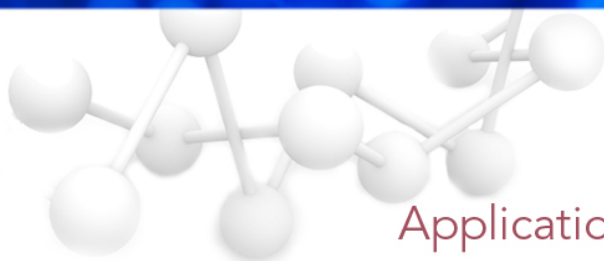


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Application Note

Measurement of Caspase Activity using the Synergy™ HT

Measuring Apoptosis Enzymes

Abstract

The orderly execution of apoptosis, or programmed cell death requires the coordinated activation of several caspase enzymes. Increases in the activity of these enzymes are indicative of apoptosis. The investigation of the response of different caspase enzymes to various stimuli can provide new insights into the regulation of this phenomenon. Here we describe the quantitation of several caspase enzymes using fluorescence and absorbance capabilities of the Synergy™ HT Multi-Detection Microplate Reader.

Introduction

Apoptosis, or programmed cell death, is a normal component of the development and health of multicellular organisms. Apoptosis is a planned, regulated event that leads to morphological changes within hours of a biochemical commitment to this pathway [1]. During apoptosis, the nuclear chromatin breaks down and the nucleus condenses, while the cytoplasm begins to shrink as lamins and actin filaments are cleaved. Apoptotic cells undergo changes to their plasma membranes, such as the translocation of phosphatidylserine to the outer surface, in order to facilitate their phagocytosis [1]. While several biochemical changes are required to accomplish this orderly transition of cell death, one of the key elements is the activation of a family of aspartate-specific cysteine proteases, also known as caspases.

Caspases exist as inactive precursor molecules called procaspases, which are activated through a proteolytic step. This proteolytic processing occurs at critical aspartic acid residues that conform to the caspase recognition sequence. As a result caspases often function in cascades. Within the caspase cascade, a model has been proposed in which an “initiator or upstream” protease, such as caspase-8, gets activated by an apoptic stimulus. This activated caspase-8 then activates an amplifier protease, such as caspase-1 by cleavage at the recognition sequence. This, in turn, activates a “machinery” protease such as caspase-3 or caspase-7. The cellular morphologic changes observed are the result of proteolytic cleavage of a number of specific proteins by caspases, including Poly(ADP-ribose) Polymerase (PARP) and DNA Fragmentation Factor (DFF).

Caspases are typically activated in the early stages of apoptosis through two main pathways. The extrinsic pathway involves the binding of “death inducing ligands” to cell surface receptors or the induction by cytotoxic T-lymphocytes by granzyme. This pathway results in the activation of caspase-8 [2]. The intrinsic pathway is initiated by cellular stress and generally involves changes to the mitochondria that release cytochrome c, which interacts with Apaf-1, dATP and multiple molecules of pro-caspase-9 to generate an active apoptosome complex that activates caspase-9

[3]. Regardless of the activation pathway, both lead to a caspase cascade, where effector caspases are activated through proteolysis. These effector caspases (e.g. caspase-3 and -7) in turn cleave many different death target proteins (Figure 1).

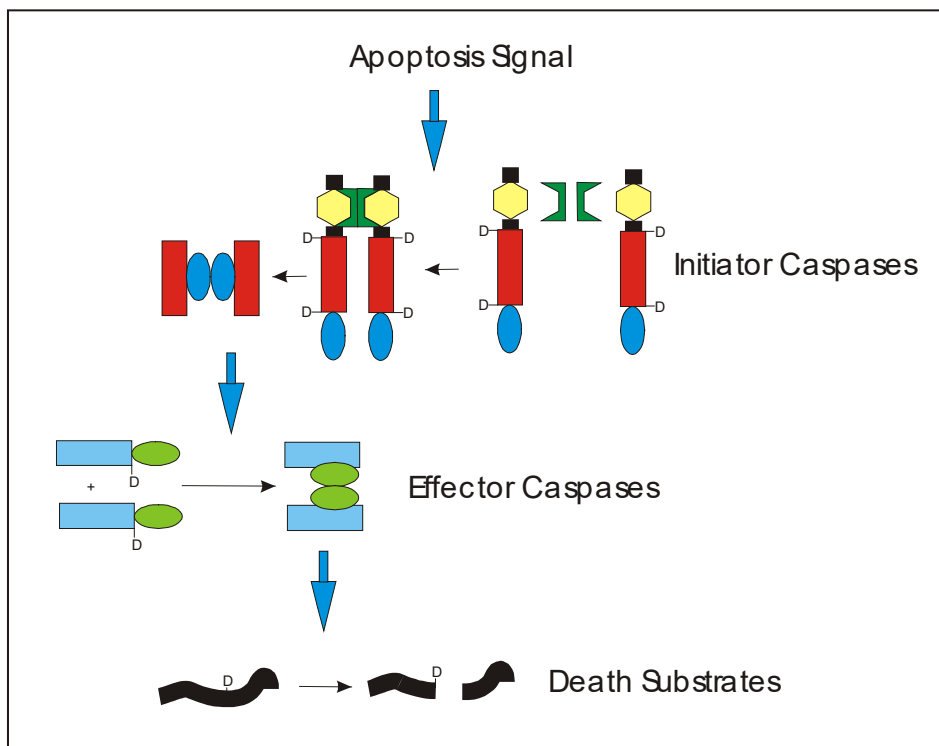


Figure 1. Schematic diagram of the Activation of the Caspase Cascade. Apoptotic signals cause oligomerization of death adaptor proteins, which in turn oligomerize initiator procaspases. Oligomerized procaspases autoproteolytic activity result in active initiator caspase enzymes. Active initiator caspases then process and activate effector procaspases. Active effector caspases cleave various substrates necessary for apoptosis to proceed.

Substrate specificity of the human caspases has been determined using a systemic approach with combinatorial synthetic peptides [4]. As a result caspases can be divided into three distinct groups. The optimal recognition motif of the first group is WEHD. This group includes the inflammatory response caspase-1. The second group cleaves the sequence DEXD (where X is V, T, or H), with a very high selectivity for Asp (D) at position P4. This caspase group includes caspase-2, -3, and -7. The recognition sequence is often found in non-caspase proteins known to be cleaved during apoptosis, further corroborating the theory that caspase-3 and -7 are effector caspases. The third group, which includes caspase-6, -8, -9, and -10, preferentially recognizes the sequence (L/V)EXD (where X is V, T, or H), the processing sequence of effector caspases and is consistent with caspase-8 being an initiator caspase.

Regardless of the caspase type, all can be monitored using a synthetic peptide, which releases a colorimetric or fluorometric moiety when cleaved by the enzyme. Here we describe the use of the Synergy™ HT Multi-Detection Microplate Reader to quantitate caspase activity using either its absorbance or its fluorescence capabilities.

Materials and Methods

Caspase-3 Colorimetric kit (catalog number 907-013) was obtained from Assay Designs, Inc (Ann Arbor, MI). Fluorometric caspase kits for caspase-3, -6, and -8 (catalog number CASF-3-F, CASP-6-F, and CASP-8-F respectively) were purchased from Sigma-Aldrich (St. Louis, MO). Black opaque 96-well microplates (catalog number 3915) were obtained from Costar

(Cambridge, MA). Assays were performed as described in the assay kit instructions. Caspase enzyme supplied in the kit was diluted using assay buffer as the diluent.

Colorimetric caspase-3 assays were performed according to the assay kit instructions. Serial dilution from 0 to 1000 U/ml of the caspase-3 enzyme standard were made using 1X Caspase reaction buffer supplied by the kit as the diluent. In replicates of 8, 50 μ l of each dilution were pipetted into 96-well half-area microplates supplied with the kit. Reactions were initiated by the addition of 75 μ l of reaction buffer containing enzyme substrate, previously prepared according to the kit instructions. Reactions were placed into a Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT) that had been preheated to 37°C. Absorbance measurements were taken kinetically at 405 nm every minute for 3 hours with the incubation temperature being maintained at 37°C.

Fluorometric caspase-3 assays were performed according to the kit instructions with the exception of the positive control. Lyophilized caspase-3 powder supplied by the kit was reconstituted with deionized water to a concentration of 100 μ g/ml. Using the stock enzyme solution, a series of dilutions ranging from 0 to 1000 ng/ml were made using 1X assay buffer (20 mM HEPES pH 7.4, 0.1% CHAPS, 5 mM DTT, 2 mM EDTA) as the diluent. In replicates of 6, 5 μ l aliquots of each dilution were pipetted into Costar 3915 microplates. Reactions were initiated by the addition of 200 μ l of reaction mix (16 μ M substrate in 1 X assay buffer). Reactions were monitored kinetically at room temperature using a Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT). The readings were taken from the top using a 360 nm, 40 nm bandpass excitation filter and a 460 nm, 40 nm bandpass emission filter. Measurements were taken every 5 minutes for a total of 120 minutes.

Fluorometric caspase-8 assays were performed according to the kit instructions with the exception of the positive control. Lyophilized caspase-8 powder supplied by the kit was reconstituted with deionized water to a concentration of 100 μ g/ml. Using the stock enzyme solution a series of dilutions ranging from 0 to 10 μ g/ml was made using 1X assay buffer (20 mM HEPES pH 7.4, 0.1% CHAPS, 5 mM DTT, 2 mM EDTA) as the diluent. In replicates of 6, 5 μ l aliquots of each dilution were pipetted into Costar 3915 microplates. Reactions were initiated by the addition of 200 μ l of reaction mix (150 μ M substrate in 1 X assay buffer). In addition to the enzyme dilution, samples containing the inhibitor peptide received 2 μ l of the 25- μ M inhibitor stock solution. Reactions were monitored kinetically at room temperature using a Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT). The readings were taken from the top using a 360 nm, 40 nm bandpass excitation filter and a 460 nm, 40 nm bandpass emission filter. Measurements were taken every 5 minutes for a total of 120 minutes.

Fluorometric caspase-6 assays were performed according to the kit instructions with the exception of the positive control. Lyophilized caspase-6 powder supplied by the kit was reconstituted with deionized water to a concentration of 100 μ g/ml. Using the stock enzyme solution a series of dilutions ranging from 0 to 10 μ g/ml were made using 1X assay buffer (20 mM HEPES pH 7.4, 0.1% CHAPS, 5 mM DTT, 2 mM EDTA) as the diluent. In replicates of 6, 5 μ l aliquots of each dilution were pipetted into Costar 3915 microplates. In addition to the enzyme dilution, samples containing the inhibitor peptide received 10 μ l of the 10- μ M inhibitor stock solution. Assay buffer (1X) was added such that all wells contained 50 μ l of fluid. Reactions were initiated by the addition of 50 μ l of reaction mix (100 μ M substrate in 1 X assay buffer). Reactions were monitored kinetically at room temperature using a Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT). The readings were taken from the top using a 360 nm, 40 nm bandpass excitation filter and a 460 nm, 40 nm bandpass emission filter. Measurements were taken every 5 minutes for a total of 120 minutes.

Results

Caspase-3 activity was measured using either a colorimetric substrate or a fluorescent substrate (Figure 2). In both cases, the caspase-3 enzyme cleaves the substrate, releasing either a colored or a fluorescent compound. When the colorimetric peptide substrate is used, *p*-nitroaniline is released, whereas when the fluorescent substrate is used the compound 7-amino-4-methylcoumarin is released. Note that regardless of the released compound the increase in absorbance or the increase in fluorescence is proportional to the amount of substrate present in the reaction.

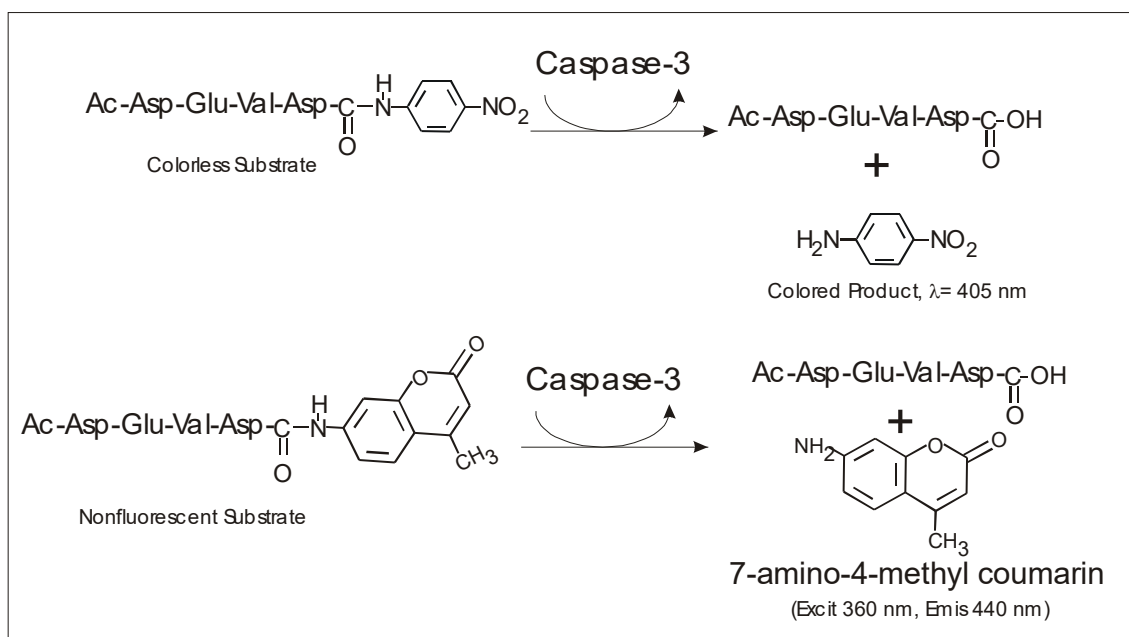


Figure 2. Caspase-3 Reaction Scheme for both colorimetric and fluorescent determinations.

Each caspase enzyme catalyzes the hydrolytic cleavage of a different substrate, differentiated by different amino acids in the recognition sequence. Note that all of the fluorescent substrates liberated 7-amino-4-methyl coumarin when cleaved, despite different peptide sequences.

When caspase-3 activity is measured using absorbance, a linear relationship between enzyme concentration and absorbance at 405 nm is observed (Figure 3) with a correlation coefficient (r^2) greater than 0.99. This indicates that quantitative estimates can be made with a high degree of confidence. Using a signal to noise criteria, a detection limit of 31 U/ml is obtained. This represents as little as 1.6 U/well can be differentiated from the blank wells.

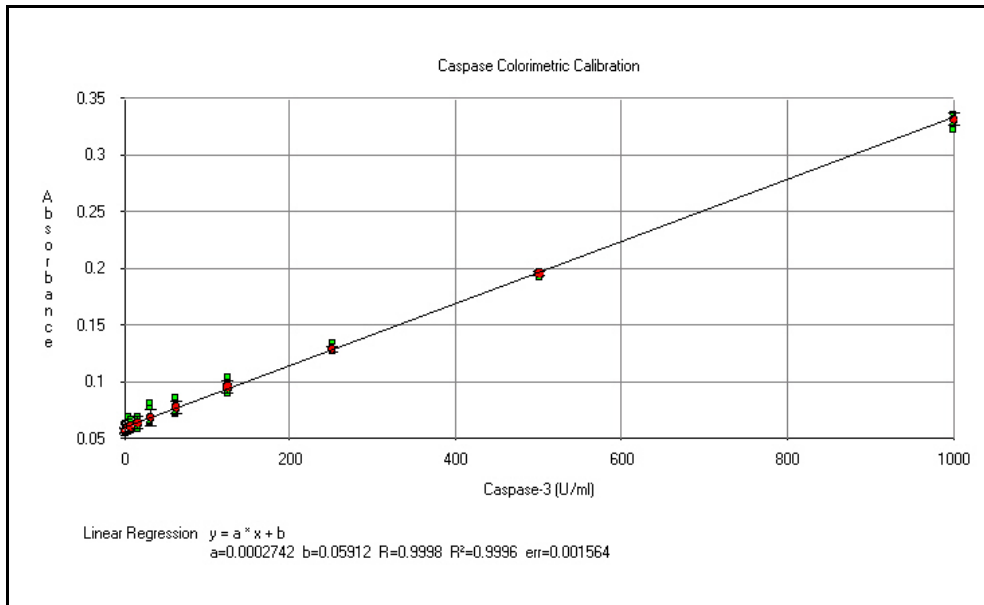


Figure 3. Caspase-3 Concentration Curve. Serial dilutions of caspase-3 enzyme were incubated with a colorimetric peptide substrate at 37°C for 3 hours and the absorbance at 405 nm measured using a Synergy™ HT Multi-Detection Microplate Reader.

When caspase-3 activity is measured using a substrate designed to produce fluorescence, a similar relationship is observed. Increasing enzyme concentrations result in an increase in signal (Figure 4). While the signal response was found to be linear at lower enzyme concentrations, the signal at higher concentrations was found to be less than expected. This phenomenon is most likely due to substrate depletion. As a result a 4-parameter logistic fit was found to provide the best means to describe the concentration curve. Using a signal to noise calculation a sensitivity limit of 15 ng/ml (75 pg/well) was determined from these data.

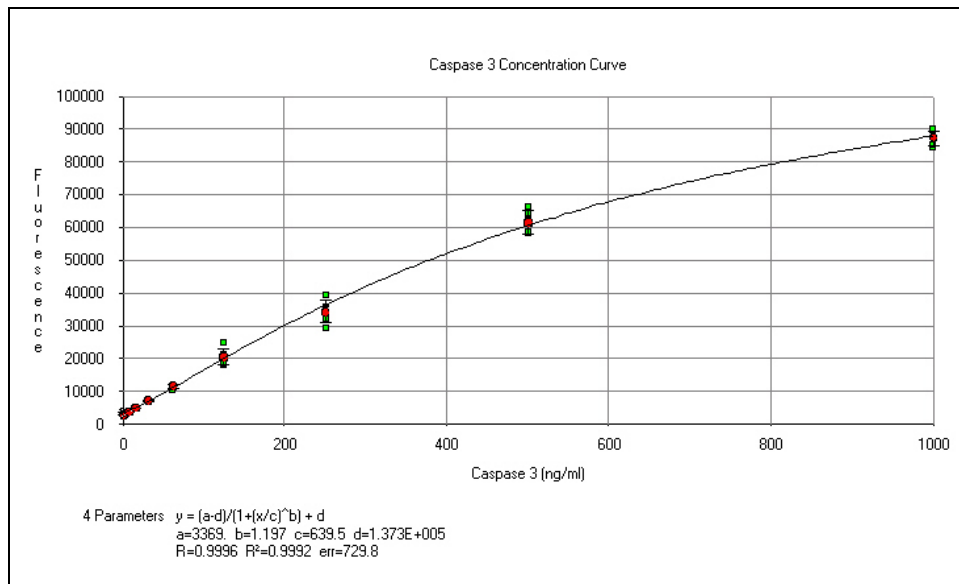


Figure 4. Fluorescent Concentration Curve for Caspase-3 Activity. Serial dilutions of caspase-3 enzyme were incubated with a Fluorometric peptide substrate for 2 hours at room temperature. The fluorescence at 460 nm from a 360 nm excitation was measured using a Synergy™ HT Multi-Detection Microplate Reader.

When caspase-3 enzyme was measured kinetically, the relationship between V_{max} and enzyme concentration was observed to be linear over the same range of enzyme concentrations measured using an endpoint determination (Figure 5). This suggests that the plateau effect observed with the higher concentrations with endpoint determinations are mostly like due to substrate depletion rather than a loss of enzyme activity.

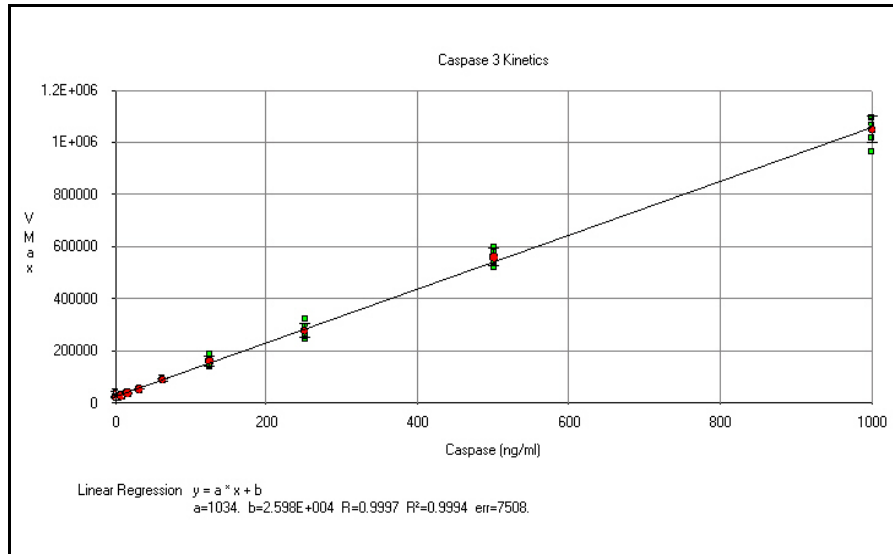


Figure 5. Comparison of V_{max} and Caspase-3 concentration. The fluorescence was measured kinetically over a period of two hours. Afterwards the V_{max} of each well was plotted against enzyme concentration. A linear regression analysis was then performed.

Caspase-8 activity was measured using a substrate that when acted upon by the enzyme produced the fluorescent product 7-amino-4-methyl coumarin. As demonstrated in Figure 6, increasing amounts of caspase-8 enzyme result in increased fluorescence after a 120-minute incubation. While a linear regression analysis provides an adequate fit of the data, a second order polynomial curve fit of the data provided a better predictive fit, as a result of lower activity observed with very low enzyme concentrations. The deviation from true linearity may be the result of nonspecific binding of the enzyme to the solid surface of the microplate well. The enzymatic reaction is specific to the substrate, as demonstrated by Figure 7.

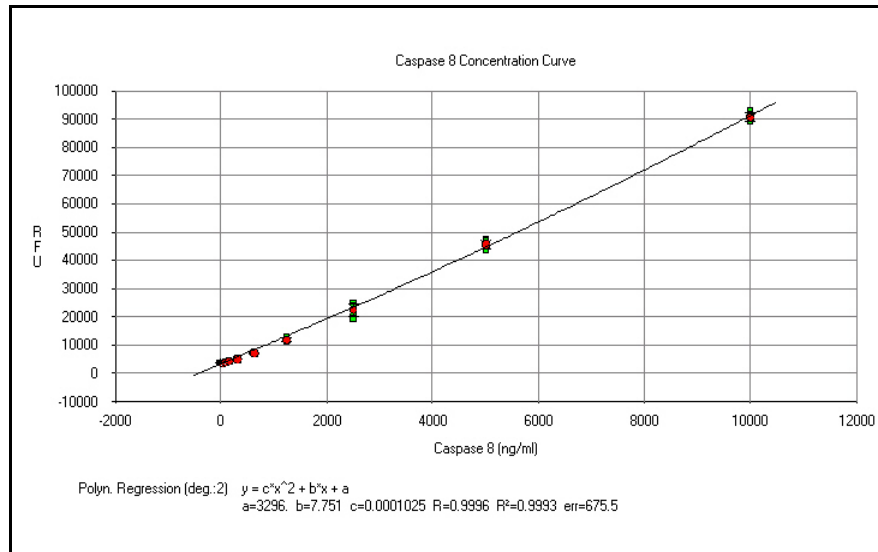


Figure 6. Caspase-8 Concentration Curve. Serial dilutions of caspase-8 enzyme were incubated with a Fluorometric peptide substrate for 2 hours at room temperature. The fluorescence at 460 nm from a 360 nm excitation was measured using a Synergy™ HT Multi-Detection Microplate Reader.

When inhibitor substrate with the same sequence is present in the reaction no activity, as measured by the production of fluorescence, is observed. At the highest enzyme concentration activity in the absence of inhibitor is approximately 900 fold greater than in the presence of the inhibitor (Figure 7).

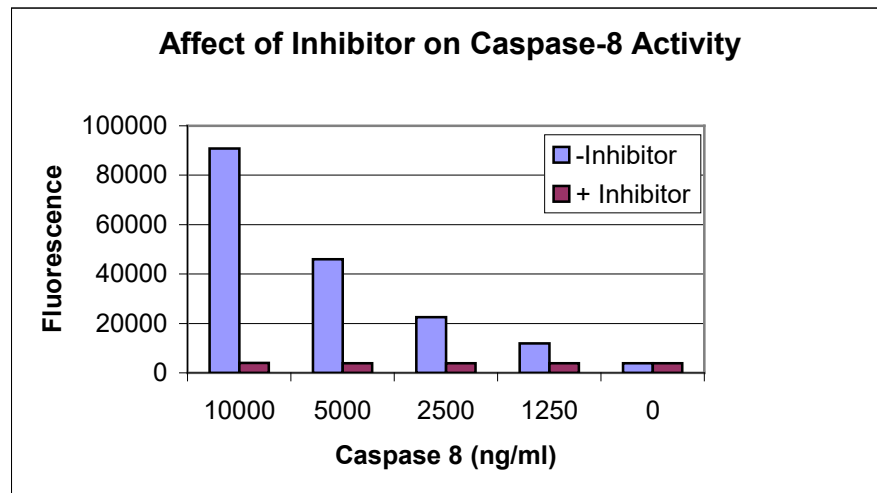


Figure 7. Affect of Inhibitor on Caspase-8 Activity. Different concentrations of active caspase-8 enzyme were measured for enzyme activity in the presence or absence of a specific inhibitor peptide. The resultant fluorescence measurements were then plotted using Microsoft® Excel®.

Caspase-6 activity was measured using a substrate that when acted upon by the enzyme produced the fluorescent product 7-amino-4-methyl coumarin. As demonstrated in Figure 8, increasing amounts of caspase-6 enzyme result in increased fluorescence after a 120-minute incubation. A second order polynomial curve fit of the data provided a better predictive fit, as a result of lower activity observed with very low enzyme concentrations. The deviation from true linearity may be the result of nonspecific binding of the enzyme to the solid surface of the microplate well.

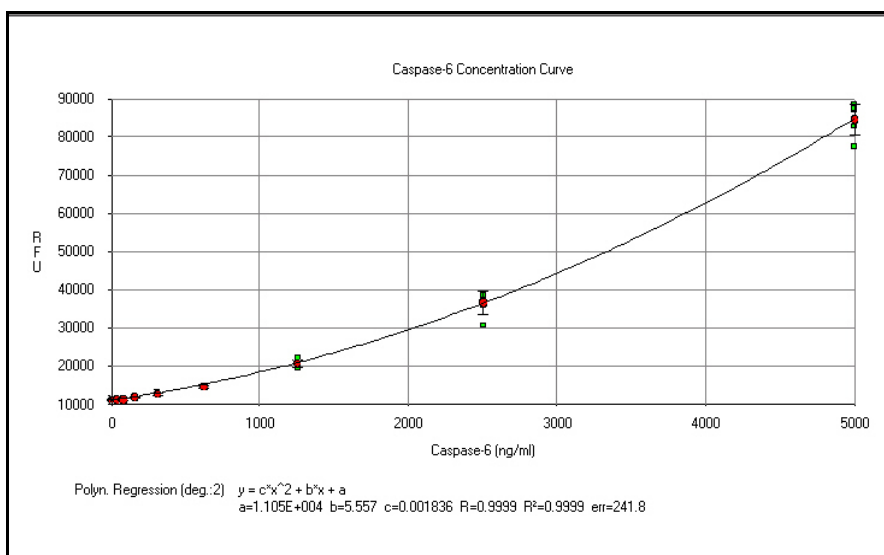


Figure 8. Caspase-6 Concentration Curve. Serial dilutions of active Caspase-6 enzyme were incubated with a Fluorometric peptide substrate for 2 hours at room temperature. The fluorescence at 460 nm from a 360 nm excitation was measured using a Synergy™ HT Multi-Detection Microplate Reader.

The caspase-6 enzymatic reaction is specific to the caspase-6 substrate. When inhibitor substrate with the same sequence is present in the reaction no activity, as measured by the production of fluorescence, is observed (Figure 9). Despite increasing enzyme concentration, virtually no activity is observed when an excess of the inhibitor peptide is present.

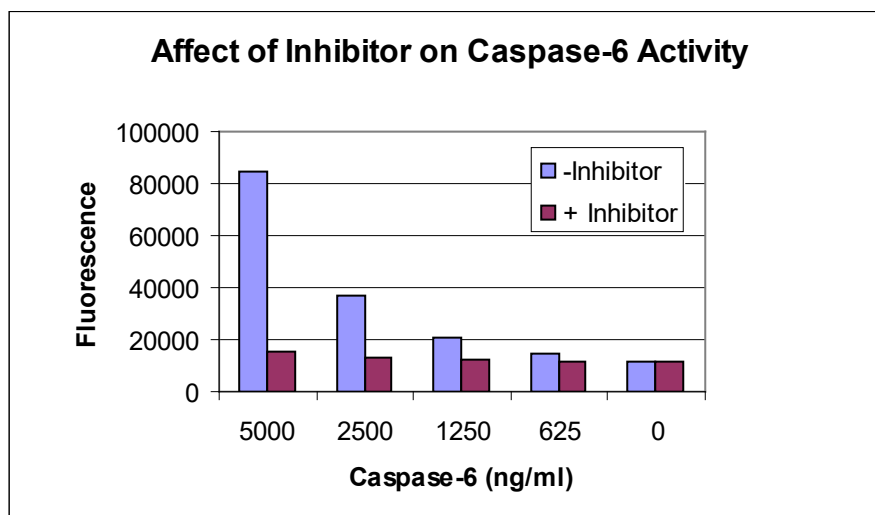


Figure 9. Affect of Inhibitor on Caspase-6 Activity. Different concentrations of caspase-6 enzyme were measured for enzyme activity in the presence or absence of a specific inhibitor peptide. The resultant fluorescence measurements were then plotted using Microsoft® Excel®.

Discussion

These data presented demonstrate the ability of the Synergy™ HT Multi-Detection Microplate Reader to measure caspase enzyme activity under a number of different conditions. The dual optics of the reader allows the investigator to measure these enzymes using either colorimetric or fluorescent substrates. This may be particularly important when trying to shift from one assay system to another. In general, fluorescent substrates provide an increase in sensitivity over the equivalent colorimetric substrate. However, they do so by sacrificing the ability to quantitate samples with very high concentration without dilution. Regardless of the mode of detection

(absorbance or fluorescence) samples can be quantified by interpolating a curve generated from a set of known standards. Alternatively a calibration curve of the product rather than the enzyme could be interpolated. For example, a series of 7-amino-4-methylcoumarin standards could be used to generate a curve rather than a series of enzyme dilutions. The resultant interpolation would return a quantity of product produced rather than the amount of enzyme present.

Kinetic examination of the reaction allows for a wide range of enzyme concentrations to be examined. This is particularly important if samples with relatively unknown amounts of caspase enzymes are being assayed. Because the assay protocol does not require the addition of a stop agent, the assays can be measured kinetically. If after a defined period of time, samples with low levels of caspase activity have not achieved a sufficient signal, the reactions can be allowed to proceed for a longer period. When sufficient amounts of substrate are converted, the reaction need go no further.

While one of the assays used for this study utilized an incubation step at 37°C, other assay kits called for room temperature incubation. It has been suggested that elevated temperature incubation provides for greater enzyme activity as compared to ambient temperature incubation. The fact that several different caspases were measured with high sensitivity using an ambient incubation suggests that elevated temperature is not an absolute prerequisite for activity. Note that comparisons between different experiments would naturally require equivalent temperatures. The use of an elevated temperature, such as 37°C, allows one to rigidly control for temperature, whereas the use of ambient temperature allows the day-to-day variation in laboratory temperature impact on the final result. Laboratories without rigid temperature control may have trouble comparing data generated on different days.

We have used several different curve fit algorithms to best describe that data generated. The ability to quickly and easily change from one curve fit to another using the KC4™ software is a distinct advantage. While the consistent use of the same curve fit on the standards is preferable, depending on the day-to-day variations of the assay, alternate curve fits may better describe the data, as monitored using the correlation coefficient (r^2) value. In regards to the caspase enzymes examined in this study, different enzymes in conjunction with their different substrates would be expected to behave differently. The ability to examine different curve fits after measurement provides the investigator added flexibility. The dual optics of the reader allow the researcher to run either absorbance based assays, as well as fluorescent substrates with no compromises in performance. This is particularly important in situations where the assay protocol may be changing from one type of assay to another.

References

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Rev. 07/28/04

RA44419.4775578704

5994-2661EN

September 1, 2021