A Flexible Bioluminescent-Quantitative Polymerase Chain Reaction Assay for Analysis of Competitive PCR Amplicons

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Aequorin-based flash-type bioluminescent methods can detect nucleic acid molecules in the attomolar range (10^{-18}) enabling improved monitoring of the polymerase chain reaction (PCR) at cycles previously considered too low for product detection. The high sensitivity of bioluminescence (BL) was used to examine the efficiency of the PCR and to assess the effect of substrate variation during the linear phase of amplification. Primer efficiency was dependent on initial template concentration, in a manner indicative of a two-component reaction. However, the rate of amplicon formation was significantly impaired at low template levels and could not be overcome by excess primer. The PCR was directly dependent upon nucleotide concentration, which was independent of tem-

plate concentration. Conditions were identified for optimal linear amplification and detection using BL. Accurate quantitative analysis was performed using competitive coamplification of a specific target standard sequence containing identical target primer recognition sites and novel internal sequences. Quantitation was most accurate when target molecule was similar in concentration to the internal standard. The Bioluminescent Quantitative-PCR (BLQ-PCR) assay has the potential to eliminate processing variability. We demonstrated high quantitative potential with a broad dynamic range. Overall, the BLQ-PCR assay is flexible and a viable alternative to contemporary Q-PCR techniques. *J. Clin. Lab. Anal.* 13:40–47, 1999. © 1999 Wiley-Liss, Inc.

Key Words: bioluminescence; quantitative PCR; BLQ-PCR; aequorin

INTRODUCTION

The polymerase chain reaction (PCR) has tremendously enhanced the value of molecular biology as a useful tool in clinical laboratory diagnostics. However, the need for rapid, convenient, and accurate DNA quantitation has not been satisfied. It has been widely considered that accurate DNA quantitation using PCR assays is not feasible because errors are exponentially amplified through multiple cycles toward a plateau before sufficient product is generated for analysis (1). Quantitative analysis must therefore be restricted to the exponential phase of product accumulation (2–4). This technical hurdle may be overcome using sensitive detection methods that measure PCR products produced prior to amplification fluctuations.

A flash type bioluminescent tag, aequorin, has been developed that can be detected in the subattomolar range. The bioluminescent photoprotein aequorin, isolated from a number of species of the jellyfish *Aequorea*, is a 22,000-molecular-weight peptide complex. The native photoprotein is characterized by bound oxygen and a heterocyclic compound called coelenterazine, a luciferin commonly found in coelenterates (5–8). Aequorin contains three calcium binding sites. Addition of calcium ($K_d = 1.4 \times 10^{-7}$) catalyzes a conformational change that affects the oxidation of the bound coelenterazine

using the protein-bound oxygen. This, therefore, is the basis of a unique detection system; it is not an enzyme, but a “self-contained,” triggerable, light-generating protein (photoprotein) (5–8). The energy from this oxidation reaction is released as a flash of blue light, centered at 469 nm. Excess calcium ions induce a rapid oxidation of the bound coelenterazine and the constant decay of activated intermediate with a $t_{1/2}$ of approximately 0.5 seconds. This provides not only a rapid signal, but also a signal that is insensitive to temperature variation during signal generation. The signal is detected in a luminometer that can inject a calcium solution and simultaneously measure light. The bioluminescence (BL) produced by an aequorin conjugate covers greater than seven logs of concentration of which approximately five logs produce a linear relationship between conjugate dilution and BL. A comparison of BL with radioimaging (2), and electrochemiluminescence and EtBr staining (9) to detect amplicons

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produced during the amplification of PCR found that: (1) BL was superior in detecting amplicons generated from small levels of template at low amplification cycles; and (2), lower cycles of amplification yield greater efficiency of quantitation. Recently, Guenther and Hart (10) compared BL with colorimetric methods for competitive quantitation of HIV-1 amplicons with similar findings.

The simplest method for quantitation of PCR products involves early detection during linear amplification with reference to external standards. The most precise quantitative methods to date utilize competitive coamplification of a specific target sequence with known concentrations of an internal standard competitor containing identical target primer recognition sequence sites (11, 12). Quantitation is thus determined by comparing relative products ensuing from the reaction; the mathematical relationships and theoretical considerations have been elaborated elsewhere (13). The possibility of measurement at low cycles during linear coamplification of both target and competitor effectively limits minor sample variation and reaction conditions that are greatly magnified during later amplification stages.

We applied the great sensitivity of aequorin to the development of a rapid, bioluminescent quantitative PCR assay (BLQ-PCR) to be used in a 96-well plate format. In this model study, we first optimized conditions for generation of biotin-labeled PCR amplicons during the linear amplification phase of PCR, and then demonstrated the utility of BL as an attractive alternative for quantitative PCR (Q-PCR) analysis.

MATERIALS AND METHODS

DNA

Plasmid pcD-IL-2 containing full-length murine IL-2 cDNA (ATCC 37553, pmut-1, Rockville, MD), and plasmid pmCK containing identical primer regions to pcD-IL-2 with unique intervening sequences (14), were grown on Luria-Bertani Medium with 60 µg/ml Ampicillin in *E. coli*. DNA was prepared using a Qiagen maxi kit (QIAGEN, GmbH, Germany), and linearized using *Pst* I or *Hind* III restriction endonuclease (Boehringer Mannheim, Germany). DNA concentration was measured by a Beckman DU 650 spectrophotometer (Arlington Heights, IL).

Nucleotide Primers and Probes

Oligonucleotides used for detection and quantitation of PCR product were synthesized at the Biotechnology Core Facility Branch, SRP, NCID, CDC (a generous gift from Janet McNicholl), or at Operon Technologies (Alameda, CA). The sense primer was biotinylated on the 5' terminal nucleotide to facilitate later capture; the oligonucleotide probes were digoxigenin-labeled on the 3' terminal. IL-2 primers and probe

sequences were based on previously published sequences (9) derived from the Genetic Sequence Data Bank (NCBI-GenBank). Primers used were (5' to 3') Biotin-AACAGCGCACCACTTCAA and TTGAGATGATGCTTTGACA. Detection of amplified PCR product from pcD-IL-2 or pmCK was accomplished using sequences (5' to 3') GTTCA-TCTTCTAGGCACTG-DIG and CTCGAGCGCGCGGCC GACTAGTT-DIG (14) respectively with solid phase BL technology (9, 15).

PCR Amplification

Template DNA was amplified using 1U recombinant Taq DNA polymerase (GibcoBRL Life Technologies, Gaithersburg, MD) in 1x PCR buffer (50 mM KCl, 10 mM Tris-HCl, 1.73 mM MgCl₂). Primer and dNTP substrate concentrations were 600 nM and 20 µM respectively, unless otherwise indicated. Denaturation, annealing, and elongation was at 94°C, 54°C, and 72°C for 1, 1, and 2 min each using a PTC-100-96V thermocycler (MJ Research, Inc., Watertown, MA) for 25 cycles. Amplicons produced were 423bp from pcD-IL-2 and 565bp pmCK, with similar efficiency of amplification (14). Quantitation experiments with internal standards used 0.005 through 18.8 attomoles (0.08 pg through 320 pg) pcD-IL-2 amplified either alone or with constant levels of pmCK (0.35 or 1.76 attomoles). Random samples were subjected to 40 cycles amplification and electrophoresis followed by ethidium bromide staining to confirm single band products.

Bioluminescent Detection of Amplicons

Biotinylated amplicons were detected using solid phase techniques according to a modified version of published methods (1,8,26,30,35). 5 µl of biotinylated amplicon was denatured for 5 min (1 M NaOH, 200 mM EDTA), neutralized (0.3 M Na₂HPO₄, pH 6.0), and added to Streptavidin-Microtiterplates® (MicroCoat, Penzberg, Germany) containing 4 ng digoxigenin-labeled probe in hybridization buffer (62.5 mM Na₂HPO₄, 0.94 M NaCl, 94 mM citric acid, 10 mM MgCl₂, 0.125% Tween-20, 0.0625% BSA, 15 mM NaN₃, pH 6.5). Hybridization at 42°C for 2 hr was followed by washing with SeaLite wash buffer (SeaLite Sciences, Inc., Norcross, GA). AquaLite® antidigoxigenin conjugate (5 ng, SeaLite Sciences, Inc.) was added for 30 min, followed by detection measuring integrated flash (469 nm) of light (2 sec) on either a ML 3000 luminometer (Dynex Laboratories, Inc., Chantilly, VA) or EG&G Berthold Microplate Luminometer (LB 96V) (SeaLite Sciences, Norcross, GA) using AquaLite® Trigger Solution (50 mM Tris, 10 mM calcium acetate, pH 7.5, 15 mM NaN₃). The limit of detection for the luminometer was determined empirically at 2.1×10^{-17} moles. The apparatus precision was determined using 2×10^{-10} moles AquaLite® and a light emitting standard luminometer check plate (SeaLite Sciences, #SL80-016) resulting in a CV (%) of 5.0. Results have been represented as Relative Light Units

(RLU). For quantitation, results are represented as a ratio of RLU obtained from both products (Target pmCK:Competitor pCD-IL-2).

Analysis of Substrate Interactions

The mathematical relationship used to evaluate component interaction was originally defined by Coppleson and Michie (16–18). The slope ($= b$) of the log substrate concentration-log RLU line indicates the number of interactions, when analysis is performed during the linear portion of amplicon generation. Analysis was accomplished using Excel 97 (Microsoft Inc., Redmond, WA) and KURV+ for Windows, ver. 4.4b (Conrad Button's Software, Arlington, WA) software. Analysis of competition equivalence was accomplished by plotting $\log_{10}$ values of corrected RLU of the Target signal over the Competitor signal, as a function of the $\log_{10}$ of the concentration of competing template. Linear regression analysis generates an equation of $y = mx + b$ where a y value of 0 ($\log_{10} 1 = 0$) results in equivalence to the competitor concentration, as demonstrated by Piatak, et al. (11), and theorized by Raeymaekers (13).

RESULTS

Optimization of Substrates for Low Cycle PCR

Effect of primer and template concentration on amplicon formation

We examined the relationship of primer and template for amplification of a 565-bp sequence from a plasmid construct (pmCK) containing the priming regions for the mouse IL-2 gene and unique intervening sequences, with a goal to define limits of template detection. In a matrix format amplification at 25 cycles was performed while varying concentration of primer (18.75 nM to 600 nM) and template DNA (0.004 to 8.81 amol) (Fig. 1). At primer concentration of 600 nM, a strong signal was generated using 0.004 amol template. The signal generated with increasing template through 0.35 amol template was linear. When the concentration of template was greater than 1.76 amol, the BL signal plateaued. Optimal amplification of template occurred only when the primer substrate was present at concentrations above 150 nM; primer concentration greater than 150 nM was detectable for amplification of the lowest level of template tested (0.004 amol). Use of 75 nM primer resulted in relatively less amplicon production. And primer concentrations below 75 nM were not sufficient for detectable amplification of template below 1.76 amol. At the highest level of template (8.81 amol), primer levels greater than 18.75 nM were sufficient for product formation. As template concentration decreased, reduced signal was generated for every primer level examined.

To further investigate why minimum levels of template

Fig. 1. Simultaneous titration of primer and template: effects on amplicon formation. From 0.004 amol to 8.81 amol template DNA (pmCK) was subjected to 25 cycles amplification using biotinylated primers, with primer concentration ranging from 18.75 to 600 nM. Following alkali denaturation and neutralization, streptavidin captured biotinylated amplicons were detected with digoxigenin-labeled probes and aequorin-conjugated antidigoxigenin. Addition of calcium acetate triggered release of a flash of light detectable at 469 nm, and represented as Relative Light Units (RLU). Mean values represented. Background levels equal 0.005 RLU. PCR was performed in duplicate and products analyzed by bioluminescence in duplicate; standard deviation was less than 5%.

were necessary for efficient amplification, we examined constant template amplification while limiting primer substrate (Fig. 2). Use of 0.04 amol template or greater resulted in rates of product formation with equivalent linear slopes ($b \approx 2.0$); the rate of product formation was similar through levels of template 1,000 times this value (not shown). Below 0.04 amol template reaction rates fell, as indicated by a reduced slope of product detection. For 0.02 amol template the reaction rate considerably slowed ($b = 1.32$). Below 0.004 amol the rate had diminished to a level indicative of a one component reaction ($b = 1.06$). Reduction in template concentration below a certain level resulted in decreased rate of amplicon production (as measured by maximal signal). Therefore, when primer is not limiting, the rate of amplification was regulated by the concentration of available template.

Effect of extraneous DNA and primer concentration

We examined whether the relationship between template and primer concentration is dependent upon sequence specificity. Thus, the efficiency of PCR was further examined in the presence of nonspecific DNA. Under excess dNTP substrate conditions (20 μ M), 1.76 amol DNA template was amplified either alone or in the presence of 1 ng extraneous (nonspecific) herring sperm DNA (Fig. 3). The rate of amplicon formation during linear amplification as a function of bioluminescent signal generated was nearly identical for plasmid alone ($b = 1.88$) or with extraneous DNA ($b = 1.87$).

Fig. 2. Effect of primer concentration on PCR product formation. Increasing amounts of template DNA (0.004–8.81 amol) were amplified 25 cycles using biotinylated IL-2 primers which varied in concentration from 0.15 nM to 600 nM. PCR was performed in duplicate, and ensuing products were

assayed in duplicate using BL. Mean values represented; for clarity, only the linear amplification phase is depicted. The linear slope ($b =$) and correlation coefficient for each template level is shown.

Fig. 3. Effect of nonspecific DNA and primer concentration on specific amplification of template. Bioluminescent signal obtained as related to IL-2 primer concentration. Using biotinylated primers (0.15 to 600 nM), 1.76 amol pmCK was amplified 25 cycles in the presence (●) or absence (■) of 1

ng herring sperm DNA. No signal above background was evident for 1 ng herring sperm DNA alone (◆). PCR was performed in duplicate, and ensuing products were assayed in duplicate using BL. The linear slope ($b =$) and correlation coefficient for each group is shown.

Effect of nucleotide concentration

PCR efficacy was evaluated while varying nucleotide (dNTP) concentration and maintaining a constant primer level (200 nM). For these experiments, plasmid pmCK was amplified 25 cycles under conditions in which nucleotide concentration varied from 20 nM to 2.5 μ M. Reactions were performed using various template concentrations (Fig. 4). For all template levels examined maximum product was produced when dNTP levels were greater than 275 nM. The PCR was directly dependent upon nucleotide concentration which was independent from template concentrations. Levels below 275 nM dNTP resulted in inefficient product formation. To examine a condition where specific template was limiting in comparison to total DNA, PCR was performed in the presence of extraneous 1 ng herring sperm DNA (Fig. 4). In the presence of varying dNTP concentration, 0.35 and 1.76 amol template DNA was amplified as above. Efficient product formation was not altered in the presence of 1 ng herring sperm nonspecific DNA.

Quantitative Analysis Using Bioluminescence

Coamplification of internal standards

Two templates of known concentration containing identical complementary primer regions and different internal detection sequences were used to examine BL quantitative analysis. Specifically, a competitive sequence (pcD-IL-2) containing the full-length murine IL-2 cDNA was coamplified in

the presence of a target sequence (pmCK) which contained primer sites identical to the IL-2 gene with different intervening DNA sequences. Amplification of a 423 base pair cDNA IL-2 PCR product from the Competitor, or a 565-bp product from the Target, was accomplished using a sense primer biotinylated on the 5' terminal nucleotide to facilitate later capture using streptavidin.

Determination of linear quantitation range

Competitor DNA (pCD-IL-2) from 0.005 to 18.8 amol was coamplified 25 cycles in the presence of either 0.35 or 0.88 amol Target DNA (pmCK), using 50 nM primer and excess dNTPs. The samples were split and probed using specific sequences for either Target or Competitor products. As depicted in Figure 5, a linear relationship was established linking the signals produced from both products ($\log_{10}$ signal ratios Target:Competitor) and $\log_{10}$ level of Competitor template used in the reaction. This relationship was linear for nearly 3 logs, centered approximately where the concentrations of the two templates were equivalent. The limit of the analysis occurred primarily at points below where product generated from the Competitor was detectable or where Competitor sequences were in large excess of Target sequences (Fig. 5). Generating a ratio of signal values for the two specific products effectively eliminated Taq polymerase variability and minimized changes in signal produced during different assay runs. It should be noted that it is possible to calculate the predicted equivalence value for the Competitor when the RLU ratio is

Fig. 4. Effect of nucleotide concentration on specific amplicon formation. 0.35 amol (■) or 1.76 amol (●) template DNA was amplified 25 cycles while varying dNTP concentration from 20 nM to 2.5 μ M, using 200 nM constant primer concentration. Amplicons were detected using biolumines-

cence. Signals increased through 275 nM dNTP. Amplification efficiency of either 0.35 amol (□) or 1.76 amol (○) specific template in the presence of 1 ng nonspecific herring sperm DNA was not affected. Mean values for 2 or 3 samples are shown; standard deviation was less than 5%.

Fig. 5. Bioluminescent quantitative analysis: coamplification with internal standards. Target DNA, 0.35 amol (◆, ■ left) or 0.88 amol (● right), was amplified 25 cycles using 50 nM primers and 2.5 μM dNTP in the presence of increasing Competitor template (pCD-IL-2, 0.005–18.8 amol). Amplified products were detected using digoxigenin-labeled probes specific for either target or competitor internal sequences. To normalize for variations in

assay efficiency, data is represented as the $\log_{10}$ ratio of bioluminescent signals obtained for Target and Competitor samples (each sample assayed in duplicate). Plotting RLU ratios versus $\log_{10}$ value of Competitor concentration (attomole) yields a linear plot, where a y value of 0 ($\log_{10} 1 = 0$) results in equivalence to the Competitor concentration. The linear equation is represented as $y = mx + b$; the correlation coefficients are indicated.

equal to 1.0 (when $\log_{10}$ ratio equals 0). In our example, for two independent experiments, use of 0.35 amol Target resulted in a predicted value of 0.40 and 0.37 amol Competitor (Fig. 5, left panel). However, when 0.88 amol Target template was used, a predicted value of 2.18 amol was generated, which was slightly higher than expected. Experiments using 200 nM primer gave similar results, with equivalent limits in quantitative potential (data not shown).

DISCUSSION

BL has demonstrated greater sensitivity than alternative technologies (2, 9) and has the advantage of being able to monitor amplicons during low amplification cycles when product levels are proportional to initial target DNA. During linear amplification the change in signal per product is at its greatest which leads to a higher quantitative potential. At high cycles the reaction rates diminish, thus rendering the PCR nonlinear and quantitatively unreliable. We used BL to examine the efficiency of the PCR and role of substrates for amplicon formation during the linear phase of the reaction. Our results help define the parameters for linear amplification at cycles normally considered too low for competitive quantitation.

The chemiluminescent-based labeling systems have held great promise for assay development (19). These systems include oligonucleotide probes labeled with acridinium esters (20), horseradish peroxidase (21), alkaline phosphatase (22), and digoxigenin (23–26). While the acridinium ester-based homogenous assays are not providing the sensitivity required for all applications, the enzyme-based systems have been used to generate assays with sensitivities greater than what can be

attained with radioisotopes. In general, the enzyme-based assays are horseradish peroxidase or alkaline phosphatase conjugate acting on a substrate whose product is chemiluminescent. Once sufficient reaction has occurred (10 to 20 min of reaction time), the glowing reaction product can be measured and compared with background to quantify amount of reaction. These methods may approach detection of femtomolar (10^{-15}) levels of amplified product. The science described here using aequorin-based methodology can successfully detect molecules in the attomolar range (10^{-18}).

The enzyme-induced chemiluminescent labels provide extremely good signal-to-noise ratios, and have been successful in applications involving detection of nucleic acids on membranes where the background reactions are masked by the film threshold. When used in microplate-based assay systems where the light reaction can be quantified by a sensitive photomultiplier tube, the backgrounds become more significant and all of the variables that affect enzymatic reactions begin to affect the precision of the assays. Thus, for quantitative measurements, these enzyme-based systems normally present high backgrounds and (unless extremely well controlled) low precision—attributes that run counter to highly sensitive assays. A sufficiently sensitive detection system could render the direct probe or signal amplified assay systems quantitative and, at the same time, permit the detection of PCR product in target amplified assays early in the reaction cycles where the reaction is still linear, so that amount of PCR product could be related to amount of target. Use of aequorin-based luminescent technology allows further lowering of signal-to-noise ratios; what is measured is released light with markedly reduced background interference.

Our bioluminescent analysis of PCR products is unique from published ELISA-PCR methods (27); markedly greater sensitivity is achieved using the aequorin-conjugated antibody than from an alkaline phosphatase conjugate (23, 28). The flexibility of the BL assay allows rapid analysis of amplicons. For example, in the single study examining changes in signal generation while varying template and primer substrate parameters, we generated over 200 samples for analysis (8 template concentrations $\times$ 13 primer concentrations $\times$ duplicates). Overall, this represented a little more than two complete microtiter plates, the complete and entire signal from which can be analyzed at 2 sec per sample.

We were able to define a relationship between primer and template suggestive of an interaction involving two components, both of which are necessary for efficient amplification (16–18). In conditions of excess primer and excess template, the rate of product formation demonstrated a linear slope of approximately 2.0. As the level of starting template DNA was reduced, increasing primer was necessary for efficient product formation. To our surprise, when template became limiting, the reaction was impaired in a manner that was not overcome by excess primer. Reaction rates, indicated by linear slopes of product formation, dropped dramatically. This finding is of paramount importance; at low template levels the quantitation potential was severely compromised.

The PCR was directly dependent upon nucleotide concentration in a manner that was independent from template. Regardless of level of template, dNTP concentration below 275 nM resulted in inefficient product formation. However, excess dNTPs resulted in no discernible effect on amplicon formation. This requirement did not change in the presence of nonspecific DNA, perhaps indicating that dNTP concentration has a direct effect on polymerase efficiency. If the nonspecific DNA template had sequestered reactants, a reduction in the PCR product produced would have led to a drop in bioluminescent signal. Similarly, although Herring Sperm DNA does not contain sequences that specifically complement the primer used, it was possible for nonspecific interactions to occur, and thus impede the reaction process. This did not occur.

We demonstrated the utility of the bioluminescent assay for quantitation of PCR amplicons during low cycle amplification. For quantitative analysis, a standard curve was produced using a ratio of BL signals generated from competitive coamplification of a specific target sequence with known concentrations of an internal competitive standard. This method is similar in theory to the competitive Q-PCR assay for HIV DNA species (11). As with PCR MIMICS (12), using an internal standard containing identical target primer recognition sites and performing the reaction in the same tube effectively eliminated sample assay variation due to Taq polymerase and normalized amplification conditions. Babu, et al. (29) demonstrated that although the target and competitor DNAs are independent of the size and sequence differences, similar ef-

iciency of amplification for competitor and target sequences is a prerequisite for proper quantitative PCR analysis. When Souzaze (30) examined the limits and accuracy of PCR quantitation, optimal analysis occurred only when target molecule was similar in concentration to the internal standard (between 0.66- and 1.5-fold); outside this range, quantitation was compromised and our results corroborate this. By changing methodologies, and analyzing products when change in signal per amplicon is greatest, it is possible to effectively broaden the quantitative range and practical limitations of Q-PCR. Analysis of competition equivalence may be accomplished according to the linear relation of plotting $\log_{10}$ values of corrected RLU of the Target signal over the Competitor signal, as a function of the $\log_{10}$ of the concentration of competing template. The linear relationship in this assay using BL covers 2 to 3 logs of Competitor concentration. Use of multiple concentrations (dilutions) of Target coamplified with increasing concentrations of Competitor sequences should allow the establishment of sets of overlapping standard curves for quantitation of an unknown over a very broad linear range. The BL assay has the potential to eliminate signal loss and variability due to processing (i.e., electrophoresis and ensuing signal assessment), which translates into higher quantitative potential.

Although 25 cycles amplification is not considered "low" for product detection, in fact, competitive quantitative PCR usually relies upon high cycles when using internal standard sequences. Previous reports using external standards (31) claimed quantitation at 20 cycles using radioactive detection methods, however 6×10^6 copies were required; 32 cycles were necessary to detect 6×10^5 copies. Wood (32) reported quantitation of HIV-1 proviral load using 30 cycles, but this was also done using an external standard and comparative methodologies. Still others reporting quantitation of HIV-1 (33) or Hepatitis C (34) required nested-amplification techniques (11); however, these reports also utilized 40 cycles amplification. To our knowledge, this is the first report evaluating competitive PCR amplicon quantitation using internal standards at 25 cycles. The obvious advantage of quantitation at lowered cycles is the increased quantitative potential. We previously demonstrated equivalent detection of amplicons using BL and radioactivity at 30 cycles, yet if amplification were lowered to 22 cycles, BL had a much higher sensitivity with greater change in signal per template (2). The recent report by Guenther and Hart (10) in essence elevates the studies of Piatak (11) comparing bioluminescent technology with that used by Piatak (HRP colorimetric evaluation). Yet although the superiority of aequorin signal over HRP was demonstrated, they did not take advantage of the exquisite sensitivity of BL as we have done here.

In summary, this is the first study to use the inherent sensitivity of aequorin to monitor the efficiency of PCR during low cycle amplification. These results define reaction conditions for maximizing the concentration of the primer set rela-

tive to template material, and as such are easily applied to similar systems for target DNA amplification. The use of BL for the detection of PCR amplicons is a rapid and highly accurate method suitable for clinical quantitative analysis. The BLQ-PCR assay design is flexible and lends itself to automation, and is a viable and sensitive alternative to contemporary techniques.

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