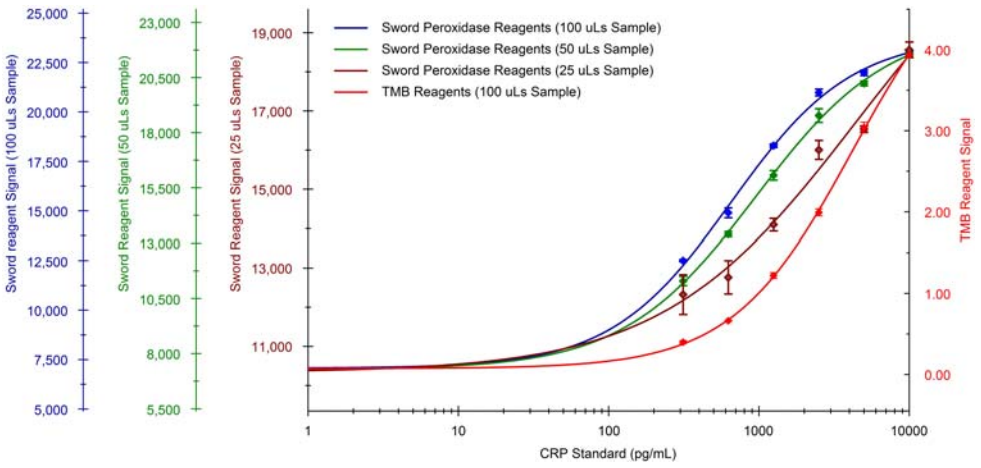

Figure 2 Dose response curves obtained in sample volume reduction

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 eBioscience CRP ELISA, an improvement in the estimated analytical LOD was observed. Specifically, CRP LODs of 6.21 and 21.37 pg/ml were observed for the Sword Peroxidase Reagents and TMB reagents respectively. The TMB-derived LOD was consistent with that cited in the CRP 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 eBioscience Human CRP ELISA assay on the Infinite M200 multimode reader using the Sword Diagnostics Peroxidase Reagents.

Sword Peroxidase Reagents were easily introduced into this TMB-optimized assay, without any modifications to the CRP 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 75 % reduction in sample volume for the CRP ELISA assay.

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

The described applications are intended for research use only.

Literature

1) eBioscience Human C-Reactive Protein (CRP) ELISA Kit Reagent Insert, (eBioscience catalog 88-7502, 10255 Science Center Drive, San Diego, CA 92121)
2) Sword Diagnostics Peroxidase Reagent Insert. Sword Catalog N818, (3440 S. Dearborn Street, Suite 260, Chicago, IL 60616)
3) Rodbard D. (1974) 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 48 174 **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

www.tecan.com

396607 V1.0, 02-2011