Real-Time Polymerase Chain Reaction Coupled Detection of Cardiac Troponin I with the Bio-Bar Code Assay

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Abstract

Every year millions of Americans go to the emergency room with symptoms of a heart attack. More than half a million Americans die each year of myocardial infarctions (MI) caused by coronary heart disease (CHD). CHD detection is currently limited to EKG analyses of electrical signals generated by the heart or to protein immunoprecipitation assays of proteins released from cardiac cell death. Neither method has a sensitivity low enough to detect minor damage to the heart or to definitively diagnose acute myocardial infarctions (AMI). False positive admissions to the emergency room for AMI waste billions of dollars annually. Unambiguous diagnosis of AMI could save millions of dollars as well as shorten the crucial time before drugs are administered to mitigate the effects of a recent heart attack. Increasing the sensitivity of protein detection specific to AMI might improve diagnosis. Using the bio-bar code assay developed in the Mirkin lab, ultrasensitive detection of proteins can be achieved. In particular, with a real-time PCR coupled bio-bar code assay, sensitivity of cardiac troponin, a protein highly specific to cardiac cell death, can be extended to 500 fM. This is 100 times more sensitive than traditional protein immunoprecipitation assays and might play a key role both in the unambiguous diagnosis of acute MI and in monitoring the progression from minor MI to acute MI.

Introduction

It might start as a numbness radiating down your left arm. It might feel as if an elephant is sitting on top of your chest. Every year millions of Americans go to the emergency room with these symptoms. Over half a million Americans die each year from heart attacks, a leading cause of death in the United States. Approximately one in five deaths can be attributed to myocardial infarctions.[1]

Ischemia characterizes the early beginnings of myocardial infarction and is clinically defined as the lack of oxygenated blood to the myocardial cells. Myocardial cells deprived of oxygen quickly die, damaging the heart. As the damage accumulates, the efficiency of the heart continues to drop; intervention at this point could halt and potentially reverse the damage, but there is currently no clinically available device with such a low threshold of detection. When the heart can no longer supply enough oxygen to itself to keep functioning, a patient experiences what is called an acute myocardial infarction, or what is better known as a heart attack.

Acute myocardial infarctions (AMI) are generally recorded as chest pain and upper body discomfort by the patient. Emergency room tests, such as electrocardiograms (EKG) and protein immunoprecipitation assays, are currently the only means of confirming the occurrence of an AMI event. An EKG monitors the electrical signals produced by the beating heart. In a patient who has experienced a heart attack, physicians look for the characteristic ST elevation peak. This peak is not always apparent, however, and EKGs cannot diagnose all cases. Thus, this method is highly subject to the opinion of the examining physician. Detection of proteins present in the plasma following a heart attack — creatine kinase, myoglobulin, or cardiac troponin I — requires extensive lab work, and results may not be available promptly. Because the two current techniques require special machinery or lab analysis, an unambiguous diagnosis of AMI may take hours. In addition, every year billions of dollars are wasted in the emergency room on false positives. Patients admitted to the emergency room exhibiting signs of myocardial infarction may in fact only be experiencing severe indigestion and heartburn, ailments with some similar symptoms.

Better detection and diagnosis of acute myocardial infarction potentially could save millions of dollars. There is a need for a quick, confident diagnosis that will not only save money by shortening the hospitalization time of misdiagnosed patients but also reduce the crucial time before patients who have suffered infarctions receive drugs to curtail the damage of a heart attack.

Increasing the sensitivity of protein detection specific to AMI is one means to potentially improve diagnosis. Using the bio-bar code assay developed in the Mirkin lab, ultrasensitive detection of proteins can be achieved.[2] This method allows for detection down to the attomolar range for a prostate specific antigen (PSA), a sensitivity that is unattainable by traditional methods such as ELISA (Enzyme-Linked ImmunoSorbent Assay). One of the goals of increased detection sensitivity is to prevent myocardial infarctions by detecting minor myocardial infarctions (MMI) that lead up to AMI.

Background

Typically, creatine kinase, myoglobulin, or cardiac troponin I (cTnI) are markers used to diagnose MI. All three are associated with myocardial damage, but cTnI is used most frequently because it

shows superior sensitivity. cTnI can be detected as early as six hours and as late as several days after myocardial damage.[3] Additionally, cTnI is highly specific to myocardial cell death. When myocardial cells undergo lysis, they release cTnI into the bloodstream. Therefore, it should only be present in individuals with myocardial damage. The other two markers, creatine kinase and myoglobulin, are both present elsewhere in the body, making it difficult to ascertain whether their presence is due to myocardial damage or to damage to other types of cells.

Damage to the heart is cumulative and can be defined based on levels of cardiac troponin I present (Table 1).[4] Troponin levels are commonly detected using an ELISA test (see Figure 1 for scheme). This method is based on a heterogeneous binding of the target protein in solution with antibodies immobilized on the bottom of a well. A second antibody tagged with an enzyme that reduces an additional reagent that fluoresces or absorbs light is then used to quantify the immobilized target protein. Of particular interest for ELISA tests are the possible level concentrations as they are defined for AMI and MMI. AMI is currently characterized in a clinical setting by a presence of cTnI greater than 1.0 ng/ml or 44 pM, a concentration currently detectable by ELISA. Meanwhile, the lowest sensitivity of a modified ELISA for cardiac troponin I achieved in the literature is 3.35 pM.[5] Although the lowest achieved sensitivity reported in the literature reaches beyond the benchmark level, the conditions and equipment with which this modified ELISA is performed are not practical for routine clinical lab work. Typically ELISA results are based on a linear relationship between protein concentration and absorbance. This linear dependence holds true for the nanomolar range of cTnI but begins to deteriorate shortly after the benchmark sensitivity. An ELISA assay, therefore, cannot be used to detect all MMI levels of cTnI, as those values lie between 27 pM and 44 pM.[4]

There is some overlap between these defined levels of cTnI in AMI and MMI.[6] These ambiguous boundaries tend not to be a problem because the ability to detect troponin more sensitively will ultimately allow for the treatment of heart damage before mild MI crosses the threshold level to acute MI.

Approach

The bio-bar code assay is an amplification scheme developed for the detection of proteins.[2,7] Two probes, a magnetic micro particle (MMP) and a gold nanoparticle, are designed to be specific to the target protein and must be synthesized prior to the assay (Figure 2). MMPs functionalized with a monoclonal antibody specific for cTnI (protocol #70004, www.polysciences.com) are incubated for one hour at 37° C with a dilutions series of the target protein in buffer. After binding of the MMP to the protein is accomplished, any unbound protein is washed away with a 0.1 M NaCl solution in PBS, while applying a magnetic field. Subsequently, a 100 nm gold particle probe is added to the magnetic particle complex. This gold nanoparticle is functionalized with a different monoclonal antibody and densely modified with thiolated DNA hybridized with complementary bar-code DNA. The gold nanoparticle probe is allowed to bind to the protein, creating a gold-protein-magnetic particle sandwich complex. Finally, water is added to dehybridize the bar-code DNA, and the sandwich complex is removed from the solution by applying a strong magnetic field. The high sensitivity of the bio-bar code assay derives in part from the thousands of bar-code DNA strands finally released for each protein-binding event (Figure 3). Since the bar-code DNA is detected rather than the target protein, the bio-bar code assay acts as a controlled intermediary step that increases the sensitivity of

Table 1: Levels of myocardial damage can be defined using key levels of troponin presence.

Condition	Level of cTnI Present	[cTnI]
Acute Myocardial Infarction (AMI)	> 1.0 ng/ml	> 44 pM
Acute Coronary Syndrome	> 0.6 ng/ml	> 27 pM
Minor Myocardial Damage (MMI)	0.6 ng/ml < cTnI < 1.0 ng/ml	27 pM < [cTnI] < 44 pM

Enzyme Linked ImmunoSorbent Assay (ELISA)

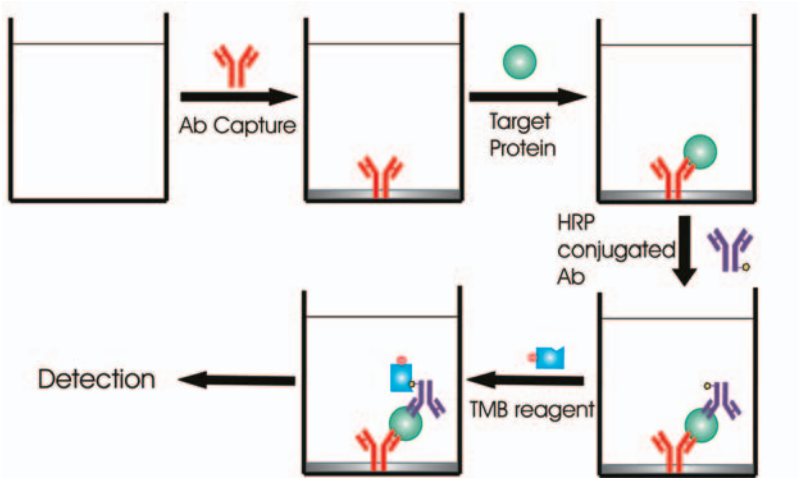

Figure 1: ELISA is a traditional protein detection method. A well is passivated and functionalized with an antibody specific to the target. The protein is then incubated to induce binding. Once bound, a second antibody that is conjugated to horseradish peroxidase (HRP) is added. Next, a tetramethlybenzidine (TMB) reagent is added. HRP reduces the TMB reagent. This reduced form of TMB absorbs light and can be detected and quantified using absorption techniques.

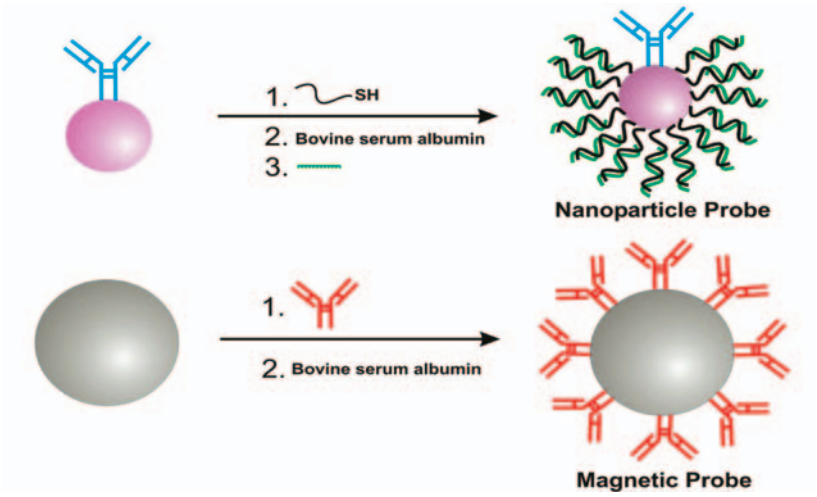

Figure 2: Activation and functionalization of the magnetic micro particles (MMP) is done according to the protocol outlined by Polysciences, Inc. The functionalized MMP has a monoclonal antibody specific to cTnI. A slow salt aging functionalizes the gold nanoparticle with thiolated DNA and another monoclonal antibody for cTnI. This slow salt aging packs the DNA onto the gold. The functionalized gold nanoparticle is then allowed to hybridize to the complementary bio-bar code DNA.

Real-Time Polymerase Chain Reaction Coupled Detection of Cardiac Troponin I with the Bio-Bar Code Assay *(continued)*

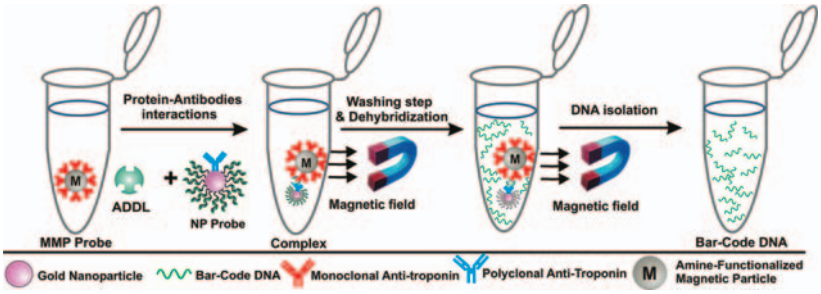

Figure 3: The bio-bar code assay allows for the amplification of the protein signal. Two binding events create a sandwich molecule. Bar-code DNA is released with the addition of water. The number of oligonucleotides released is proportional to the number of sandwich complexes formed. Here, the DNA isolated from the assay is detected rather than the protein, significantly amplifying the signal.

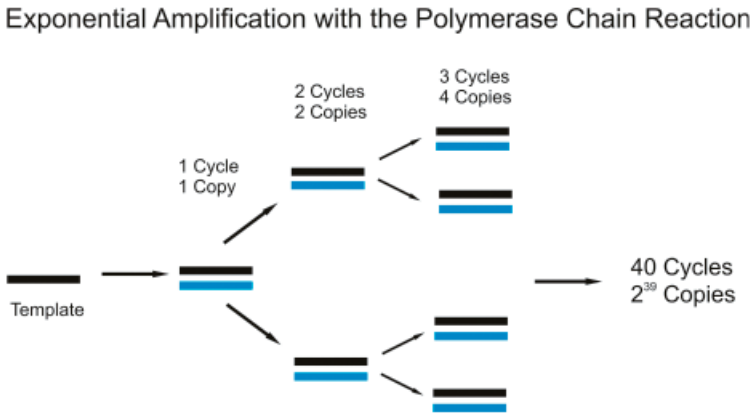

Figure 4: The exponential amplification scheme of real-time PCR.

detection by amplifying the signal as well as taking advantage of the numerous DNA detection techniques available, such as the real-time PCR technique.

Real-time PCR is a dynamic technique used for DNA quantification; it obtains readings at every amplification cycle, taking advantage of an exponential amplification scheme (Figure 4). The more initial bar-code DNA present, the sooner exponential amplification begins, indicated by an increasing fluorescent curve with time. Amplification begins in an activation step to deprotect the polymerase enzyme at 95° C. Once the polymerase has been activated, the components are cycled through a 95-60-72° C temperature scheme to amplify the DNA present. At 95° C any double-stranded DNA becomes dehybridized. Primers are then allowed to anneal at 60° C to the template DNA isolated from the bio-bar code assay. The polymerase is finally allowed to extend the DNA at 72° C. The SYBR green fluorophore from the master mix binds selectively to double-stranded DNA. Once the polymerase has completed extension of the template, SYBR green binds to the double-stranded DNA, and a fluorescence reading is obtained at this step to ascertain the amount of DNA present. The fluorescence signal varies exponentially with DNA concentrations (Figures 5, 6). This cycle is repeated as many times as necessary to amplify the sequence. Amplification can be completed in less than 30 minutes using the Light Cycler.

Real-time PCR offers a practical and reproducible way to quantify the bio-bar-code DNA isolated from the bio-bar code assay. Increased experience with real-time PCR results in minimal differences in results obtained from duplicates. Optimization is crucial for real-time PCR,

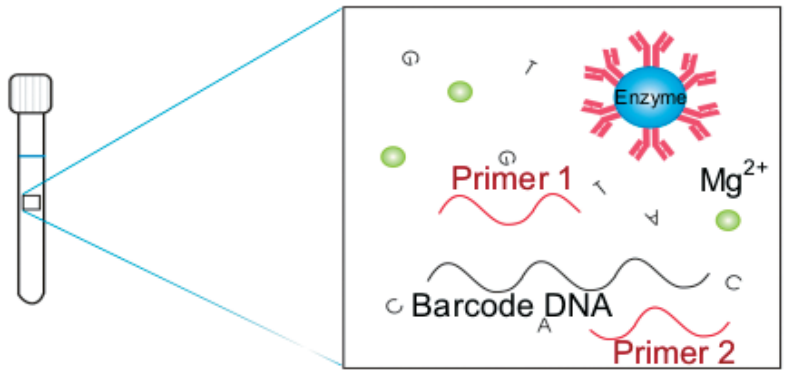

Figure 5: The components of real-time PCR include a protected Taq polymerase suspended in a reaction buffer, an Mg^{2+} cofactor, free nucleotides, template DNA isolated from the bio-bar code assay, primers, and an SYBR fluorophore.

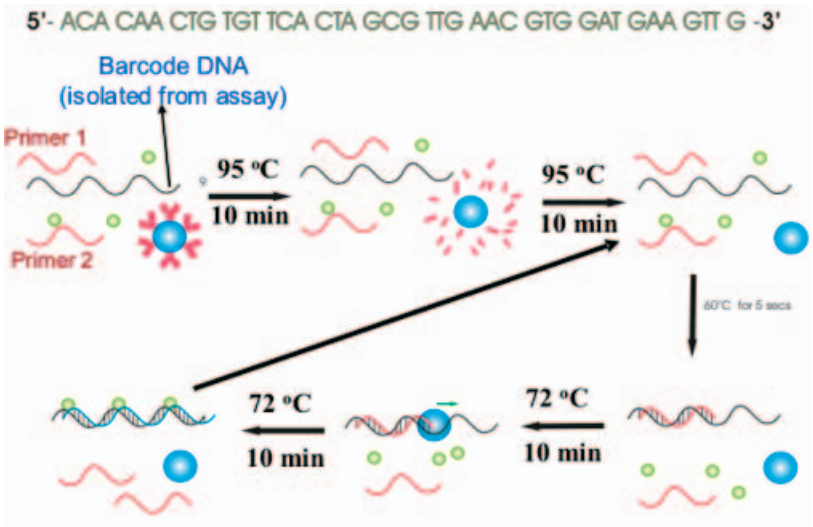

Figure 6: Amplification of DNA occurs exponentially, as the DNA doubles each time. A deprotecting step precedes the amplification to activate the polymerase at 95° C. Next, the components are cycled through a 95-60-72° scheme for amplification. The first leg of the cycle causes any double-stranded DNA to dehybridize. At 60° C the primers anneal to the template. Finally, at 72° C the polymerase extends the DNA, adding new nucleotides in accordance with the template DNA.

however. For each sequence and for each machine, the reaction needs to be optimized to generate ideal amplification curves. Factors that need to be optimized include the optimum annealing temperature, primer concentration, and the Mg^{2+} cofactor concentration. Each of these factors plays an important role for well-spaced amplification curves with minimal primer amplification. If the annealing temperature is too high, the primers will not anneal to the DNA, thus preventing the sequence from being amplified. If the annealing temperature is too low, there will be a high incidence of nonspecific binding, leading to primer dimerization and thus nonspecific amplification of the primers. A similar logic exists for optimizing the primer concentrations. If the primer concentration is too high, a high incidence of nonspecific amplification will be seen. If the primer concentration is too low, the DNA may not be doubling with every cycle. Optimization of the Mg^{2+} cofactor is also based on minimizing the nonspecific amplification of the primers.

It is well known, meanwhile, that real-time PCR also has some limitations. One of its pitfalls that has already been noted is the nonspecific amplification of the primers used. There are ways to avoid the dimerization that causes the amplification of the primers. One is to design the bar-code DNA such that primer dimerization is minimized. There are limitations to this, however; we want our bar-code DNA to be a sequence that is as stable as possible. The only other means to limit nonspecific amplification is to optimize the reaction. A second drawback of real-time PCR is high sensitivity to contaminants, such as DNAses and RNAses, which exist commonly in the environment and on the human body. If these agents come in contact with the template DNA isolated from

the bio-bar code assay, they will cleave it and render it useless in an amplification scheme. Thus, it is extremely important to work under meticulously clean environments using pipets specifically designated for PCR. Finally, real-time PCR cannot be reliably used for detecting fewer than 10 copies of DNA. Although the bio-bar code assay is designed to release thousands of oligonucleotides per protein-binding event, this number is highly dependent on the integrity of the gold colloid and can vary. Despite its limitations, however, real-time PCR is a means of quantification that is consistent and reproducible.

Results and Discussion

Optimization experiments were performed for both the Biorad iCycler and the Roche Light Cycler using the sequence from Nam et al.[2] For the iCycler, optimal annealing temperature was determined to be 61.3° C (Figure 7). Optimal final primer concentrations in the reaction mixture were determined to be 100 nM of primer 1 and 250 nM of primer 2 (Table 2). Optimal Mg^{2+} concentration was determined to be the one optimized within the Biorad master mix. Each of these factors was determined such that nonspecific primer amplification was minimized. For the light cycler system, optimal annealing temperature, final optimal primer concentration, and final optimal Mg^{2+} concentration were determined to be 60° C, 0.5 μM of each primer, and 2 mM, respectively.[8] The calculated Mg^{2+} concentration combines both the Mg^{2+} present in the reaction buffer and the Mg^{2+} used to spike the reaction mixture. Optimal reaction conditions are summarized in Table 3.

Using the optimized reaction protocol for the Biorad iCycler, real-time PCR was calibrated with a serial dilution of the bar-code DNA from Nam et al,[2] a highly stable sequence isolated from a successful bio-bar code amplification. Serial dilutions of 10^8, 10^7, 10^6, and 10^4 strands of template DNA were used. A negative control containing only the primers was used to control for nonspecific amplification. Amplification curves generated modeled the expected data (Figure 8). Since the threshold cycle for each amplification curve can be difficult to determine, a second derivative analysis was performed for each curve. The x-coordinate of the peak of the second derivative, shown in Figure 9, corresponds to the threshold cycle. A threshold fluorescence value can also be evaluated by examining the fluorescence at the threshold cycle. Typically, this value is about one-sixth of the final fluorescence. The threshold cycle is where the amount of DNA increases most rapidly, indicating time of maximum amplification rate. The relationship between the threshold cycle and DNA strands is

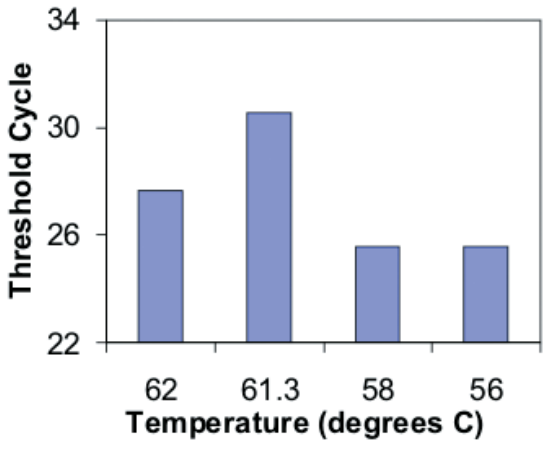

Figure 7: Optimal annealing temperature was chosen in order to minimize nonspecific amplification of the primers. 61.3° C had the highest threshold cycle, indicating little nonspecific amplification.

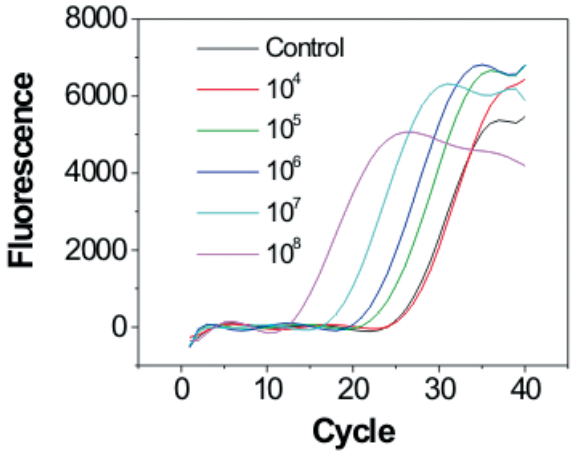

Figure 8: Amplification curves are shown using the optimized protocol for the Biorad iCycler. A trend of decreasing amplification time can be seen for increasing strands of DNA.

exponential (Figure 10); as the amount of DNA increased, the threshold cycle decreased exponentially. There is some deviation at lower levels of DNA because of the complex kinetics involved in real-time PCR amplification. These results, however, were difficult to obtain, as the iCycler was not very consistent. Optimization of the Roche Light Cycler provided results that were much more consistent, with very little nonspecific amplification of the primers (Figure 11). Results shown were typical of an amplification of the serial dilution of the bar-code DNA.

Amplification of the bio-bar-code DNA isolated from the bio-bar code assay showed sensitivity of cTnI to previously unattained levels. Amplification curves were well spaced down to femtomolar concentrations (Figure 12a). Below 500 fM, amplification curves were no longer consistently well spaced. Amplification of the negative protein control occurred at 36.3 cycles, however, indicating that not all of the unbound gold colloid was washed away. Threshold cycle analysis (Figure 12b) shows an exponential trend between the protein concentration and the threshold cycle at the upper end of cTnI concentrations. At concentrations lower than 500 fM, amplification curves were no longer consistently spaced, and threshold cycle analysis no longer showed a clear relationship, leading us to believe that we had reached a new detection limit at 500 fM. A negative control for nonspecific amplification had a threshold cycle of 38 cycles. Because nonspecific amplification did not occur until much after amplification of samples from the bio-bar code assay, nonspecific amplification likely did not play a role in the amplification observed in the samples.

At 500 fM, detection sensitivity is two orders of magnitude more sensitive than traditional ELISA methods. With a detection limit reaching into the femtomolar range, detection in the low picomolar range becomes more reliable, and the progression from minor myocardial infarctions (MMI) to acute myocardial infarctions (AMI) eventually can be monitored accurately. Additionally, increased sensitivity could also increase accuracy of AMI diagnosis, decreasing the annual number of false positives.

An increase in the sensitivity of the assay is possible but will require further optimization of the real-time PCR reaction. Currently, optimization of the primer length is under way. The primers used in this experiment were half complementary at 20 bases. Complete and exact hybridization with a primer that is half complementary may be difficult; shorter primers are being investigated for the use in real-time PCR.

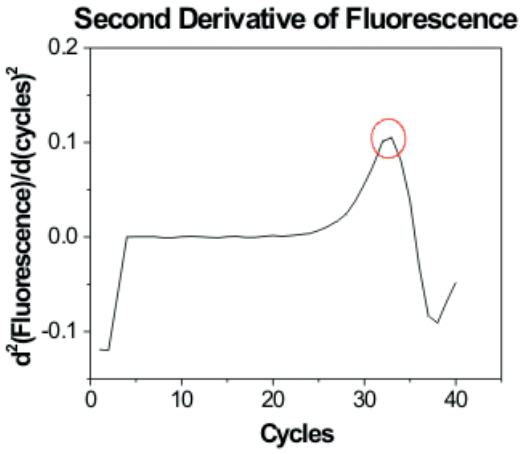

Figure 9: A typical second-derivative analysis for each of the amplification curves from real-time PCR curves shown in Figure 8. The peak for the second derivative is circled for clarity and is taken as the threshold cycle representing the highest rate of amplification.

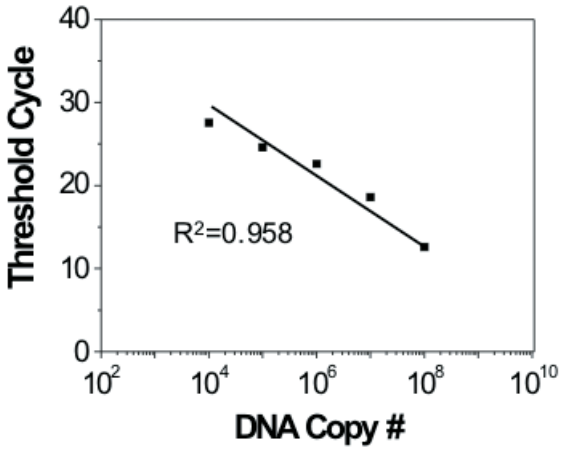

Figure 10: Threshold cycle analysis shows that as the amount of DNA increases the threshold cycle decreases exponentially.

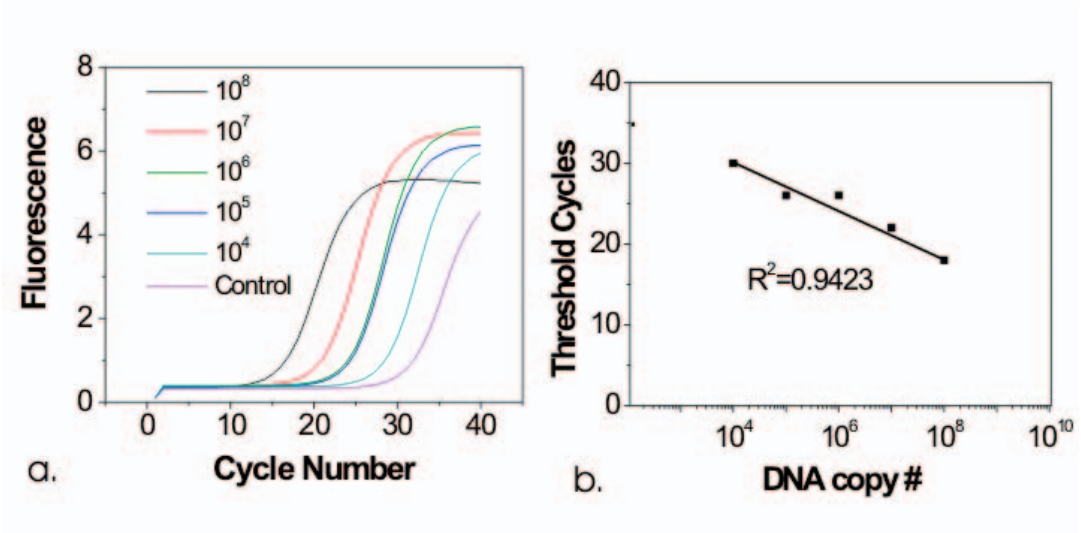

Figure 11: (a) Results from an optimized amplification reaction for the Roche Light Cycler. (b) Threshold cycle analysis of the data in (a) using the Roche Light Cycler. The amount of DNA increases as the threshold cycle decreases exponentially.

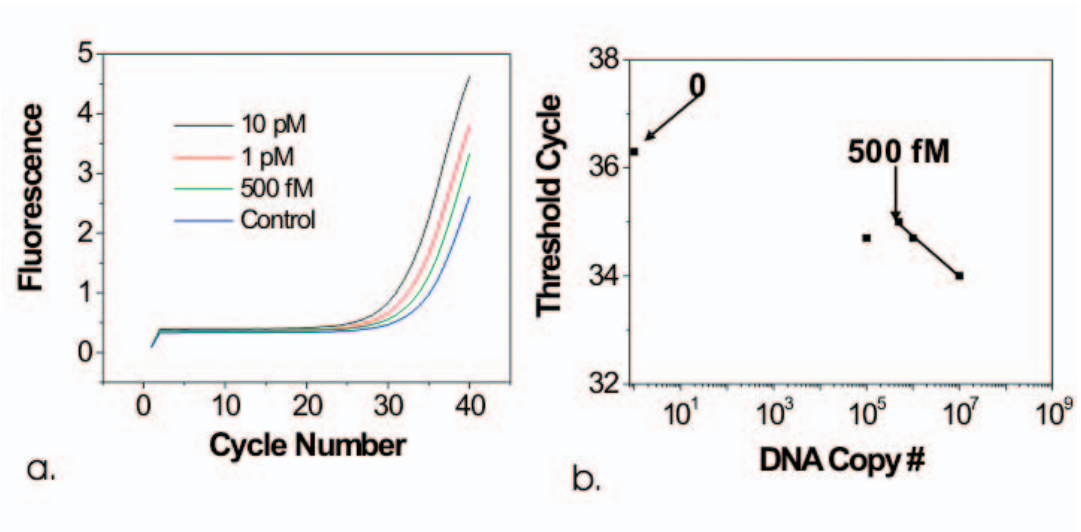

Figure 12: (a) Amplification curves obtained for bio-bar code assay done with known protein concentrations. Well-spaced amplification curves were observed up to 500 fM. For clarity, curves beyond our detection limit were omitted. The control curve corresponds to the negative control used in the cTnI dilution series. (b) Threshold cycle analysis reveals an exponential trend between cTnI concentration and threshold cycle down to about 500 fM. Below 500 fM, however, data no longer show a consistent relationship. 500 fM is noted in the figure.

Table 2: Primer concentrations were varied and used in a negative control. Primer concentrations were chosen such that nonamplification was minimized but present. Threshold cycle analysis was done to determine the optimal primer concentration.

Concentration of Primer 1	Concentration of Primer 2	Threshold Cycle
100 nM	100 nM	> 40
100 nM	250 nM	35.56
100 nM	500 nM	31.58
250 nM	100 nM	> 40
250 nM	250 nM	28.58
250 nM	500 nM	25.58
500 nM	100 nM	> 40
500 nM	250 nM	25.58
500 nM	500 nM	17.59

Table 3: Light Cycler and iCycler optimization results.

	iCycler	Light Cycler
Optimal Annealing Temperature	61.3° C	60° C
Optimal Final Concentration of Primer 1	100 nM	0.5 μM
Optimal Final Concentration of Primer 2	250 nM	0.5 μM
Optimal Final Mg^{2+} Concentration	Concentration present in Biorad master mix	2.0 mM

Conclusions

Following extensive experimentation with the real-time PCR detection method, we were able to detect cardiac troponin I concentrations down to 500 fM using the bio-bar code assay. This level of detection is 100 times lower than traditional ELISA results for detecting cardiac troponin I. Continued optimization of real-time PCR detection with varying lengths of primers has hopes of increasing this sensitivity further. Promising data already obtained with samples from known protein concentrations lay the framework for future analysis of serum samples obtained from patients who have already suffered from AMI. It also establishes a benchmark concentration for the levels of naturally occurring cardiac troponin I in healthy people.

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