



## Quantitation of Hydrogen Peroxide using the Synergy HT™

Several biological processes create or utilize hydrogen peroxide. Using Amplex® Red, reagent hydrogen peroxide can be measured in a one step process using the Synergy HT™ multi-detection Microplate reader. This monograph describes the quantitation of hydrogen peroxide using either the absorbance or fluorescence modes of the Synergy HT reader.

### Introduction

Hydrogen peroxide is produced in vitro under a variety of circumstances. Activated lymphocytes release hydrogen peroxide as part of an oxidative burst as a means to kill bacteria (1). Several enzymatic reactions produce hydrogen peroxide ( $H_2O_2$ ) as a bi-product, while other reactions consume the molecule (2, 3). Regardless of the reaction, Amplex Red reagent can be utilized to measure  $H_2O_2$  levels. Amplex Red, in the presence of peroxidase enzyme, reacts with  $H_2O_2$  in a 1:1 stoichiometry to produce resorufin, a red fluorescent compound (Figure 1). Resorufin has an absorption and fluorescence emission maxima of 563 nm and 587 nm respectively (Figure 2).

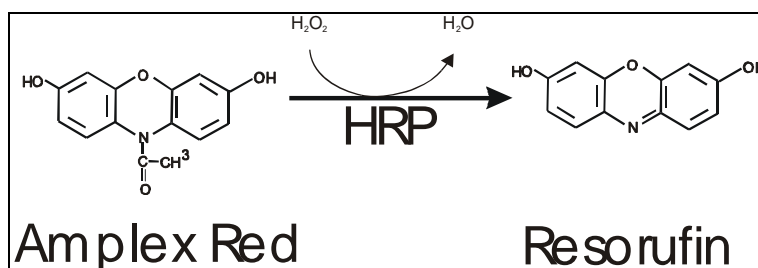
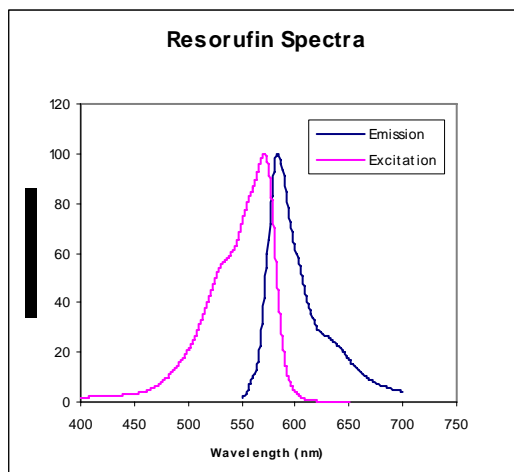


Figure 1. Conversion of Amplex Red into Resorufin.

### Materials and Methods

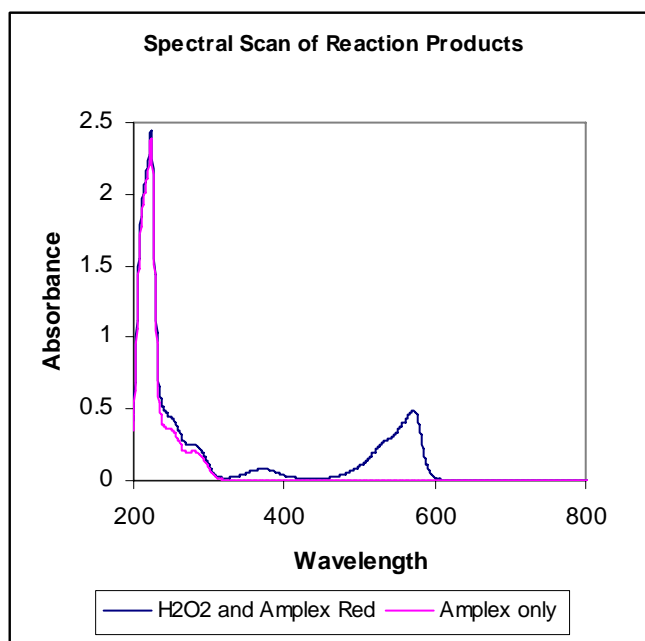
An Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (A-22188) was purchased from Molecular Probes (Eugene, Oregon). Several stock solutions were prepared according to the assay kit instructions. A 10 mM stock solution of Amplex Red was prepared by dissolving Amplex Red reagent in dimethyl sulfoxide (DMSO). Reaction buffer (1X) is comprised of 0.05 M sodium phosphate (pH 7.4) and was prepared from a 5X concentrate supplied by the kit. Horseradish Peroxidase (HRP) 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%  $H_2O_2$  solution, a 20 mM working solution was prepared using (1X) reaction buffer as the diluent. A series of dilutions of hydrogen peroxide were made using 1X reaction buffer as the diluent. After the dilutions were prepared 50  $\mu$ l of each concentration were placed into wells of a black opaque microplate (Corning-Costar 3915). The reaction was initiated by the addition of 50  $\mu$ l of a working solution that contained 0.1 mM Amplex Red reagent and 0.2 U/ml HRP in 1X reaction buffer. This

results in a final concentration of 50  $\mu\text{M}$  Amplex<sup>®</sup> Red reagent and 0.1 U/ml of HRP along with the various concentrations of  $\text{H}_2\text{O}_2$  in the reaction mix and a final reaction volume of 100  $\mu\text{l}$  per well.



**Figure 2. Resorufin Excitation and Emission Spectra.** Excitation and Emission scan data provided by Molecular Probes (Eugene, Oregon) was plotted using Microsoft Excel. Note that the data represents relative intensities as measured by fluorescent signal.

In the case of spectral scans, duplicate samples either lacking  $\text{H}_2\text{O}_2$  or with 10  $\mu\text{M}$   $\text{H}_2\text{O}_2$  were incubated for 30 minutes with Amplex Red reagent (50  $\mu\text{M}$ ) and HRP (0.1 U/ml). After incubation, spectral absorbance scans from 200 nm to 800 nm in 1 nm increments were performed using a BioTek Synergy HT<sup>™</sup> Multidetection Microplate Reader. After scanning, the data was exported to Microsoft<sup>®</sup> Excel. The absorbance values of duplicate samples for each wavelength were averaged and the absorbance of an empty well was subtracted. For kinetic analysis, dilutions of  $\text{H}_2\text{O}_2$  from 0 to 1.25  $\mu\text{M}$  were made using 1 X reaction buffer as the diluent and 50  $\mu\text{l}$  aliquoted into microplates in replicates of eight. Kinetic reactions were initiated by the addition of 50  $\mu\text{l}$  of a working solution containing 0.1 mM Amplex Red reagent and 0.2 U/ml HRP in 1X reaction buffer using an eight channel multichannel pipette. Using a 530/25 excitation and a 590/35 emission filter set and a sensitivity setting of 50, the fluorescence was measured kinetically every 90 seconds for 30 minutes. For endpoint absorbance determinations, dilutions of hydrogen peroxide from 0 to 20  $\mu\text{M}$  (10  $\mu\text{M}$  final concentration) were made using 1 X reaction buffer as the diluent. Aliquots of 100  $\mu\text{l}$  of each dilution were placed in Costar 3635 microplates and the reaction initiated by the addition of 100  $\mu\text{l}$  of a working solution containing 0.1 mM Amplex Red (0.05 mM final) and 0.2 U/ml HRP (0.1 U/ml final) in 1 X reaction buffer. Samples were incubated for 30 minutes and the absorbance at 570 nm was measured using a Synergy HT<sup>™</sup> Multidetection Microplate Reader. Endpoint fluorescence determinations were performed in a similar fashion except the concentration range was from 0 to 2.5  $\mu\text{M}$  final  $\text{H}_2\text{O}_2$  concentration. Fluorescence was measured using a 530/25-excitation filter and a 590/35-emission filter with a sensitivity setting of 50. Samples were incubated for 5 minutes rather than 30 minutes as described for absorbance measurements. Using either detection mode, the reader was controlled and the data collected and analyzed using KC4<sup>™</sup> Data Reduction Software (BioTek Instruments, Winooski, VT).

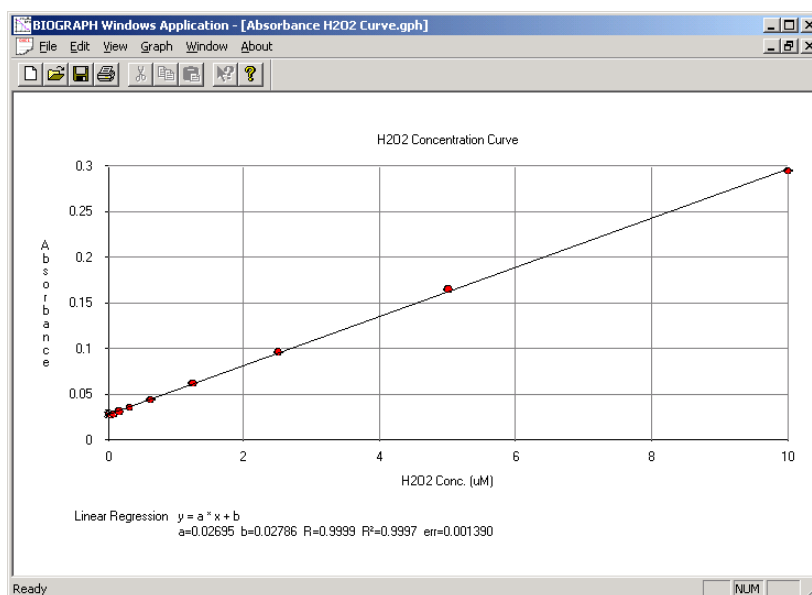


**Figure 3. Spectral Absorbance Scans of Amplex® Red Reagent and Reacted Products.** Spectral scans from 200 nm to 800 nm in 1-nm increments of samples containing Amplex Red and HRP that either have or do not have H<sub>2</sub>O<sub>2</sub> present.

## Results

Spectral scan analysis reveals the formation of the brightly colored product, resorufin, when hydrogen peroxide is present in the reaction. Resorufin demonstrates a significant absorbance peak at 570 nm (Figure 3), while reactions that are performed in the absence of peroxide have virtually no absorbance at this wavelength. Both reacted and un-reacted samples demonstrate significant absorbance in the UV range reflecting the presence of protein and the aromatic nature of Amplex Red and/or resorufin. Using this absorbance peak, several dilutions of hydrogen peroxide were reacted and the absorbance measured. The resultant concentration curve demonstrates a linear relationship between H<sub>2</sub>O<sub>2</sub> concentration and absorbance (Figure 4). Concentration determinations can be made with a high degree of confidence, as the correlation coefficient ( $r^2$ ) for the linear regression analysis was calculated to be 0.9997. Using absorbance, the limit of detection of H<sub>2</sub>O<sub>2</sub> was found to be 0.3  $\mu\text{M}$  or 60 pmoles/well.

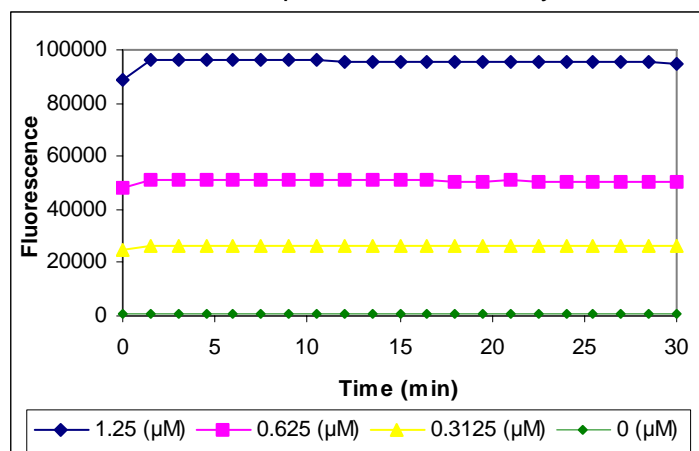
Initial fluorescence experiments measured the production of resorufin from Amplex Red kinetically. As demonstrated in Figure 5, different starting concentrations of H<sub>2</sub>O<sub>2</sub> result in different amounts of fluorescence signal. Surprisingly, the reaction reached a steady state very quickly, usually within 2 minutes, suggesting that the excess Amplex Red substrate quickly exhausts the peroxide present and HRP enzyme (Figure 5). Because the reaction is not stopped, a kinetic measurement can be terminated whenever desired and treated as an endpoint reaction. When using fluorescence as a means for detection, a linear relationship between fluorescent emission and H<sub>2</sub>O<sub>2</sub> concentration is observed. As demonstrated in Figure 6A, peroxide concentrations up to 1.25  $\mu\text{M}$  are quite linear, with a slight tailing at 2.5  $\mu\text{M}$  concentrations. Note that a second order polynomial equation very nicely describes the relationship (Figure 6A). The advantage of fluorescence, in terms of detection, is demonstrated in Figure 6B. Using a linear regression, concentrations from 0 to 0.08  $\mu\text{M}$  concentrations can be described with a high degree of confidence, as the correlation coefficient ( $r^2$ ) was calculated to be 0.993. The detection limits for H<sub>2</sub>O<sub>2</sub> when using fluorescence were calculated to be 0.004  $\mu\text{M}$ . Taking into account the reaction volume of 100  $\mu\text{l}$ , this concentration can be converted to a detection limit of 400 fmoles/well.



**Figure 4. Hydrogen Peroxide Absorbance Calibration Curve.** Hydrogen Peroxide samples (in duplicate) ranging from 0 to 10  $\mu\text{M}$  final concentration were reacted with HRP and Amplex<sup>®</sup> Red reagent and the absorbance at 570 nm determined. The subsequent data was plotted and linear regression analysis performed using BioTek's KC4 Data Reduction Software.

## Discussion

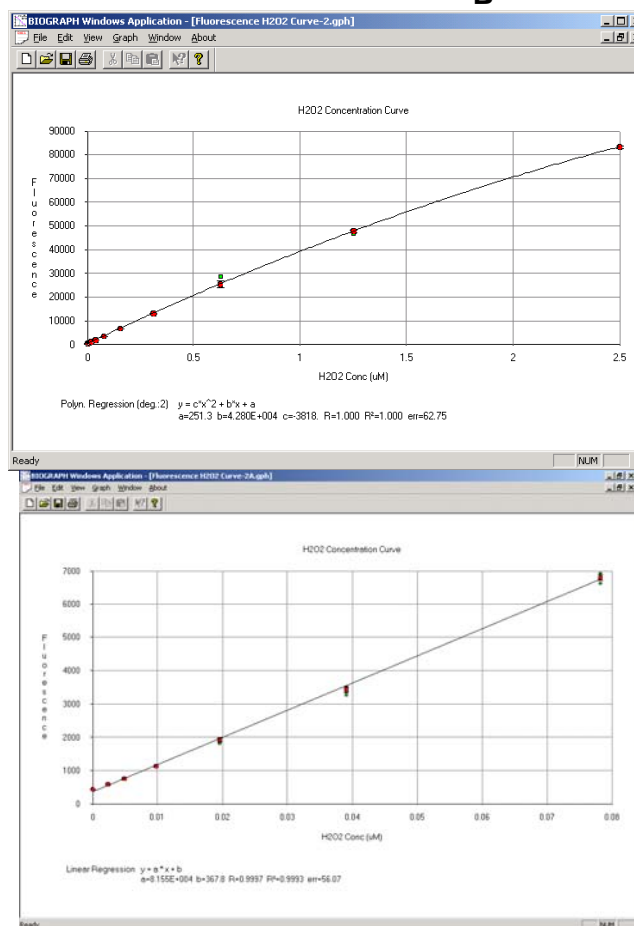
We have demonstrated that the Synergy HT<sup>™</sup> multi-detection Microplate Reader can capably and reliably quantitated  $\text{H}_2\text{O}_2$  in samples. The ability to measure the reaction using either absorbance or fluorescence allows the investigator a number of different options experimentally. Samples that are expected to contain large amounts of hydrogen peroxide can be determined by measuring the absorbance at 570 nm, while samples with lower levels can be determined by fluorescence. The dual optics of the Synergy HT allow for either absorbance or fluorescence measurements without any optical compromise. Note that as with many compounds that can be measured by both absorbance and fluorescence, the detection limits were found to be much lower with the use of fluorescence. While the reaction is not stopped by the addition of any reagent, in our hands, an incubation of 5 minutes, rather than the 30 minutes recommended by the kit manufacturer, was sufficient for sample to reach a steady state.



**Figure 5. Kinetic Analysis of  $\text{H}_2\text{O}_2$  Reaction with Amplex Red.** The indicated concentrations of  $\text{H}_2\text{O}_2$  were reacted with HRP and Amplex Red as described previously. Data represent the mean of eight replicates at each time point.

A

B



**Figure 6. Hydrogen Peroxide Fluorescence Calibration Curve.** Hydrogen Peroxide samples (in duplicate) ranging from 0 to 2.5  $\mu\text{M}$  final concentration (graph A) or 0  $\mu\text{M}$  to 0.08  $\mu\text{M}$  (graph B) were reacted with HRP 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 ability to measure  $\text{H}_2\text{O}_2$  levels stoichiometrically has many advantages. Because the assay utilizes hydrogen peroxide, other reactions that either produce or utilize peroxide can also be measured. For example, the assay as utilized in this dissertation uses HRP in excess to measure peroxide levels; however if HRP was the limiting agent and  $\text{H}_2\text{O}_2$  was in excess the signal produced would be related to HRP levels (2). Other compounds that can be measured using Amplex Red in conjunction with  $\text{H}_2\text{O}_2$  include: catalase (3); cholesterol (4); glutamate (5) and phospholipase C (6). Besides requiring that hydrogen peroxide be a component of the reaction there are some limitations. Amplex Red is unstable in the presence of thiols such as dithiothreitol (DTT) or 2-mercaptoethanol. Therefore, the measurement of enzymes or compounds that require reducing agents will be difficult. In addition, while Amplex Red reagent is unstable at high pH (>8.5), the absorption peak of the reaction product, resorufin, shifts considerably below its  $\text{pK}_a$  (~6.0) requiring that the reactions be performed at pH 7-8.

This treatise demonstrates that the Synergy HT™ Multidetector Microplate Reader is an ideal microplate reader for this assay. As stated previously, the Synergy HT uses two different optics systems for reading fluorescence and absorbance. When measuring fluorescence the reader uses a tungsten-halogen light source and excitation and emission filters to provide wavelength specificity. Absorbance measurements are carried out using a xenon flash lamp light source and a monochromator for wavelength selection. The reaction product for this assay has a very high

absorbance extinction coefficient, which allows for its quantitation by not only fluorescence, but absorbance as well. This allows the investigator the option to measure samples with very high concentrations using absorbance and using fluorescence for low concentrations.

### References

- (1) Zhou, M., Diwu, Z., Panchuk-Voloshina, N., and Haugland, R.P. (1997) A Stable Nonfluorescent Derivative of Resorufin for the Fluorometric Determination of Trace Hydrogen Peroxide: Applications in detecting the Activity of Phagocyte NADPH Oxidase and other Oxidases, *Analytical Biochemistry*, **253**:162-168.
- (2) Mohanty, J.G., Jaffe, J.S., Schulman, E.S., and Raible, D.G. (1997) A Highly Sensitive Fluorescent Micro-assay of H<sub>2</sub>O<sub>2</sub> Release from Activated Human Leukocytes Using a Dihydroxyphenoxazine Derivative, *Journal of Immunological Methods*, **202**:133-141.
- (3) Mueller, S., Riedel, H.D., and Stremmel, W., (1997) Determination of Catalase Activity at Physiological Hydrogen Peroxide Concentrations, *Analytical Biochemistry*, **245**:55-60
- (4) Amundson, D.M. and Zhou, M. (1999) Fluorometric Method for the Enzymatic Determination of Cholesterol, *Journal Biochemical and Biophysical Methods*, **38**:43-52.
- (5) McElroy, K.E., Bouchard, P.J., Harpel, M.R., Horiuchi, K.Y., Rogers, K.C., Murphy, D.J., Chung, T.D., and Copeland, R.A. (2000), Implementation of a Continuous, Enzyme-coupled Fluorescence Assay for High-throughput Analysis of Glutamate-producing Enzymes, *Analytical Biochemistry*, **284**:382-387.
- (6) Molecular Probes Inc. (2002) Amplex<sup>®</sup> Red Phosphatidylcholine-Specific Phospholipase C Assay Kit Instructions, Molecular Probes, Inc. Eugene, Oregon.

**Paul Held PhD**  
**Senior Scientist and Applications Lab Manager**

Rev. 8-20-02