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Application Note

Quantitation of Cholesterol using a Fluorometric Assay and the Synergy™HT Multi-Detection Microplate Reader

Introduction

Cholesterol and Cholesterol esters are significant constituents of blood born lipoproteins. Elevated levels of these components have been linked to atherosclerosis and heart disease. As a result, a large research effort has been put into understanding cholesterol homeostasis and ways of lowering cholesterol levels. Quantitation of cholesterol in experimental samples is paramount to this research. Here we describe a fluorescent method to quantitate cholesterol in biological samples using a Synergy™ HT Multi-Detection Microplate Reader.

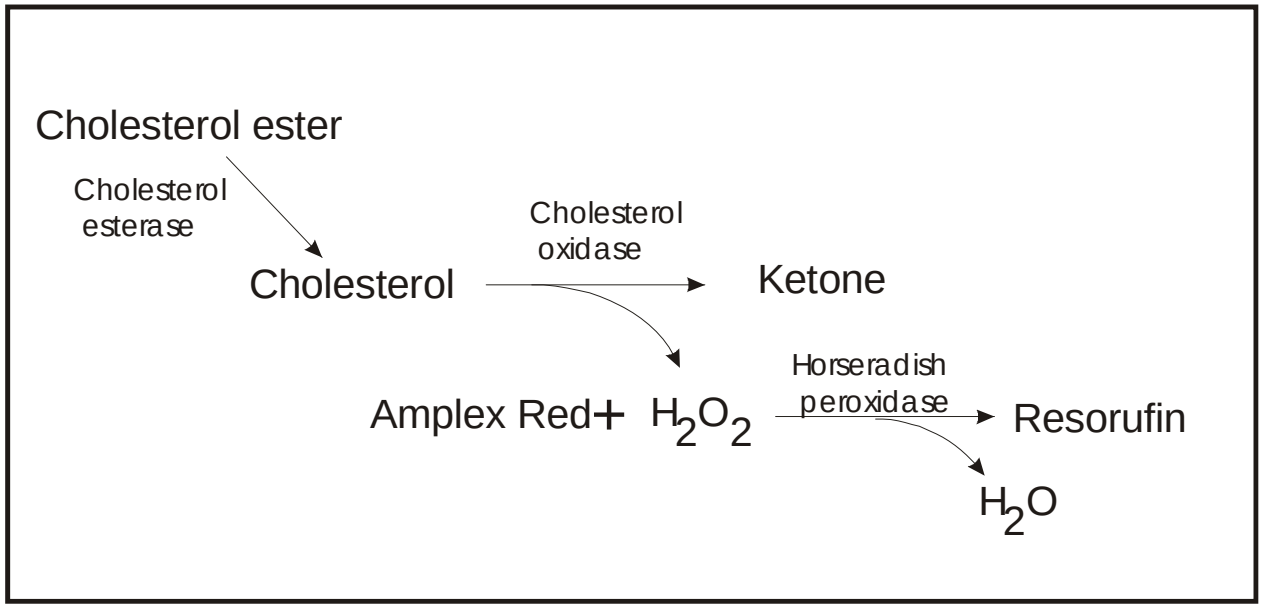

Figure1. Enzymatic Reaction for Cholesterol Quantitation. Free cholesterol is oxidized by cholesterol oxidase to form a cholesterol ketone and hydrogen peroxide. The peroxide is reacted with Amplex Red with HRP to produce the fluorescent product resorufin.

There are several methods used to measure cholesterol. The Abell-Kendall method reacts sulfuric acid, acetic anhydride, and acetic acid with free cholesterol to produce a chromogen with an absorption maximum at 620 nm [1]. This method has largely been abandoned in favor of other methods that are easier to perform and that do not require the use of caustic materials. Other analytical approaches used primarily in research include the use of gas chromatography and high performance liquid chromatography (HPLC). Unfortunately these methodologies do not lend themselves to the testing of large numbers of samples. Clinical samples are almost

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universally tested using enzymatic methods, which first hydrolyze cholesterol esters to free cholesterol, and then oxidize cholesterol to cholest-4-ene-3-one and peroxide. In regards to detection, most assays measure peroxide formation with another enzymatic reaction. Commonly this reaction uses peroxidase to catalyze the oxidative coupling of 4-aminoantipyrene and phenol by hydrogen peroxide to form a quinoneimine dye, which has absorption maxima at 500 nm [1]. The cholesterol assay used in this study also employs an enzyme-linked process. Because cholesterol is often found in an esterified state, cholesterol esterase is used to hydrolyze any cholesterol ester present to free cholesterol. Free cholesterol is converted to cholest-4-ene-3-one ketone product by the action of cholesterol oxidase and in doing so generates hydrogen peroxide (H_2O_2). Amplex red reagent and H_2O_2 are converted to resorufin and water in a one-to-one stoichiometry by horseradish peroxidase (Figure 1). Resorufin is a highly fluorescent compound with absorption-maxima of 563 nm and a peak emission wavelength of 587 nm. Because the product generated is a fluorescent compound rather than a colorimetric moiety, it would be expected to be more sensitive.

Materials and Methods

An Amplex® Red Cholesterol Kit (catalog number A-12216) was obtained from Molecular Probes (Eugene, OR). Several stock solutions were prepared according to the assay kit instructions. A 20 mM stock solution of Amplex Red was prepared by dissolving Amplex® Red reagent in dimethyl sulfoxide (DMSO). Reaction buffer (1X) is comprised of 0.1 M sodium phosphate (pH 7.4), 0.05 M sodium chloride, 5 mM cholic acid and 0.1% Triton® X-100 was prepared from a 5X concentrate supplied by the kit. Horseradish Peroxidase (HRP) (200 U/ml), cholesterol oxidase (200 U/ml) and cholesterol esterase (200 U/ml) stock solutions were prepared by dissolving the respective lyophilized powders in the necessary amount of 1X reaction buffer. A series of dilutions of cholesterol/cholesterol ester standard were made using 1X reaction buffer as the diluent. After the dilutions were prepared 50 µl of each concentration were placed into wells of a black opaque Costar 3915 microplate (Corning-Costar, Corning, NY) in replicates of 8. The reaction was initiated by the addition of 50 µl of a working solution that contained 300 µM Amplex® Red reagent and 2 U/ml HRP, 2 U/ml cholesterol oxidase, and 0.2 U/ml cholesterol esterase in 1X reaction buffer. This results in a final concentration of 150 µM Amplex® Red reagent in the reaction mix and a final reaction volume of 100 µl per well. Reactions were monitored using a Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT) in fluorescence mode. Kinetic assays were incubated at 37°C inside the Synergy™ HT Multi-Detection Microplate Reader with measurements every 5 minutes for a total of 60 minutes. Endpoint determinations were made after a 60-minute incubation at 37°C in the Synergy™ HT Multi-Detection Microplate Reader. Determinations were made from the top using a 530 nm, 25 nm bandwidth excitation filter and a 590, 35 nm bandwidth emission filter.

Results

As demonstrated in Figure 2, samples containing a 25-µM mixture of cholesterol and cholesterol ester develop a significant amount of fluorescent signal over a period of 60 minutes. Fluorescence in these samples increased approximately 25 fold. Samples that lack any cholesterol develop considerably less signal, increasing less than 2 fold. The increase in signal of the samples lacking cholesterol is most likely due to nonspecific hydrolysis of the Amplex Red substrate.

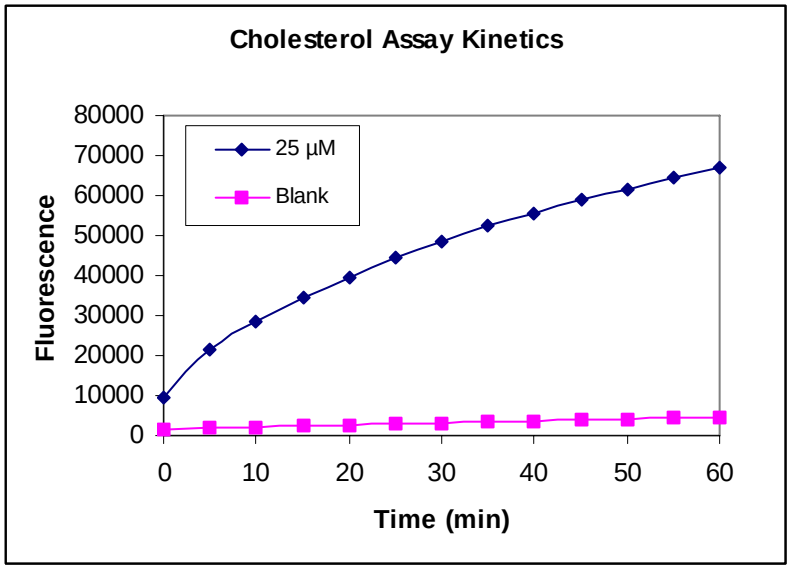

Figure 2. Kinetic measurement of the fluorescent signal generated by the assay. Samples containing either 25-µM cholesterol or no cholesterol were reacted and the fluorescence monitored every 5 minutes for a total of 60 minutes. The resultant data was exported to Microsoft® Excel and plotted.

Samples containing different concentrations of cholesterol developed fluorescence at different rates. As shown in Figure 3, the rate of the reaction, as measured by the Mean Velocity (Mean V) is proportional to the cholesterol concentration. These data can be described using a 4-parameter logistic fit with a high degree of confidence.

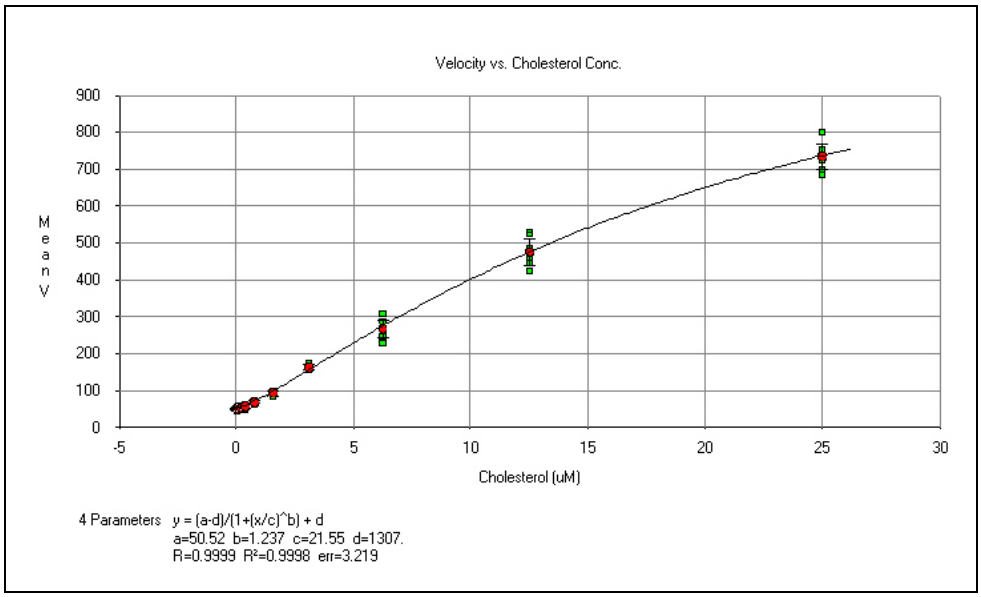

Figure 3. Relationship between Mean V and cholesterol concentration. Various concentrations of cholesterol were assayed kinetically over a period of 60 minutes. The resultant Mean V for each concentration was plotted against cholesterol concentration and 4-parameter logistic fit of the data performed using KC4™ Data Analysis Software (BioTek Instruments, Winooski, VT).

Because the kinetic experiments determined that increasing fluorescent signal was generated by the assay for at least one hour after the initiation of the reaction, an incubation time of 60 minutes was used for all subsequent experiments. Using the reader for temperature control (37°C), incubation timing, and measurement, endpoint determinations were performed on various

concentrations of cholesterol as described previously. As depicted in Figure 4, as cholesterol concentration increases so does the fluorescence generated. Samples with cholesterol concentrations as low as 0.2 µM can be detected (Figure 4 inset), which equates to approximately 4 ng/well. Using a polynomial regression analysis to describe these data, one can determine concentrations from unknown samples with a high degree of confidence, as the correlation coefficient (R^2) is 0.9996 (Figure 4).

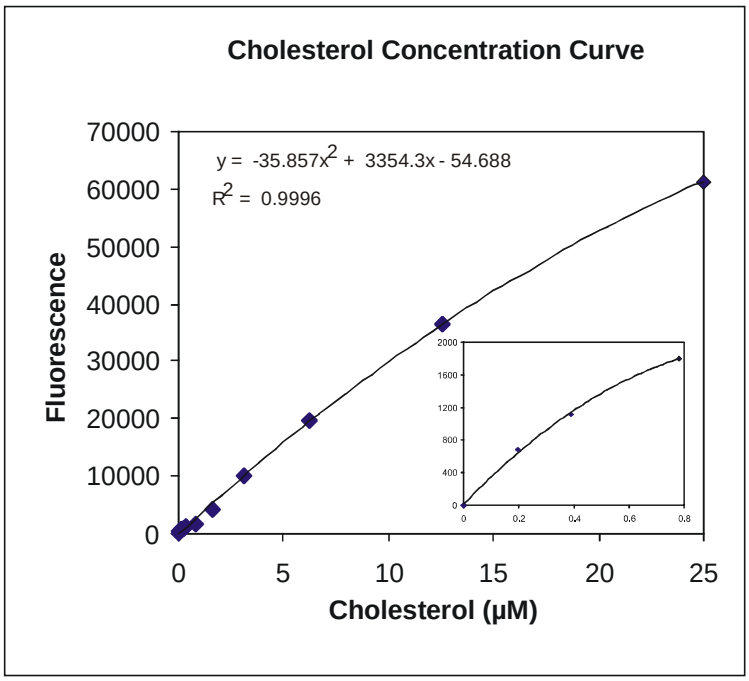

Figure 4. Cholesterol Concentration Curve. Various concentrations of cholesterol (0-25 µM) were assayed as described previously and the fluorescent signal plotted against cholesterol concentration. The inset graph depicts cholesterol concentrations from 0 to 0.79 µM.

Discussion

We have demonstrated that the Synergy™ HT Multi-Detection Microplate Reader can capably and reliably quantitate cholesterol in samples. Although these assays utilized opaque black plates and fluorescence determination from the top, the Synergy™ HT Multi-Detection Microplate Reader is capable of either top or bottom fluorescence detection via software selection. While this assay required fluorescence for detection, the product of the reaction, resorufin, can also be measured by absorbance due to its high absorbance extinction coefficient [3]. 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.

In addition to improved detection limits, this cholesterol assay has many advantages. The assay can be performed as a continuous reaction containing all of the constituents, including enzymes, from the beginning. This eliminates the need for reagent addition during the assay and once the reaction mix is added the assay is set to run to completion. This feature makes the assay well adapted for automation and HTS. The sensitivity of the assay offers advantages in regards to required sample volume. For example, samples of normal serum cholesterol levels need only be 0.01 µl in order to be detected using this assay, saving on precious experimental samples [2]. Simple alterations to the assay can provide more information for the investigator. Running parallel assays of samples with and without cholesterol esterase has been suggested by the kit manufacturer as a means to determine the fraction of cholesterol that is in the form of cholesterol

esters of a particular sample. Alternatively, by supplying an excess of free cholesterol, and not introducing exogenous cholesterol oxidase enzyme, one can measure cholesterol oxidase activity in samples [4]. Under these conditions the formation of hydrogen peroxide would be rate-limited by the level of endogenous cholesterol oxidase present in the sample.

References

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