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Application Note

Determination of Horseradish Peroxidase (HRP) Using Amplex® Red and the Synergy™HT Microplate Reader

In order to improve detection limits, many ELISA assays now incorporate fluorogenic substrates in lieu of colorimetric substrates. Here we describe the use of the Synergy HT multi-detection microplate reader for the detection of horseradish peroxidase (HRP) using the fluorogenic substrate Amplex® Red.

Introduction

Enzyme linked immunosorbent assays (ELISA) have been used to quantitate a wide range of compounds and pathogens for almost 40 years. Initially radioactivity was used to quantitate the assays, but radioimmunoassays (RIA) have been replaced with assays utilizing enzymes to obtain colorimetric results. Recently new substrates have been developed to produce fluorescent and luminescent products. The basic tenet of the new assays remains the same as colorimetric assays. The substrate is converted into a measurable compound by the enzymatic activity of proteins conjugated to an antibody, which confers specificity.

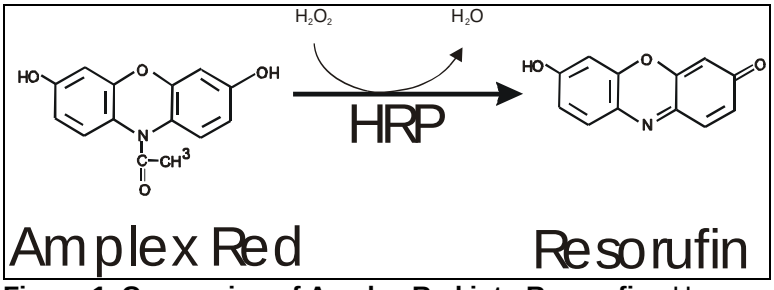

Figure 1. Conversion of Amplex Red into Resorufin. Horseradish peroxidase uses Amplex red as an electron donor during the reduction of hydrogen peroxide to water. The resultant product, resorufin, is a highly colored and fluorescent compound.

One commonly used enzyme conjugate in ELISA is horseradish peroxidase. Horseradish peroxidase (HRP) is a 40,000 Dalton protein, which catalyzes the reduction of hydrogen peroxide (H2O2) to water (H2O) [1]. In the presence of specific substrates, which act as hydrogen donors, the action of HRP converts colorless or nonfluorescent molecules into colored and/or fluorescent moieties respectively. Amplex Red is a substrate for use with HRP containing assays [2]. Amplex Red, in the presence of peroxidase enzyme, reacts with H2O2 in a 1:1 stiochiometry to produce resorufin, a red fluorescent compound (Figure 1) which has an absorption and fluorescence emission maxima of 563 nm and 587 nm respectively [2].

Materials and Methods

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An Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (A-22188) was purchased from Molecular Probes (Eugene, Oregon). Several stock solutions were prepared according to the assay kit instructions. A 10 mM stock solution of Amplex Red was prepared by dissolving Amplex Red reagent in dimethyl sulfoxide (DMSO). Reaction buffer, is comprised of 0.05 M sodium phosphate (pH 7.4) was prepared from a 5X concentrate supplied by the kit.

Horseradish Peroxidase stock solution (10 U/ml) was prepared by dissolving HRP powder in 1x reaction buffer. Note that 1 unit (U) is defined as the amount of enzyme that will form 1.0 mg of purpurogallin from pyrogallol in 20 seconds at ph 6.0 and 20°C. Using the supplied ~3% H2O2 stabilized solution, a 20 mM working solution was prepared using reaction buffer as the diluent on the day of the experiment and discarded after 24 hours. A working solution containing 100 µM Amplex red and 2 mM H2O2 was prepared immediately before use from the previously described stock solutions. A series of dilutions of HRP, using 1X reaction buffer as the diluent, were prepared prior to use. Aliquots of each concentration (50 µl) were pipetted into black opaque microplates (Catalog # 3631) from Costar. Reactions were initiated by the addition of 50 µl of the Amplex Red/H2O2working solution previously described. Fluorescent measurements were made using a BioTek (Winooski, VT), Synergy HT multi-detection microplate reader with a 530/25-excitation and a 590/35-emssion filter. Absorbance measurements used a working solution of 200 µM Amplex Red and 4 mM H2O2 and were made at 570 nm. Endpoint measurements, fluorescent or absorbance, were made at either 5 or 30 minutes after the initiation of the reaction, while kinetic reactions were measured every 90 seconds for a total of 30-60 minutes.

Results

The fluorescence was determined for reactions containing HRP concentrations ranging from 0 to 5 x 10-3 U/ml. Over this range the fluorescence increased in a hyperbolic fashion, with HRP concentrations above 5 x 10-3 U/ml per well resulting in very little increase in signal (Figure 2A). Using KC4 data reduction software (BioTek Instruments; Winooski, VT), a 4-parameter logistic best-fit equation describing the standard curve can be generated. When lower concentrations are examined (0 to 3.9 x 10-5 U/ml), the relationship between fluorescence and HRP concentration is linear (Figure 2B). Under these conditions a least means squared linear regression can be utilized to describe the data. The coefficient of determination (r2=0.991) value indicates that concentration determinations can be made with a high degree of confidence at these concentrations. In terms of sensitivity, as little as 4.8 x 10-6 U/ml of HRP can be reliably detected (data not shown).

A B

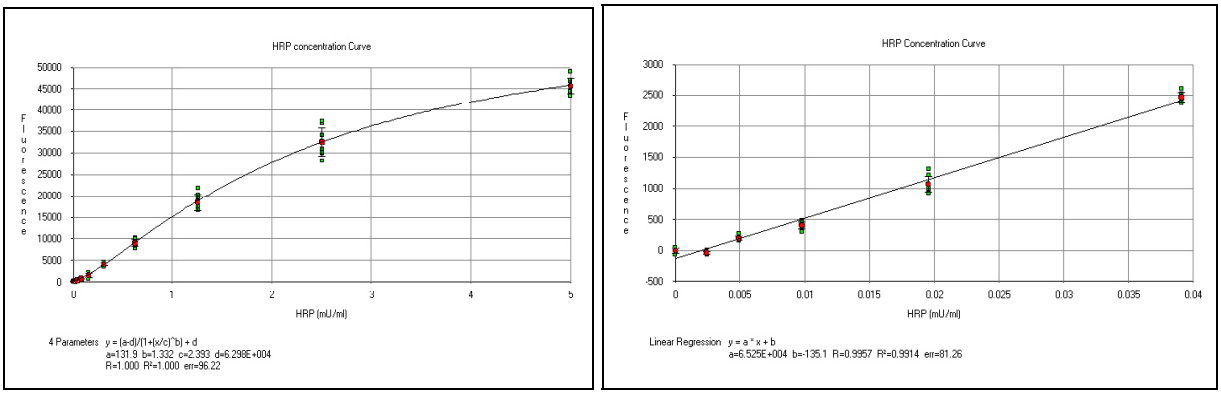

Figure 2. Horseradish Peroxidase Calibration Curve. HRP samples, in replicates of 8, ranging from 0 to 5 x 10-3 U/ml final concentration (graph A) or 0 to 4 x 10-5 U/ml (graph B) were reacted with H2O2 and Amplex Red reagent and the fluorescence using a 530/25 excitation and a 590/35 emission filter

determined. The subsequent data was plotted and linear regression analysis performed using KC4 Data Reduction Software.

The Fluorescence of samples with HRP enzyme concentrations ranging from 0 to 5 x10-3 U/ml were read kinetically. When the fluorescence values are plotted as a function of time, enzyme concentrations up to 1.25 x 10-3 U/ml are linear for at least 30 minutes (Figure 3). Enzyme concentrations above 1.25 x 10-3 U/ml result in over range values prior to 30 minutes, with the 5 x 10-3 U/ml samples reaching maximal values by 5 minutes. When Vmax values are calculated from these data and plotted against enzyme concentration using KC4, a linear relationship is observed (Figure 4). Using a 4-parameter logistic regression analysis of these data an equation describing this relationship can be used with a high degree of confidence, as the coefficient of determination (R2) is >0.9999.

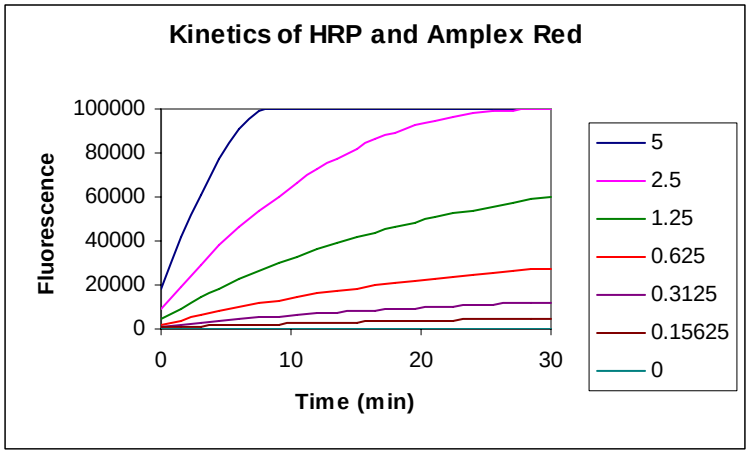

Figure 3. Kinetic Analysis of Fluorescent signal production with different HRP concentrations. HRP samples, in replicates of 8, ranging from 0 to 5 x 10-3 U/ml final concentration were reacted with H2O2 and Amplex® Red reagent and the fluorescence using a 530/25 excitation and a 590/35 emission filter determined kinetically. Fluorescent measurements were made every 90 seconds from the top using a Synergy HT multi-detection reader with a PMT sensitivity setting of 50. The subsequent data was exported to Microsoft© Excel and the mean of the replicates at each time point plotted.

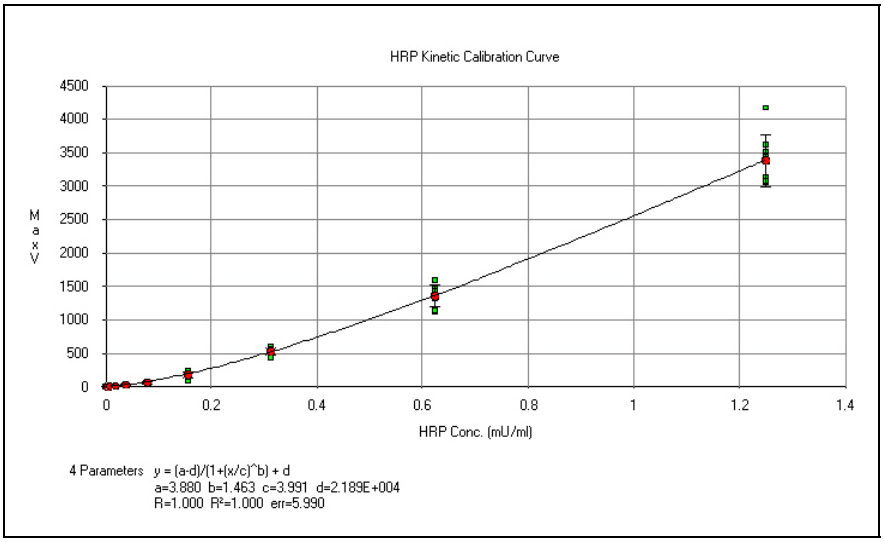

Figure 4. Kinetic Calibration Curve. Using the data presented in Figure 3, the MaxV was calculated using KC4 data reduction software and plotted against enzyme concentrations ranging from 0 to 1.25 x 10-3 U/ml of HRP using a 4-parameter logistic fit.

The reaction product of Amplex® Red substrate, resorufin, has been reported to be a colored compound with a high extinction coefficient (54,000 cm-1M-1) [3]. Previous experiments have indicated that it can be measured using absorbance [4]. This suggests that high levels of HRP, which may be outside the linear range of fluorescence, can be quantitated using absorbance. Figure 5 demonstrates the ability to quantitate horseradish peroxidase via the absorbance of reacted resorufin. At high enzyme concentrations, the absorbance quickly utilizes all of the substrate, despite having twice the concentration of Amplex Red and H2O2 as the fluorescent assays. This can be observed as a plateau in the curve depicted in Figure 5A. At lower concentrations, the increase in absorbance is linear with respect to HRP concentration, with a correlation coefficient > 0.99 using a linear regression analysis (Figure 5B).

A B

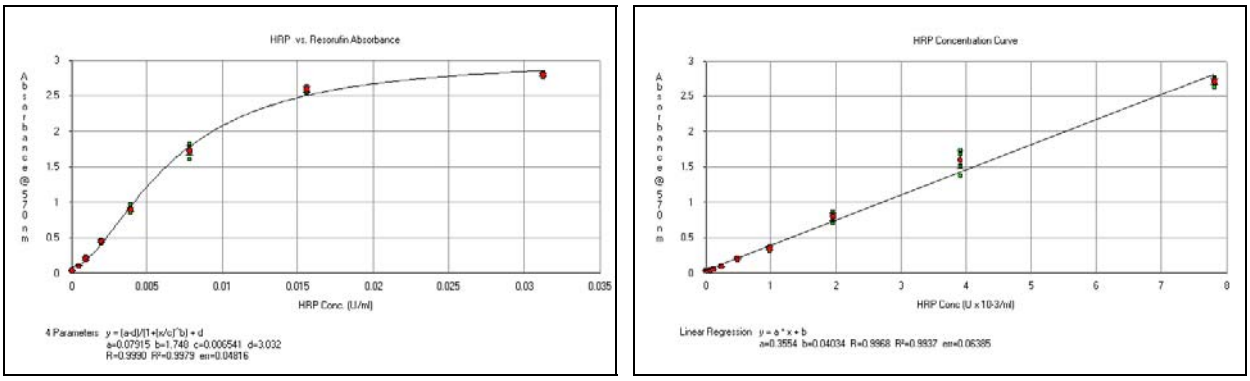

Figure 5. HRP Concentration Measured by Absorbance. Figure A: HRP samples, in replicates of 8, ranging from 0 to 3.12 x 10-2 U/ml final concentration were reacted with H2O2 and Amplex Red reagent and the absorbance at 570 nm determined after a 5-minute incubation at room temperature. The subsequent data was plotted using a 4-parameter logistic fit analysis using KC4 Data Reduction Software. Figure B: HRP samples ranging from 0 to 7.8 x 10-3 U/ml final concentrations were reacted with H2O2 and Amplex Red reagent and the absorbance at 570 nm determined after a 30-minute incubation at room temperature using a Synergy HT multi-detection reader. The subsequent data was plotted and linear regression analysis performed using KC4 Data Reduction Software.

Discussion

In order to achieve increased sensitivity, chemifluorescence is increasingly being used for ELISA reactions. The use of photomultiplier tubes with fluorescence detection has typically resulted in an order of magnitude increase in sensitivity. With the advent of stable, highly fluorescent substrates this trend will only increase. Horseradish peroxidase and alkaline phosphatase enzymes are the most commonly used enzymes conjugated to antibodies for ELISA. New substrates have been developed for these enzymes that result in formation of fluorescence. In this application note we have demonstrated the capability of the Synergy HT to detect the fluorescence and absorbance of Amplex Red, a substrate for Horseradish peroxidase with independent optical systems. The Synergy HT multi-detection reader is unique in its ability to measure both absorbance and fluorescence. This provides an almost 10 fold increase in the concentration range that can be analyzed with one instrument. Samples with low levels of HRP can easily be measured using fluorescence, while those which exhibit a fluorescence signal that is over-range can still be quantitated using the absorbance mode of the reader.

Amplex Red substrate has several qualities that make it a good ELISA substrate. The reagent has very little background fluorescence or absorbance in its unreacted state. In addition, it does not require any stop-like additives in order to fluoresce. The substrate is resistant to photo-bleaching and can, therefore, be read repeatedly in order to obtain appropriate detection levels. This applies to both absorbance and fluorescence. Likewise, the measurement of very high

enzyme concentrations can be accomplished by either dilution of the sample or measurement using absorbance.

The use of KC4 software to control the reader allows the user a great deal of flexibility in regards to data reduction capabilities. The software allows the user to define any configuration of plate map necessary and as demonstrated in this application note, with several different curve fit algorithms to choose from, regression analysis of the standards and the subsequent concentration determinations of samples can be accomplished with a high degree of confidence. KC4 is also capable of performing statistical analysis on sample groups, as well as any mathematical calculation required by the user.

References

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