



Peroxidase detection using Sword™ Peroxidase Reagents

Enhanced performance using the Tecan Infinite® M1000 multimode reader

Introduction

Use of horseradish peroxidase in ELISAs

For almost 40 years, enzyme-linked immunosorbent assays (ELISAs) have been used to quantify a wide range of compounds and pathogens. These assays initially utilized enzymes bound to specific antibodies, or binding proteins as enzyme conjugates to produce colorimetric signals whose presence was proportional to the targeted assay analyte concentration.

One commonly used enzyme conjugate in ELISA is horseradish peroxidase (HRP). Horseradish peroxidase is a 40,000 Dalton protein which catalyzes the reduction of hydrogen peroxide (H₂O₂) to water (H₂O). In the presence of specific substrates, acting as hydrogen donors, the action of HRP converts colorless molecules into a colored moiety. One of the most common colorimetric substrates is 3,3',5,5'-tetramethylbenzidine (TMB). When oxidized by HRP, this substrate turns a blue color, and is transformed into a yellow product on acidification.

Recently, new peroxidase substrates have been developed where the products are fluorescent or luminescent rather than colored. The compound luminol and its several derivatives represent one class of peroxidase substrates which, when enzymatically oxidized, produce a chemiluminescent product. Again, the presence of the chemiluminescent product is proportional to the targeted assay analyte concentration. In several applications, the replacement of the colorimetric product with a chemiluminescent product has led to improved peroxidase detection and enhanced ELISA performance.

This technical note describes the performance of Sword Diagnostics' new and more sensitive peroxidase detection system on the Tecan Infinite M1000 multimode reader. The performance of this detection methodology is compared to currently used colorimetric (TMB), fluorescent (Amplex® Red and QuantaRed™) and chemiluminescent (Glo) detection methodologies.

Materials and methods

Instrument

Tecan Infinite M1000 multimode reader

Reagents

- Sword Peroxidase Reagents (Sword Diagnostics, IL)
- SureBlue Reserve™ (3,3',5,5'-tetramethylbenzidine, TMB; KPL, Gaithersburg, MD)
- TMB Stop Solution (KPL, Gaithersburg, MD)
- Glo Substrate Reagent Pack (DuoSet® Ancillary Product; R&D Systems, MN)
- QuantaRed Enhanced Chemifluorescent HRP Substrate (Thermo Scientific, IL)
- Amplex Red Peroxidase Detection Kit (Invitrogen™, CA)
- Peroxidase Type VI-A from Horseradish (Sigma, MO)
- Peroxidase Diluent (PBS, 0.10 %; BSA, pH 7.2)

Microplates

Flat bottom, clear, medium binding, polystyrene 96-well microplates (Costar, NY)

Flat bottom, solid black, medium binding, polystyrene 96-well microplates (Greiner Bio-one, Germany)

Assay procedures

Preparation of peroxidase standards

- 1) Concentrated stock solutions (2 µg/ml) of horseradish peroxidase were prepared in Peroxidase Diluent (PBS, 0.1 %; BSA, pH 7.2), aliquoted into 150 µl single use aliquots, and stored frozen at -20 °C. Aliquots were discarded after one freeze-thaw cycle.
- 2) All subsequent horseradish peroxidase standards were prepared in Peroxidase Diluent as serial dilutions of the 2 µg/ml stock HRP solution. All standards were used within two hours of preparation.

Peroxidase reactions using Sword Peroxidase Reagents

- 1) Sword Peroxidase Substrate / Peroxide Mixture and Sword Development Solution were prepared as described in the peroxidase assay insert¹. The reagents were brought to room temperature prior to use. Preparation of the Substrate / Peroxide Mixture consists of mixing specified volumes of water, concentrated Substrate Diluent, concentrated Substrate, and concentrated Stabilized Peroxide, storing the reagent in the dark.

The mixture is stable at room temperature for over 8 hours. Development Solution is prepared by mixing specified volumes of water and concentrated Development Solution together. The mixture is stable at room temperature for over 24 hours.

- 2) 150 µl of Sword Substrate / Peroxide Mixture was added to 50 µl of Peroxidase Standard in a flat bottom, clear, medium binding, polystyrene microplate well.
- 3) The microplate well reactions were incubated at room temperature in the dark for 15 minutes.
- 4) After this incubation, 150 µl of Development Solution was added to each well. To ensure adequate mixing, 50 µl of each well was gently aspirated and dispensed 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.
- 6) The signal from each well was measured in a Tecan Infinite M1000 multimode reader (fluorescence intensity mode) within 60 minutes of the completed development reaction.

Peroxidase reactions using KPL SureBlue Reserve TMB Reagent

- 1) The KPL SureBlue Reserve TMB Reagent was brought to room temperature prior to use.
- 2) 100 µl of the TMB reagent was added to 50 µl of peroxidase standard in a flat bottom, clear, medium binding, polystyrene microplate well.
- 3) The microplate well reactions were incubated for 20 – 25 minutes in the dark at room temperature.
- 4) 100 µl of KPL TMB Stop Solution was added to each reaction well.
- 5) The absorbance of each well at 450 nm and 540 nm was measured in a Tecan Infinite M1000 multimode reader (absorbance mode) within 30 minutes of adding the stop solution.

Peroxidase reactions using R&D Systems Glo Substrate Reagents

- 1) The R&D Systems Glo Substrate Chemiluminescent Reagents were brought to room temperature prior to use.

- 2) A Glo Reagent Mixture was created by mixing one part of Glo Reagent A with two parts of Glo Reagent B. This mixture was stored in the dark prior to use.
- 3) 100 µl of the Glo Reagent Mixture was added to 50 µl of Peroxidase Standard in a flat bottom, solid black, medium binding, polystyrene microplate well.
- 4) The microplate well reactions were incubated for 10 minutes in the dark at room temperature.
- 5) The luminance of each well was measured in a Tecan Infinite M1000 multimode reader (luminescence mode) within 2 – 5 minutes of the addition of the Glo Reagent Mixture.

Peroxidase reactions using the Thermo Scientific QuantaRed Enhanced Chemifluorescent HRP Substrate

- 1) The QuantaRed Reagents were brought to room temperature prior to use.
- 2) The QuantaRed Substrate Solution was prepared by mixing 50 parts QuantaRed Enhancer Solution with 50 parts QuantaRed Stable Peroxide and 1 part QuantaRed ADHP Concentrate.
- 3) 100 µl of QuantaRed Substrate Solution was added to 50 µl of Peroxidase Standard (diluted in 1X reaction buffer rather than the Peroxidase Diluent cited earlier) in a flat bottom, solid black, medium binding, polystyrene microplate well.
- 4) The microplate well reactions were incubated for 10 minutes in the dark at room temperature.
- 5) At the end of the incubation, 10 µl of QuantaRed Stop Solution was added to each reaction well and the fluorescence of each well was measured in a Tecan Infinite M1000 multimode reader (fluorescence intensity mode).

Peroxidase reactions using Invitrogen Amplex Red Substrate Reagents

- 1) The Amplex Red Reagents were brought to room temperature prior to use.
- 2) The contents of the Amplex Red Reagent vial (Component A) were dissolved in 60 µl of dimethylsulphoxide (DMSO, Component B). This solution was stored in the dark prior to use.
- 3) A 20 mM hydrogen peroxide solution was prepared by adding 22.7 µl of a 3 % H₂O₂ solution to 977 µl of 1X

reaction buffer. The 1X reaction buffer was prepared from a vendor-supplied 5X reaction buffer solution.

- 4) An Amplex Red working solution was prepared by adding 50 µl of the dissolved Amplex Red to 500 µl of the 20 mM H₂O₂ solution and 4.45 µl of the 1X reaction buffer.
- 5) 50 µl of the Amplex Red working solution was added to 50 µl of Peroxidase Standard (diluted in 1X reaction buffer rather than the Peroxidase Diluent cited earlier) in a flat bottom, solid black, medium binding, polystyrene microplate well.
- 6) The microplate well reactions were incubated for 20 minutes in the dark at room temperature.
- 7) On completion of the incubation, the fluorescence of each well was measured in a Tecan Infinite M1000 multimode reader (fluorescence intensity mode).

Table 1

Fluorescence intensity parameters	Setting
Mode	Fluorescence intensity
Reads	Top
Flash number (frequency)	25 (100 Hz)
Integration	20 µsec
Gain	150 – 160 (manual)
Z height (top read)	25,000 (manual)
Absorbance parameters	Setting
Mode	Absorbance
Measurement wavelength	450 nm
Bandwidth	9 nm
Reference wavelength	540 nm
Bandwidth	9 nm
Flash number (frequency)	25 (100 Hz)
Settle time	0 msec
Luminescence parameters	Setting
Mode	Luminescence
Attenuation	Automatic
Integration time	100 msec
Settle time	0 msec

Table 1 Infinite M1000 multimode reader measurement parameters

Note: Auto settings of the gain and Z height may also be used in the fluorescence intensity mode, although for more sensitive readings the Z height should be optimized to the microplate and unit being used.

Measurement settings for each detection system

- The Sword Peroxidase Reagents signal was measured as relative fluorescence units (RFU) detected by the fluorescence detection channel, using an excitation wavelength of 530 nm (9 mm bandwidth) and an emission wavelength of 700 nm (20 mm bandwidth).
- The TMB Reagent colorimetric signal was measured as the optical density at 450 nm with a correction at 540 nm (A_{450} minus A_{540}).
- The QuantaRed Reagent signal was measured as relative fluorescence units (RFU) detected by the fluorescence detection channel, using an excitation wavelength of 540 nm (9 mm bandwidth) and an emission wavelength of 600 nm (20 mm bandwidth).
- The Amplex Red Reagent signal was measured as relative fluorescence units (RFU) detected by the fluorescence detection channel, using an excitation wavelength of 540 nm (9 mm bandwidth) and an emission wavelength of 600 nm (20 mm bandwidth).
- The Glo Reagent chemiluminescent signal was measured as relative luminescence units (RLU) detected by the luminescence detection channel.

Data analysis

The experimental data and the measurement parameters were exported automatically by the i-control™ software to Microsoft Excel® for further analysis. Horseradish peroxidase (HRP) response curves were fitted to a four parameter logistic curve (4PLC) using the equation communicated by D. Rodbard².

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

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 calibration 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

In this evaluation we examined the relative impact of the different horseradish peroxidase detection methodologies in microplate wells. HRP samples at differing concentrations (0 – 3,000 pg/μl) were measured using the Sword Peroxidase Reagents, TMB Reagents (colorimetric substrate), QuantaRed Reagents, Amplex Red Reagents (both fluorescent substrates) and Glo Reagents (chemiluminescent substrate). These results are shown in Tables 2 – 6.

Table 2

Peroxidase (pg/ml)	Mean	SD	N	% CV	SE
0.00	2,630.25	74.880	8	2.8	26
7.81	3,122.50	32.147	8	1.0	11
15.63	3,435.75	57.186	4	1.7	29
31.25	3,895.00	53.784	4	1.4	27
62.5	4,851.50	17.059	4	0.4	9
125	6,517.25	17.576	4	0.3	9
250	9,165.75	141.912	4	1.5	71
500	12,888.00	151.453	4	1.2	76
1,000	18,192.75	441.877	4	2.4	221
3,000	26,343.75	368.881	4	1.4	184

Table 2 Response signal generated with Sword Peroxidase Reagents

Table 3

Peroxidase (pg/ml)	Mean	SD	N	% CV	SE
0.00	0.00665	0.00531	8	79.9	0.00188
7.81	0.00818	0.00679	8	83.1	0.00240
15.63	0.00332	0.00215	4	64.7	0.00108
31.25	0.00665	0.00112	4	16.9	0.00056
62.5	0.02955	0.01159	4	39.2	0.00580
125	0.06678	0.00609	4	9.1	0.00304
250	0.16225	0.01352	4	8.3	0.00676
500	0.34907	0.00684	4	2.0	0.00342
1,000	0.78350	0.02119	4	2.7	0.01059
3,000	2.30727	0.03843	4	1.7	0.01922

Table 3 Response signal generated with TMB reagents

Table 4

Peroxidase (pg/ml)	Mean	SD	N	% CV	SE
0.00	814	160	8	19.7	57
3.91	794	159	4	20.1	80
7.81	926	115	4	12.4	58
15.63	931	73	4	7.9	37
31.25	1,124	154	4	13.7	77
62.5	1,664	133	4	8.0	67
125	3,025	194	4	6.4	97
250	8,351	467	4	5.6	233
500	62,946	1,821	4	2.9	910
1,000	225,416	8,578	4	3.8	4,289
3,000	1,201,285	42,392	4	3.5	21,196

Table 4 Response signal generated with Glo Reagents

Table 5

Peroxidase (pg/ml)	Mean	SD	N	% CV	SE
0.00	424	23	8	5.4	8
3.91	420	32	4	7.5	16
7.81	468	20	4	4.3	10
15.63	550	54	4	9.9	27
31.25	802	34	4	4.2	17
62.5	1,325	18	4	1.4	9
125	2,456	33	4	1.3	16
250	4,631	794	4	17.2	397
500	10,307	1,521	4	14.8	761
1,000	18,707	589	4	3.1	294
3,000	43,437	595	4	1.4	297

Table 5 Response signal generated with QuantaRed Reagents

Table 6

Peroxidase (pg/ml)	Mean	SD	N	% CV	SE
0.00	149	8.48	8	5.7	3.0
3.91	154	5.74	4	3.7	2.9
7.81	157	8.08	3	5.1	4.7
15.63	160	8.50	4	5.3	4.3
31.25	184	8.76	4	4.8	4.4
62.5	232	6.90	4	3.0	3.4
125	331	23.39	4	7.1	12
250	479	22.13	4	4.6	11
500	915	56.57	4	6.2	28
1,000	2,051	226.88	4	11.1	113
1,500	5,516	739.82	4	13.4	370
2,500	24,314	955	4	3.9	478
3,000	46,495	1,802	4	3.9	901

Table 6 Response signal generated with Amplex Red Reagents

These results demonstrate that the Tecan Infinite M1000 multimode reader is capable of measuring the response of the Sword Peroxidase Reagents over the entire HRP range with a precision greater than that seen with the other HRP detection reagents.

The mean response values were plotted against the HRP standard concentrations on semi-log plots. The data was then fitted to a 4PLC. These fitted curves were shown on the plots, with standard error bars, to demonstrate the relative fit of the observed data (Figure 1).

The signals from all the detection methodologies (Sword Peroxidase, TMB, QuantaRed, Amplex Red and Glo) were drawn on the sample plot, scaling the response axis (Y axis) such that the negative control (0 pg/ml) and highest positive (3,000 pg/ml) values from each respective curve overlapped.

The data obtained from all three types of detection reagent yield smooth dose response curves that fit well to a 4PLC (with R² values of 0.9998, 0.9997, 9978, 0.9988 and 0.9996 respectively for the Sword, TMB, QuantaRed, Amplex Red and Glo data).

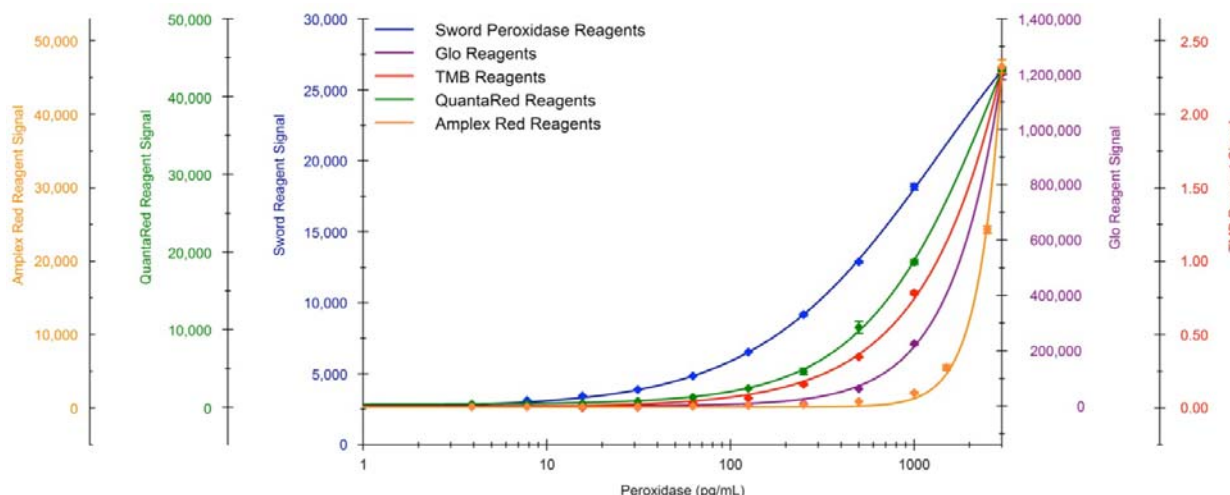


Figure 1 HRP dose response curves

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 colorimetric, fluorescent and chemiluminescent reagent derived curves. The magnitude of this shift is described by evaluating the concentration at $\frac{1}{2}$ the maximum response of the highest calibrator (Concentration $\frac{1}{2} \text{Max}$).

This suggests that the Sword Peroxidase Reagents are capable of detecting three to four fold lower HRP concentrations relative to the TMB results, and perhaps as much as five fold lower HRP concentrations relative to the Amplex Red results, when only half of the available signal is expended. Evaluation of the plots in Figure 1 and the data in Table 7 indicates that when the Sword Peroxidase Reagents are used in HRP detection, 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.

Table 7

Detection system	Concentration $\frac{1}{2} \text{Max}$
Amplex Red Reagents curve	2,437
Glo Reagents curve	1,928
TMB Reagent curve	1,534
QuantaRed Reagents curve	1,171
Sword Peroxidase Reagents curve	510

Table 7 Concentration at $\frac{1}{2}$ maximum signal

The shifted curves demonstrate an improvement in the estimated LODs. Specifically, HRP analytical limits of detection of 2.7, 16.8, 4.38, 284 and 15.8 pg/ml were observed for the Sword Peroxidase Reagents, TMB, QuantaRed, Amplex Red and Glo reagents.

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.

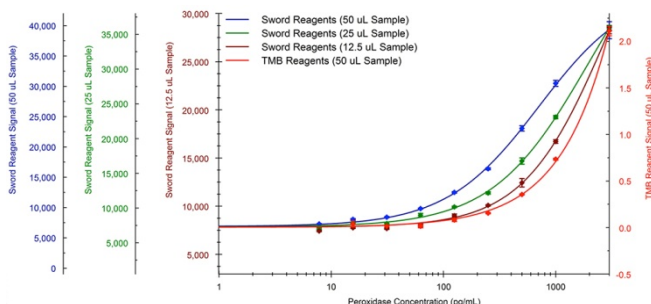


Figure 2A HRP volume reduction study (relative to TMB reagents)

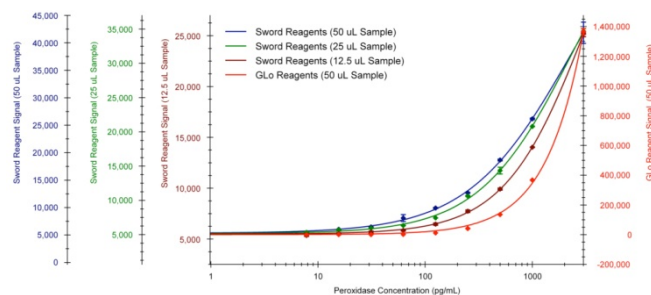


Figure 2B HRP volume reduction study (relative to Glo Reagents)

To demonstrate the power of this added optimization flexibility, a sample volume reduction study was performed. In this study, the original sample volume (50 μ L) was reduced by 50% (25 μ L) and 75% (12.5 μ L). The remaining sample volume was replaced with diluent. HRP samples were 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 (Figures 2A and 2B).

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

Using peroxidase solutions as samples, this technical note demonstrates the performance improvements inherent with the use of Sword Peroxidase Reagents. While the detection of

free peroxidase may be of interest, the true strength of these reagents in ELISA is realized in the detection of bound conjugate associated peroxidase. Sword Peroxidase Reagents have been introduced into several established assays capable of detecting human TNF- α , IL-2 (Interleukin-2), IL-4, IL-6, IL-8, C-reactive protein, interferon gamma, soluble TNF receptor II and mesothelin. In each case the native colorimetric peroxidase substrate (TMB) or chemiluminescent peroxidase substrate (Glo) was replaced with the Sword Peroxidase Reagents without additional assay optimization. Characteristic curve shifts were observed for each assay, with associated benefits.

Three specific examples of this enhanced performance in human TNF- α ³, C-reactive protein⁴ and IL-6⁵ assays using the Infinite M1000 multimode reader, have been described in greater detail.

Conclusion

This technical note documents the performance of Sword Peroxidase Reagents in the measurement of horseradish peroxidase, using the Tecan Infinite M1000 multimode reader. The performance was compared against a commonly used peroxidase colorimetric substrate, TMB, and a peroxidase chemiluminescent substrate, Glo.

Use of the Sword Peroxidase Reagents results in a shift in the dose response curve to a lower concentration than the typical curves generated with the other HRP detection substrates studied. This shift gives assay developers more flexibility in their optimization efforts, for example sample volume reduction, as demonstrated in this report. Sword detection reagents provide improvements that will allow assay developers more acceptable trade-offs between competing critical performance attributes than those available with existing detection reagents.

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

Literature

- 1) Sword Diagnostics Peroxidase Reagent Kit Insert. Sword Catalog N818, (3440 S. Dearborn Street, Suite 260, Chicago, IL 60616)
- 2) Rodbard D. Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assays. Clin Chem. 1974 Oct; 20 (10):1255–1270.
- 3) Human TNF- α ELISA using Sword Diagnostics Peroxidase Reagents, M1000 (Tecan Technical Note 396610)
- 4) Human C-reactive protein ELISA using Sword Diagnostics Peroxidase Reagents, M1000 (Tecan Technical Note 396606)
- 5) Human IL-6 chemiluminescent ELISA with Sword Diagnostics Peroxidase Reagents, M1000 (Tecan Technical Note 396608)

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